THE INCORPORATION OF GLYCINE-2-C^{14} INTO URINARY URIC ACID AND SERUM BILIRUBIN IN NORMAL AND PORPHYRIC SUBJECTS.

With a review of the literature on porphyrin, haem, bilirubin and purine metabolism and abnormal haem-purine relationships in porphyria.

By

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A THESIS

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TO FAY

A C K N O W L E D G E M E N T S .

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CONTENTS.

ABBREVIATIONS USED IN THESIS.

AA - Aminoacetone

ADP - Adenosine diphosphate

AIA - Allylisopropyl acetamide

AIGAR - 5-amino-4 imidazole carboxamide ribonucleotide

AIR - 5-amino imidazole ribonucleotide

ALA - Delta-aminolaevulinic acid
Delta-aminolaevulic acid

AMP-S - Adenylosuccinic acid

ATP - Adenosine triphosphate

B.D.H.- British Drug Houses

C-AIR - 5 amino 4 imidazole carboxylic acid ribonucleotide

CMP - Cytosine monophosphate

CoA - Coenzyme A

DDC - Dicarbethoxy dihydrocollidine
- 3,5-diethoxycarbonyl-1,4 dihydro-2,4,6-trimethyl pyridine.

DNA - Deoxyribonucleic acid

DPN - Diphosphopyridine nucleotide

DPNH - Reduced diphosphopyridine nucleotide

EDTA - Ethylene diamine tetra-acetic acid

FAD - Flavin adenine dinucleotide

$FADH_2$ - Flavin adenine dinucleotide (reduced form)

FGAM - α -N formylglycinamidine ribonucleotide

PGAR	-	α-N formylglycinamide ribonucleotide
GAR	-	β-glycinamide ribonucleotide
GDP	-	Guanosine diphosphate
GMP	-	Guanosine monophosphate; guanylic acid
GTP	-	Guanosine triphosphate
HCB	-	Hexachlorobenzene
IMP	-	Inosine monophosphate; inosinic acid.
NAD	-	Nicotinamide-adenine dinucleotide
$NADH_2$	-	Dihydronicotinamide-adenine dinucleotide
NADP	-	Nicotinamide-adenine-dinucleotide phosphate
$NAD\ PH_2$	-	Dihydronicotinamide-adenine-dinucleotide phosphate
O.D.	-	Optical density
PBG	-	Porphobilinogen
Pi	-	Phosphate
PIA	-	2-propyl-2-isopropyl acetamide
PPi	-	Pyrophosphate
PP-ribose-P-	-	5-phosphoribosyl-1-pyrophosphate
RNA	-	Ribonucleic acid
S.A.C.P.	-	South African genetic porphyria
SAICAR	-	5 amino-4 imidazole-N-succino carboxamide ribonucleotide
S.P.	-	Symptomatic porphyria
S-RNA	-	Soluble ribonucleotide
THFA	-	Tetrahydrofolic acid
TPN	-	Triphosphopyridine nucleotide
TPNH	-	Reduced triphosphopyridine nucleotide
XMP	-	Xanthosine monophosphate; xanthylic acid.

CHAPTER I.

INTRODUCTION.

The porphyrias are a group of disorders characterised by the excessive excretion of haem precursors in urine and stool. In the past two decades, isotopic studies have led to impressive progress in our understanding of both normal and disordered porphyrin metabolism but despite these advances, many aspects of the latter are still unexplained.

In the biosynthesis of porphyrins, succinate and glycine condense to form δ aminolaevulinic acid (fig. 1-1). δ aminolaevulinic acid (ALA) can be metabolised along two pathways, one leading to the synthesis of porphyrin and haem and the other to the formation of non-porphyrin compounds including purines.

Experimental evidence suggests that an expanded hepatic ALA pool is available for porphobilinogen (PBG) and porphyrin synthesis in acute human porphyria (392,402). This could result from a block in the non-porphyrin metabolic disposition of ALA or a block beyond PBG in the biosynthesis of haem by the liver. The major aims of this thesis have been to explore these two possible basic defects and a brief outline of how this problem has been tackled, will now be given.

A. BLOCK IN THE NON-PORPHYRIN METABOLIC DISPOSITION OF ALA.

(1) Experimental evidence in favour of block.

It has been a popular theory for many years that difficulty in the metabolism of ALA along non-porphyrin pathways is the fundamental biochemical lesion of human porphyria. The sequelae of such a lesion may account for both the clinical and biochemical features of an acute attack. As ALA is a donor of formyl groups in purine biosynthesis, a block at this level could result in a deficiency of purine nucleotides and nucleic acids to a degree sufficient to cause a serious disturbance of body metabolism.

With a fall in body purines, a negative feed back mechanism might operate whereby more succinic acid condenses with glycine in an attempt to overcome the block. The common precursor, ALA, would then accumulate and be disposed of via the porphyrin pathway (see Fig. 1-1).

Evidence for a disturbance in haem/purine relationships in porphyria rests upon the following observations:-

(a) In chick embryos with Sedormid-induced porphyria, there is a significant reduction in purine synthesis and rate of turnover (234,437).

(b) Purine nucleosides and nucleotides exert a strong

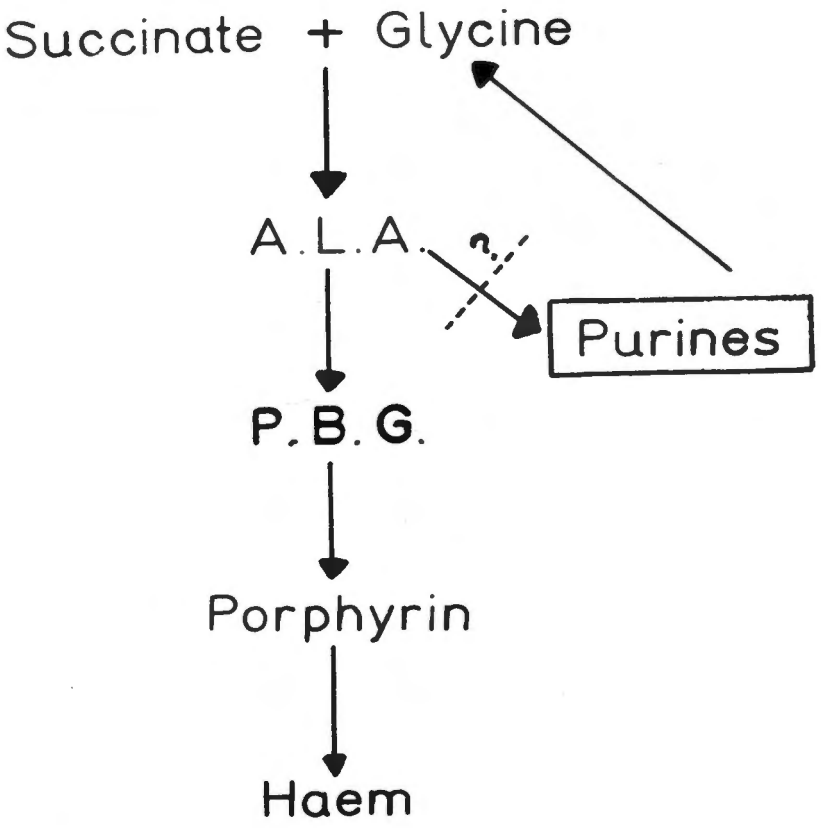

Fig. 1-1: Framework of porphyrin biosynthesis.

inhibitory effect on porphyrin synthesis by Rhodopseudomonas spheroides (112).

(c) Beneficial effects on both the clinical and biochemical manifestations of porphyria in animals and man have been claimed after treatment with purine compounds (109,111,113,234,278,279,437).

(d) Drugs such as orotic acid (115),ethionine (335) and AIA (74) all reduce hepatic ATP levels and are porphyrinogenic in the experimental animal.

On the other hand, de Matteis (74) showed no impairment of purine synthesis in Sedormid, AIA or HCB induced animal porphyria and showed that other compounds could reduce hepatic ATP levels without producing disturbance of porphyrin metabolism. During the course of this thesis, evidence will be reviewed to the effect that the beneficial effects of purine treatment in no way substantiate the hypothesis of Talman et al (234,437) that the symptoms and biochemical features of acute porphyria are due to impaired synthesis of purines from ALA.

Hitherto, no-one has measured the formation of purines from common haem/purine precursors in human porphyria. For this reason, the incorporation of glycine-2-C^{14} into urinary uric acid and its constituent carbons has been studied in normals and patients with symptomatic or S.A. genetic porphyria, the latter in remission or during an acute attack.

(2) Rationale of uric acid degradation experiments.

In fig. 1-2, the synthesis of purines from glycine and ALA has been set out in slightly more detail. The glycine-1-C (Carboxyl carbon) has been marked by a cross and the glycine-2-C (α carbon) by a black circle. The latter has been labelled isotopically in this study. Note how in the formation of ALA, the glycine-1-C is lost as CO_2 whilst the glycine-2-C becomes the 5th (δ) carbon of the ALA molecule. ALA can go on to haem synthesis or its δ carbon may be incorporated via the single carbon atom pool into carbons 2 and 8 (the ureido carbons) of the purine nucleus. The glycine molecule is incorporated intoto to provide the carbons in position 4 and 5 and nitrogen in position 7.

Hence, if there were a defect in the conversion of ALA to purine without a concomitant defect in the direct incorporation of glycine one would expect to find relatively less radioactivity in C2+8 than in C4+5 following glycine-2-C^{14} administration.

70 to 80 μc of glycine-2-C^{14} were administered I.V. to 4 normal controls, 4 symptomatic porphyrics and 6 who suffered from S.A. genetic porphyria of whom 5 were studied during an acute attack. Uric acid was isolated from daily specimens of urine for the ensuing two weeks and degraded in such a manner as to allow for separate counting of C4+5 as glyoxylic acid semicarbazone and C2+8 as

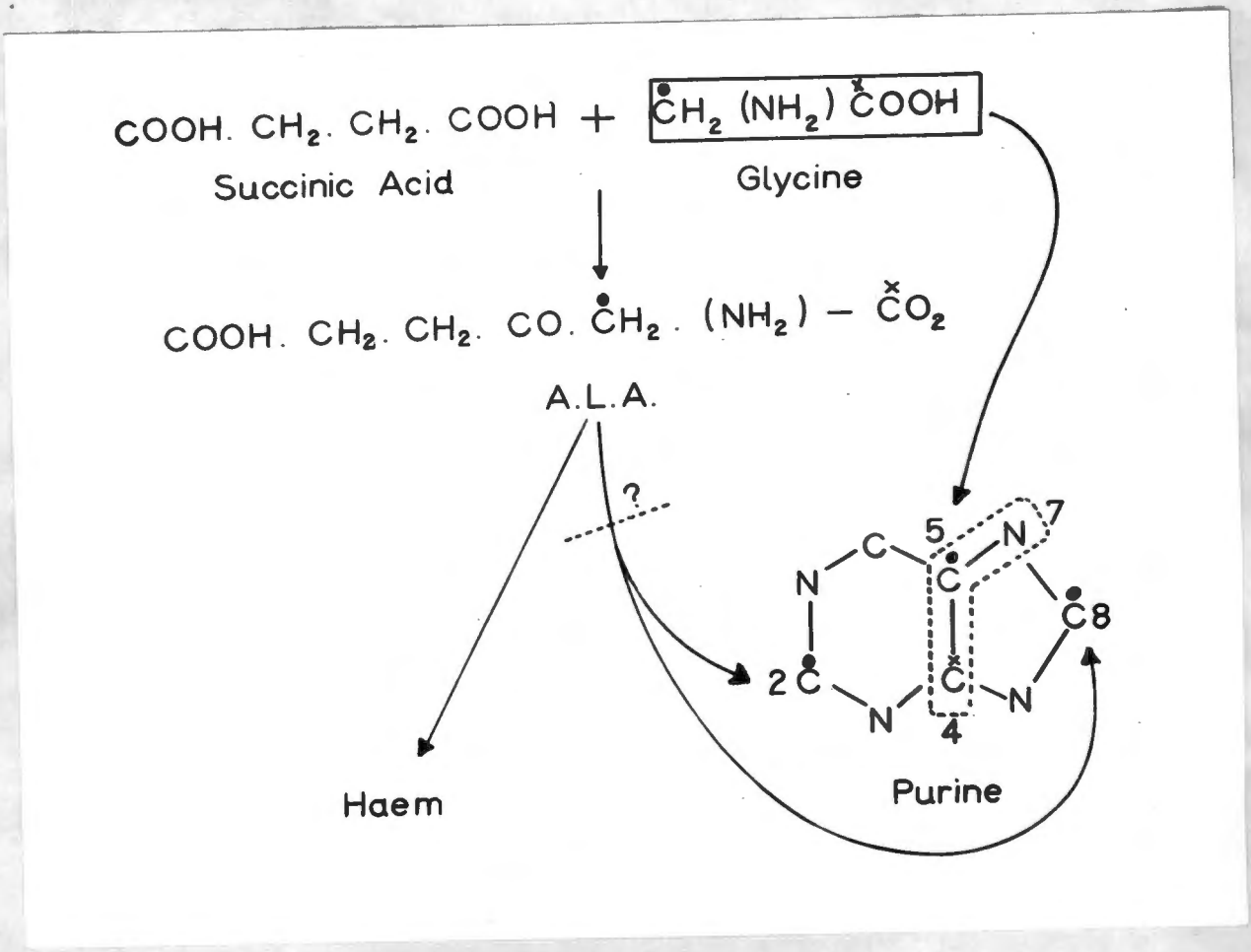

Fig. 1-2: The incorporation of the glycine carbons into the purine nucleus.

urea. In this manner, the percentage dose glycine-2-C^{14} incorporated into the component carbons of the urinary uric acid-C^{14} moiety, the C4+5: C2+8 ratios and the disposition of radioactivity within the urate molecule have been ascertained.

B. BLOCK BEYOND PBG IN BIOSYNTHESIS OF HAEM.

(1) Liver haem production and turnover in porphyria.

Rimington (362,363) and Heikel et al (187) have suggested that when liver cells are damaged, e.g. in symptomatic porphyria, failure of the intracellular reducing systems to keep porphyrinogen intermediates in their reduced state, may account for their escape from the porphyrin biosynthetic pathway and their excretion in excessive quantities. From the findings of Lascelles and her co-workers in photosynthetic bacteria (51,244,247), it is most likely that any difficulty in making haem would result in enhancement of ALA and porphyrin synthesis.

In experimental animal porphyria, synthesis of liver catalase (130,131,382,383,443) but not of its haem moiety (239,279,382,383) is impaired. In fact, there is overwhelming evidence from Fe^{59}, glycine-2-C^{14}, acetate-2-C^{14} and succinate-2-3-C^{14} incorporation studies that liver haem turnover is increased in AIA induced rat porphyria (239,279).

To date, no-one has investigated the turnover of liver haem in human porphyria. The technical difficulties involved are considerable when dealing with the minute amounts of liver obtained by conventional biopsy. However, the observations of Israels et al (205,207,498) who have followed the incorporation of porphyrin precursors into serum bilirubin have suggested valid indirect methods of

investigating liver haem production.

(2) Rationale of serum bilirubin-C^{14} incorporation studies.

Bilirubin is continuously produced from the breakdown of haemoglobin and other haem-proteins in the body. With the advent of tracer techniques, the role of the various haem-proteins in bilirubin formation has been more accurately assessed. Following the administration of glycine-N^{15} to normal humans, London et al (273) and Gray et al (151) have shown that the N^{15} label appears in faecal stercobilin in two distinct maxima of activity, the smaller initial peak during the first week and the larger second peak between the 80th and 140th day after giving glycine.

The later peak, accounting for 70 to 80% of the total stercobilin activity excreted, is derived from the breakdown of senescent red cells at the end of their life span (461). The source of early-labelled or "shunt" bilirubin is not known for certain. It is increased in pernicious anaemia (274), congenital porphyria (151,275) thalassaemia (162) and after haemorrhage (154), suggesting a relationship with haemopoiesis. Yet it is also increased in patients with erythroid aplasia (400) indicating that this fraction in part may arise from non-haemoglobin haem.

Studies on bile bilirubin-C^{14} excretion in bile fistula dogs and on the serum bilirubin-C^{14} patterns in humans have

added to our understanding of the metabolic sources of early-labelled bilirubin and show that it is made up of more than one separable fraction (42,202,203,205,207,498) (this thesis).

Following glycine-2-C^{14} administration serum bilirubin-C^{14} activity exhibits two peaks within the first week of administration, the first within 12 to 24 hours and the second at 3 to 5 days (205,207,498, this thesis). The 2nd peak coincides with maximal increments in circulating haem activity and is increased in haemolytic and post-haemorrhagic states where there is marrow hyperplasia (206, 498, this thesis). It is absent in bile fistula dogs with aplastic anaemia (206) and following ALA-C^{14} where there is minimal incorporation of the label into red cell haem (78,201,202,207,400,498). This fraction almost certainly reflects ineffective erythropoiesis.

In this thesis, special attention has been focused on the first peak of early-labelled serum bilirubin-C^{14} activity. This peak is readily labelled by glycine-2-C^{14} before activity enters circulating haem and, in contrast to the second peak above, is highly active after ALA-C^{14} (78,201, 202,205,207,400,498) and in bile fistula dogs with marrow aplasia (206). It almost certainly arises from non-erythropoietic sources and experimental evidence from bile-fistula dogs (400) and isolated rat liver perfusion studies (364) to be reviewed indicates that is derived in the main,

from the catabolism of non-haemoglobin liver haem. This is not surprising as the liver is an organ rich in rapidly turning over haem-proteins such as catalase and the cytochromes with life spans of a few days (70,81,82,237,383).

In the hepatic forms of human porphyria, disordered porphyrin metabolism should be reflected in changes in liver haem production and turnover. It is for this reason that the first peak of early-labelled serum bilirubin-C^{14} following glycine-2-C^{14}, has been studied in 5 normals, 3 symptomatic porphyrics and 3 South African genetic porphyries, the latter during an acute attack.

C. OUTLINE OF CONTENTS OF THESIS.

Chapter II deals mainly with a review of the biosynthesis of porphyrins, special emphasis being placed on the enzymatic synthesis of ALA, the metabolic fate of ALA and the formation of protoporphyrin and haem.

In Chapter III, aspects of haem-protein and bilirubin metabolism will be discussed, particular attention being directed to sections on the turnover and distribution of haem-proteins, the catabolism of haem and the metabolic sources of bile pigment.

Chapter IV is a comprehensive review of the metabolism of purines and uric acid. Included in the discussion are aspects of liver nucleic acid turnover, a large section on the incorporation of isotopic glycine into uric acid and a brief review of the one-carbon unit pool and its relationship to purine biosynthesis.

In Chapter V, research work done in the field of haem purine relationships in porphyria will be correlated and discussed relevant to the work being done in this thesis.

The details of the experimental procedures adopted in this thesis will be outlined in Chapter VI, the results of the experiments presented in Chapter VII and correlated and interpreted in Chapter VIII.

In the final Chapter, Chapter IX, an attempt will be made to place my findings in perspective and to assess how

much my thesis has contributed to the understanding of the fundamental biochemical lesions of human porphyria.

D. DESIGNATION OF HUMAN PORPHYRICS STUDIED.

In designating the human porphyrics studied, the classification scheme in current use in Cape Town has been adopted. According to this scheme (90) the forms of human porphyria studied in this thesis are South African genetic porphyria (porphyria variegata, protocoproporphyria, mixed porphyria, porphyria cutanea tarda hereditaria) and symptomatic porphyria (urocoproporphyria, acquired porphyria hyperporphyrinuria, porphyria cutanea tarda symptomatica).

CHAPTER II.

THE BIOSYNTHESIS OF PORPHYRINS.

A. PIONEER EXPERIMENTAL WORK.

The elucidation of the porphyrin nucleus is probably the greatest achievement in the field of experimental isotopic chemistry. In 1945 and 1946 Shemin and Rittenberg (411,412) fed N^{15} glycine to humans and rats and demonstrated incorporation of the label into circulating haem. As other labelled nitrogenous compounds including ammonia, glutamic acid, proline, leucine (411,441), ethanolamine (306), histidine (441) and aspartic acid (485) were not significantly incorporated, it was concluded that glycine was the sole provider of nitrogen atoms for porphyrin synthesis. By degrading haemin synthesized from N^{15} glycine, Muir (306) and Wittenberg (481) were able to show that the label was distributed equally between pyrroles A & B and pyrroles C & D. These findings suggested a common pyrrole as the precursor of the four pyrrole rings of the porphyrin nucleus.

These initial studies were performed in-vivo in humans, rats, rabbits and ducks. Shemin, in 1948 (414),

reported an in-vitro system in which the nucleated red cells of ducks incubated with N^{15} glycine could synthesize labelled haem. Subsequently, haemolyzed preparations (358,416) and cell-free extracts (418,421) of these erythrocytes provided the source of the necessary enzymes.

Using C^{14} labelled glycine as the subtrate in in-vivo and in-vitro experiments, it soon became apparent that whereas the glycine-2-C was utilized for porphyrin synthesis (6,307,352,482), the glycine-1-C was not (161, 352). Furthermore, by degrading haemin synthesized from doubly labelled glycine ($N^{15}H_2C^{14}H_2COOH$), Radin (352) and Muir (307) were able to show that for every nitrogen atom utilized, 2 carbon atoms from the glycine-2-carbon entered the porphyrin molecule. Since glycine is the precursor of all 4 nitrogen atoms of the porphyrin nucleus, 8 carbons must originate from the glycine-2-C. By using glycine-2-C^{14} as substrate, the location of these 8 carbons was established, 4 in the methene bridges of the porphyrin (307,482), and 4 in carbon 2 of each pyrrole nucleus (482).

The source of the remaining 26 carbon atoms of protoporphyrin had yet to be determined. The first clue came from Bloch and Rittenberg (31) who, in 1945, had observed the incorporation of deuterium into haem synthesized from deuterioacetic acid (CD_3 COOH). Muir (307) and

Shemin (415), using carboxyl and methyl-labelled acetate, found activity in the remaining 26 carbons, the methyl carbon of acetate being more efficiently incorporated than the carboxyl carbon. Absence of activity in the methene bridges provided negative confirmation of the role of the glycine-2-C. By degrading the porphyrin nucleus, and noting the pattern of radioactivity within each pyrrole ring, Shemin (415) concluded that an unsymmetrical 4-carbon compound, an intermediate in the α ketoglutarate-succinate reaction of the tri-carboxylic acid cycle (possibly a succinyl-coenzyme A complex) was the likely precursor of those carbon atoms not derived from the glycine-2-C. Support for this concept was obtained from the demonstration by Shemin and Rumin (416) and Wriston et al (483,484) that haem synthesized from various isotopic forms of succinate, α ketoglutarate, and citrate, was labelled in the manner predicted. Further, by inhibiting the tricarboxylic acid cycle with malonate, it was evident that "active" succinate could arise not only from α ketoglutarate, but from succinic acid as well, the latter reaction being reversible (416).

It had now been established that the first step in the biosynthesis of porphyrins was the condensation of glycine with "active" succinate (probably succinyl coenzyme A), the latter dependent upon a functioning

tricarboxylic cycle for its generation.

B. THE ENZYMATIC SYNTHESIS OF δ AMINOLAEVULINIC ACID.

The first step of porphyrin biosynthesis, the condensation of glycine and "active" succinate to form ALA, is catalysed by the enzyme, ALA synthetase. This reaction and the nature of the enzyme involved, have been extensively studied in avian erythrocytes (43,125, 144,251), in micro-organisms (51,123,124,127,220,221,222, 246,422) and in mammalian liver mitochondrial preparations (130,131,148,445). Many details of the enzymatic synthesis of ALA have been elucidated, but before these are discussed, the in-vitro systems of study will be briefly reviewed.

(1) In-vitro Systems.

(a) Avian Erythrocyte Systems.

Shemin (414), in 1948, reported his findings that nucleated avian red cells could synthesize haem from glycine in-vitro. In 1954, Shemin, Abramsky and Russel (418) showed that while intact or gently haemolysed duck erythrocytes could synthesise protoporphyrin from glycine and succinate, on homogenization, this did not occur. However, homogenates and soluble cell-free extracts were still able to synthesize porphyrins and haem from ALA.

It had already been established by Shemin (420,421) that in-vitro, ALA was a much more efficient precursor

of haem than glycine and that the labelling of the protoporphyrin molecule by ALA-5-C^{14} was identical with that by glycine-2-C^{14}. These and isotope-dilution experiments pointed to this 5-carbon aminoketone as being the intermediate formed by the condensation of glycine and succinate. In the available in-vitro systems, however, evidence was all inferential as the little ALA formed was so rapidly metabolized that it could not be measured.

In 1958, Lever et al (251) and Brown (43) developed a system whereby the ALA synthesized from glycine and tricarboxylic acid intermediates, was not metabolised further.

Lever, Neuberger and Udenfriend (251) centrifuged the haemolysates of red cells of chickens, made anaemic by the injection phenylhydrazine, at 3,500g for 30 minutes and were able to demonstrate that washed particles derived from the centrifugate could synthesize ALA from glycine and succinate or α-ketoglutarate. Gibson, Lever and Neuberger (125) freeze-dried similarly prepared particles for their experiments and Brown (43) observed enzyme activity in the homogenized centrifugate of lysed red cells of normal chicks having centrifuged the haemolysate at 1,800g for 15 minutes. ALA, readily formed, was not metabolised further by any of these systems and could be

measured directly, thus permitting the study of its synthesis, in more detail.

(b) Micro-organisms.

In 1956, Lascelles (243,244) demonstrated that in Rhodopseudomonas spheroides, not only does porphyrin biosynthesis proceed along the same lines as in other cells, but under particular growth conditions, intermediates of porphyrin synthesis accumulate in the medium.

In 1958, Gibson (123) and Kikuchi, Shemin et al (220,221,422) prepared particle-free extracts of Rhodopseudomonas spheroides and Rhodospirillum rubrum by centrifugation at 105,000g for 1-3 hours and showed that these extracts were capable of synthesizing ALA from glycine and succinate in the presence of certain co-factors. Kikuchi et al (220) demonstrated that whereas extracts of Rhodopseudomonas spheroides containing very small particles (centrifuged for 30 minutes) synthesized a fair amount porphyrin from the formed ALA, little synthesis of porphyrins occurred in particle-free extracts - possibly due to the inactivation of ALA dehydrase. A year later, Kikuchi et al (222) purified ALA-synthetase 60-80 fold from Rhodopseudomonas spheroides, and, by kinetic analysis, the same authors put forward the sequence of events by which this enzyme catalyses the condensation of succinate and glycine.

Thus another system was developed whereby the enzymatic synthesis of ALA could be studied.

(c) Mammalian Liver Mitochondria Preparations.

Liver mitochondria from dicarbethoxy-dihydrocollidine (DDC) treated guinea pigs (148) and from rats rendered porphyric with DDC (130,131), allyl-isopropyl-acetamide ALA (131,302) and hexachloro-benzene (HCB) (302) all show striking increases in ALA synthetase activity when compared with similar preparations from normal animals. Electron microscopic studies in guinea pig preparations (148) indicate that in the first few days of poisoning, mitochondria get wider and their cristae increase in area. Whether this relates to the increased enzyme activity observed, is not known. Granick and Urata (148) have further demonstrated that in DDC treated guinea pigs, fresh mitochondrial preparations synthesize ALA at a rate comparable with that observed by Gibson, Laver and Neuberger (125) with particles from chicken reticulocytes.

(2) Substrates.

(a) Succinyl Coenzyme A.

Pioneer work by Shemin and Wittenberg (415), Shemin and Rumin (416) and Wriston, Lack and Shemin (483 and 484) had already established the importance of a functioning tricarboxylic acid cycle in porphyrin biosynthesis. The unsymmetrical 4-C unit derived from

the cycle, was thought by these authors to be an active form of succinate, possibly succinyl CoA. The more recent studies by Gibson, Laver and Neuberger (125), Kikuchi, Shemin et al (220,221), Granick and Urata (148) and Brown (43), have provided conclusive evidence that succinyl-coenzyme A is the tricarboxylic acid cycle intermediate that condenses with glycine.

Two reactions in this cycle generate succinyl coenzyme A (43,148,416). One is the oxidative decarboxylation of αketoglutaric acid; the other is the activation of succinate by a succinyl coenzyme A synthetase, succinate thiokinase. For this latter reaction, Kikuchi et al (220) and Brown (43) have demonstrated the need for coenzyme A and ATP. In addition, magnesium ions (220) potentiate the activation while pyridoxal phosphate has no effect. Both authors observed that, not only could succinyl coenzyme A replace succinate, ATP and coenzyme A as a substrate, but it was in addition a far more efficient precursor of ALA. Gibson et al (125) and Granick and Urata (148) also reported the synthesis of ALA from succinyl coenzyme A.

The second source, from αketoglutarate (43,125,148, 251,359,372) is catalyzed by αketoglutaric acid decarboxylase. This reaction involves NAD (125,359), thiamine, lipoic acid (43,245,359) and coenzyme A. Arsenite (144) is thought to inhibit this reaction by combining with lipoic acid.

In an avian red cell system, however, Brown (43) showed that ALA could be synthesized in decreasing order of effectiveness from isocitrate, citrate, α ketoglutarate, succinate, oxaloacetate, malate and fumarate. Further, high concentration of α ketoglutarate inhibited the reaction, probably interfering with the condensation of succinyl coenzyme A and glycine. The efficacy of isocitrate and citrate could possibly be related to the slow generation of α ketoglutarate at optimal concentrations. Brown also demonstrated the inhibition of ALA synthesis from citrate by adding dicarboxylic acid intermediates of the cycle. This, he inferred, interfered with the oxidative decarboxylation of α ketoglutarate.

It can be seen how varying concentrations of tricarboxylic acid cycle intermediates can influence the synthesis of ALA in-vitro. Their importance in regulating porphyrin synthesis in-vivo, is speculative.

The use in particular, of malonate, (416), parapyruvate and fluoricitrate (43) and arsenite (148), substances which inhibit specific reactions in the citric acid cycle, has provided confirmatory evidence that in avian red cells and mitochondrial systems, succinyl coenzyme A is derived from succinate or α ketoglutarate, the latter source probably being the more important one.

(b) Glycine.

(i) Pyridoxalphosphate.

The importance of pyridoxal phosphate in porphyrin synthesis is well established. Studies in animal nutrition have shown that in the deficiency of this vitamin, defective haemopoiesis results in a hypochromic anaemia in pigs, dogs and rats (61,105,225). Cartwright and Wintrobe (59,477) found that the small pale red cells of pyridoxin-deficient pigs were lacking both haem and free protoporphyrin, findings which suggested impaired synthesis of protoporphyrin.

Shulman and Richert, in 1957 (395,396) demonstrated quite clearly that the rate of haem synthesis from glycine-2-c^{14} or succinate-C^{14} by red cells or haemolysates of B deficient ducklings was much lower than normal controls. Yet haem synthesis from ALA 2-3-C^{14} was normal, indicating that the succinate-glycine condensation was the one affected. Addition of pyridoxal-5-phosphate or pyridoxamine phosphate (but not pyridoxal, pyridoxamine or pyridoxine) to the in-vitro system stimulated the B deficient cells to synthesize haem from glycine and succinate but did not stimulate haem synthesis by normal cells or from ALA.

In 1957, Lascelles (245) noted that when media had growth limiting amounts of pyridoxal, synthesis of porphyrins by cell-suspensions of Tetrahymena vorax(a protozoon) was reduced to 30% that of the controls.

Impairment of ALA synthesis on witholding pyridoxal phosphate, varies with the in-vitro system used - 90 - 95% in bacterial systems (221), 70-80% in liver mitochondrial preparations (148) and 20-40% in avian red cell systems (43,125,251). Studying the co-factor requirements in these systems indicates that pyridoxal phosphate is involved in the activation of glycine prior to its condensation with succinyl coenzyme A.

Finally, compounds such as L penicillamine, L cysteine and cyanide, known to inhibit reactions catalysed by pyridoxal-phosphate-containing enzymes, have been shown to inhibit the synthesis of ALA (43,148,251). The substances react with the aldehyde group of the coenzyme thus preventing the formation of an aldimine (see below) (126).

(ii) Activation of Glycine.

Available evidence suggests that the amino group of glycine reacts with pyridoxal phosphate (attached to the enzyme protein) to form an aldimine or Schiff's base (126). This facilitates the loss of a proton from the carbon of glycine leading to the formation of a carbanion. The glycine-2-C thus has a high electron density and will react readily, with electrophilic carbonyl carbon of succinyl coenzyme A (126,221). The sequence of events in the activation of glycine and its subsequent condensation with succinyl coenzyme A to form ALA has been set out by Kikuchi et al (222).

(3) α Amino β Ketoadipic Acid.

The primary condensation product of succinyl coenzyme A and glycine is probably α amino β ketoadipic acid (320,417,419,420,421) but spontaneous decarboxylation with the formation of ALA occurs so rapidly that this compound has not been isolated. However, if the diethylester of α amino β ketoadipic acid is injected, a rise occurs in the urinary excretion of porphobilinogen (419,420). Kinetic studies by Kikuchi (222) probably indicate that it is still linked to the pyridoxal-phosphate-ALA synthetase complex when decarboxylation occurs. That the glycine-1-C is lost after and not before condensation with succinyl coenzyme A, is conlusively shown by the glycine-1-C^{14} experiments of Kikuchi et al (221).

All enzymes known to contain biotin catalyse reaction s in which carbon dioxide is activated or transferred (124, 127). Gibson et al (124,127) demonstrated that when Rhodopseudomonas spheroides was cultured in biotin-deficient media, both their growth was impaired and their ALA and porphyrin production was reduced. For the reasons mentioned by these authors (124,126,127) it was concluded that the role of biotin in the decarboxylation of α amino β ketoadipic acid was not important.

(4) Inhibitors of ALA Synthesis.

The complexity of ALA synthesis allows inhibitors to act at a variety of sites. In the course of describing ALA synthesis, many inhibitors have been mentioned and their biological implications discussed. These may be important biologically as ALA synthesis is generally thought to be the rate controlling reaction of porphyrin biosynthesis. Inhibitors of this reaction include:-

(a) Inhibitors of the tricarboxylic acid cycle, e.g. arsenite, malonate.

(b) Dicarboxylic acid intermediates of this cycle.

(c) Agents combining with pyridoxal phosphate thus preventing the activation of glycine e.g. L penicillamine, L cysteine and cyanide.

(d) Inhibition by iodacetamide and chloromercuribenzoate, thus indicating the need for coenzyme A (125).

(e) Certain amino acids inhibit ALA synthesis. Granick (144) thought that this might biologically regulate porphyrin synthesis. In erythropoiesis, during rapid globin synthesis, amino acid level might fall, thus promoting rapid ALA synthesis. Thus the formation of haem would keep pace with the synthesis of globin.

(f) Lead is known to impair haemopoiesis and Goldberg et al suggested, from their studies on the incorporation of Fe^{59} into haem, that lead acts at at least two sites,

impairing both the synthesis of ALA (134) and the incorporation of iron into the protoporphyrin molecule (95,134,210).

Dressel and Falk (85,86) using intact and haemolysed chicken enthrocyte systems, demonstrated that addition of lead acetate resulted in an almost total inhibition of haem synthesis from glycine, considerable inhibition of porphyrin formation from ALA and scarcely any inhibition when PBG was used as the substrate. They concluded that the main site of action was on ALA synthesis, although ALA dehydrase activity was also impaired. However, conversion of PBG to haem was efficient, no apparent difficulty in the incorporation of iron to protoporphyrin being noted in this in-vitro system. The findings were compatible with the known anaemia of lead poisoning but could not account for the excessive excretion of porphyrins which accompanies this condition(86).

Vannotti (450) discussed the observation that in lead intoxication, although haemoglobin levels fall, the concentration of cytochrome C can increase in the tissues. This indicates that, in-vivo, many factors affecting haem turnover of different haem-proteins are at play. Thus, while lead can inhibit porphyrin synthesis in the erythroblast and induce anaemia, the latter may, in turn, provoke an adaptive increase in the turnover of other

respiratory haem, proteins, eg. cytochrome C.

Rubino (367) presented his findings that in lead poisoning, both in bone marrow and in circulating erythrocytes, while all porphyrin intermediates are in excess of normal, the levels of free protoporphyrin are disproportionately increased, evidence favouring an impairment of haem synthesis from protoporphyrin in-vivo in humans.

(g) Iron most certainly can inhibit ALA and haem synthesis in-vitro. However, its mode of action is complex and it can stimulate ALA synthesis in certain circumstances. The influence of iron on the formation of ALA will be discussed in detail in the following section.

(B) Iron - Influence on Ala Synthesis.

The influence of ferrous iron on porphyrin, haem and bacteriochlorophyl synthesis is well documented. However, its site and mode of action is not clear. As will be shown later, the enzymatic incorporation of ferrous iron into protoporphyrin is the last stage in the synthesis of haem. The present discussion will be centred on how the ferrous iron affects the first stage, the synthesis of ALA.

Pappenheimer (336) in 1947, showed that the synthesis of porphyrin in Corynebacterium diphtheriae was stimulated by ferrous sulphate in concentrations up to 0.1 mg/litre of culture medium, although concentrations in excess of this

were inhibitory.

In 1956, Lascelles (244) studied the effect of iron on pophyrin, haem and bacteriochlorophyll synthesis in cell suspensions of Rhodopseudomonas spheroides. In induced iron deficiency, porphyrins accumulated in the medium (244,247). However, the addition of small amounts of ferrous iron almost completely suppressed porphyrin formation from glycine and α ketoglutarate and, although there were increases in bacteriochlorophyll and haem components, these could only account for 10% and 1% respectively of the porphyrins which failed to appear. In other words, diversion of porphyrins to form iron-porphyrin complexes was only a small factor and iron, therefore, in some other way inhibited porphyrin synthesis. The observation that iron did not affect porphyrin synthesis from ALA pointed to impairment of ALA synthesis as the basis of the inhibition.

Burnham and Lascelles (51) noted that adding haem to a suspension of Rhodopseudomonas spheroides markedly inhibited porphyrin synthesis from glycine and α ketoglutarate, but not from ALA. They postulated that control of porphyrin synthesis could be explained on a negative feed-back mechanism whereby, with the addition of iron, the synthesis of a haem-compound was promoted which in turn controlled the activity of that enzyme

catalysing the first step of the biosynthetic pathway.

The next logical step was to study the effects of haem and other metal-protoporphyrin complexes on ALA synthetase activity. It was shown that haem or haemin markedly inhibited enzyme activity and that free protoporphyrin, copper or magnesium protoporphyrin also did this, but in very much higher concentrations. While haemoglobin and myoglobin also inhibited ALA synthetase activity, catalase and cytochrome C did not. Inhibition was not reversed by the addition of succinyl coenzyme A, glycine or pyridoxal phosphate. These findings supported the negative feed-back concept (51).

It was also shown that ferrous iron itself was a potent inhibitor of ALA synthesis while the ferric form at the same concentration, had no effect (51). However, this inhibition was reversed by adding pyridoxal phosphate or o-phenanthroline. Whether the ferrous iron was bound with pyridoxal phosphate preventing its activation of glycine or whether pyridoxal phosphate simply chelated the iron preventing it acting elsewhere, is not certain.

Brown (44) studied the effect of ferrous iron in a chicken red cell particulate system. He confirmed that the addition of ferrous iron inhibited ALA synthesis by a fresh red cell preparation. However, in an aged preparation (48 to 72 hours) where ALA synthesis had already

fallen, addition of ferrous sulphate more than doubled the amount of ALA formed. He implied that the ferrous iron in the fresh system enhanced ALA synthesis but on ageing, it was progressively oxidised to the inactive ferric form, hence the stimulatory effect of adding ferrous iron at this stage. Ascorbic acid, presumably by its reducing action, also stimulated aged preparations.

Further evidence for the role of ferrous iron was obtained by the demonstration that α,α' dipyridyl, a potent chelating agent for ferrous iron, inhibited the synthesis of ALA. This could be reversed by adding ferrous sulphate. (44).

With the aid of a hydroxylamine-succinohydroxamic acid system, the synthesis of ALA and succinyl coenzyme A was studied in a 48 hour old preparation. While succinyl coenzyme A synthesis was unaffected by the addition of ferrous iron, ALA synthesis was increased almost twofold. The inhibition of ALA formation by 5:6 dimethyl benzimidazole, thought to interfere with the activation of glycine, is consistently diminished by ferrous iron. These two observations lead Brown (44) to the conclusion that ferrous iron may act by stabilising the pyridoxal phosphate-glycine complex by chelation.

Patwardhan (338) also showed that the ferrous iron was necessary in the pyridoxal phosphate dependent aspartic-glutamic transaminase reaction.

It therefore appears that iron, in its reduced form and in optimal concentration, is essential for ALA synthesis, probably in the activation of glycine. In higher concentrations however, it clearly inhibits the reaction. Biologically, ferrous iron by promoting the formation of haem, may be important in regulating ALA (and therefore porphyrin) synthesis by a negative feed-back mechanism.

(6) Specificity of ALA Synthetase.

Gibson (125) showed that when glycine was incubated with ALA synthetase (avian red cell system) in the presence of acetyl coenzyme A, aminoacetone (A.A.) was formed although its synthesis was slower than that of ALA. Other aminoketones were formed from propionyl and glutaryl-coenzyme A but these reactions were extremely sluggish. Granick and Urata (148) found a number of differences between the synthesis by liver mitochondria of ALA and aminoacetone from glycine and the respective acylcoenzyme A. They made the following observations:-

(a) Normal mitochondria, although they had a good activity for A.A. synthesis, barely synthesised ALA.

(b) Pyridoxal-phosphate inhibitors inhibited the synthesis of ALA more than that of AA.

(c) The addition of pyridoxal-phosphate stimulated the formation of ALA more than that of AA indicating a greater dissociation of pyridoxal-phosphate from

ALA synthetase.

(d) Other inhibitors and stimulators affected the syntheses of the two compounds differently.

These authors therefore inferred that in liver mitochondria at least two specific aminoketone synthetases are present.

(7) Recapitulation.

An enzyme, ALA synthetase, which needs pyridoxal phosphate as a co-factor, synthesizes the condensation of succinyl coenzyme A with glycine to form α amino β ketoadipic acid which decarboxylates spontaneously to form ALA. This reaction occurs in mitochondria (144, 148,374) where a functioning tricarboxylic acid cyle and, therefore, aerobic conditions are necessary for the generation of succinyl coenzyme A. The condensing enzyme system can operate under anaerobic conditions.

This reaction is thought to be the rate controlling reaction in porphyrin biosynthesis in the mammalian liver (148,445).

C. METABOLIC FATE OF δ AMINOLAEVULINIC ACID.

The metabolism of ALA has been extensively studied in humans, animals and a variety of in-vitro systems. It appears that ALA can be metabolized along two pathways:

(1) as an obligatory intermediate in the biosynthesis of porphyrin and haem, and

(2) in the succinate-glycine cycle, a series of reactions whereby succinyl-coenzyme A and glycine condense to form ALA, whose carbon is utilized for incorporation as a "one-carbon-fragment" following the removal of the amino group and the formation of α Ketoglutaralde.

(1) ALA in Porphyrin Biosynthesis.

It is now generally accepted that ALA is an obligatory intermediate in the biosynthesis of porphyrins and haem. It is probably also the precursor of the dehydroporphyrin of chlorophyll, the tetrahydroporphyrin of bacteriochlorophyll (126) and the porphyrin-like moiety of vitamin B_{12} (66).

ALA can replace glycine and succinate as substrates in porphyrin synthesis (24,25,83,84,320,417,418,419,420,421). In duck red cell systems, Shemin et al have demonstrated that haem synthesized from ALA-5-C^{14} was about 45-65 times more active than that formed from equimolar amounts of glycine-2-C^{14} of the same specific activity. In-vivo,

ALA is not nearly as efficient a precursor of erythrocyte haem. In ducks, Nemeth, Russell and Shemin (319) showed that the activity of circulating haem after the intraperitoneal injection of ALA-5-C^{14} was only twice that of haem synthesised from an equivalent amount of glycine-2-C^{14}. Neuberger and Scott (32) observed that after oral or intraperitoneal administration, rats incorporated labelled glycine five times more efficiently than ALA into red cell haem. Scott (402), Berlin, Neuberger and Scott (24,25), more recently Israels and his group (205, 207,498) and Dowdle (78) have consistently shown the poor incorporation of ALA-C^{14} into circulating haem in humans, after oral or intravenous administration. By contrast, after the intravenous injection of glycine-2-C^{14} to humans (76,77,205,207,498, this thesis) and dogs (206) red cell haem is readily labelled.

Scott (402) and Berlin, Neuberger and Scott (24,25) isolated active protoporphyrin in the stools of humans fed ALA-N^{15}, ALA-4-C^{14} and ALA-2-4-C^{14} while Dowdle (78) similarly detected high faecal porphyrin activity in humans who had ingested ALA-5-C^{14}. Scott (402) also observed that rats, whose bile ducts had been cannulated, rapidly excreted protoporphyrin in the bile following the administration of ALA by stomach tube. Stool stercobilin activity was also very much higher than that observed in

circulating haem (24,25,78,402-).

In their studies on early-labelled bilirubin, Israels et al (205,207,498), Dowdle (78) and Ibrahim et al (201,202,400) have shown that the activity detected in that bilirubin fraction thought to result from the catabolism of liver haem, is far higher when ALA-C^{14} is the precursor (oral or I.V.) than when glycine-2-C^{14} is administered intravenously. Schwartz, Ibrahim and Watson (400) using bile fistula dogs, have shown that the total activity excreted in this "non-erythropoietic" bilirubin fraction after administering ALA-C^{14} is about 500 to 900 times more than that following glycine-2-C^{14}. However, the fraction of bilirubin thought to be derived from ineffective erythropoiesis, is only labelled by glycine-2-C^{14} (78,205,207,498) (this thesis).

Thus ALA appears a more efficient porphyrin precursor than glycine, both in-vitro and in-vivo. Its failure as red cell haem precursor in mammals is almost certainly related to its failure to penetrate haemopoietic cells.

In duck red cell in-vitro systems, Shemin et al has shown that ALA serves as the source of all the nitrogen and carbon atoms of the porphyrin moiety. ALA-5-C^{14} labelled the same carbon atoms of the porphyrin molecule as

glycine-2-C^{14} (418,421) while the distribution of activity in protoporphyrin synthesized from ALA-1-4-C^{14} was identical to that synthesized from succinate 1-4-C^{14} (380,419).

The radioactivity of haem obtained from either C^{14}- labelled succinate or glycine-2-C^{14} in a red cell system, was reduced by 80-90% by the addition of any equimolar amount of unlabelled ALA to the system (84, 417,421). When N^{15} - labelled ALA was added, the excellent simultaneous incorporation of N^{15} into haem indicated that the observed effects were due to dilution of the newly formed ALA-C^{14} rather than inhibition of its synthesis (421). These findings further established the role of ALA as an obligatory intermediate in the biosynthesis of haem.

(2) <u>The Succinate-Glycine Cycle.</u>

The succinate-glycine cycle, first put forward by Shemin and his group (419,420,421) is a series of reactions whereby, following the condensation of succinyl coenzyme A and glycine, the carboxyl carbon of glycine is metabolised to CO_2 and the α carbon is utilised as a C_1 fragment in the synthesis of the ureido carbons of purines (217,319) (this thesis), βcarbon of serine (9,10,370,476), methyl groups (8,471) and formate (319). In the process, succinate is regenerated. Thus the cycle is interrelated with the

tricarboxylic acid cycle with which it is similar in that both cycles are concerned with the metabolism of a 2-carbon compound and intermediates of both are utilized for synthesis of other compounds.

The proposed cyclic pathway involves the following reactions:-

(a) Condensation of succinyl coenzyme A and glycine to form α amino β ketoadipic acid.

(b) Decarboxylation of the latter to form ALA. In this reaction, the glycine-1-C (carboxyl) is lost as CO_2 and the glycine-2-C becomes the δ carbon of ALA.

(c) The trans- or deamination of ALA to form α ketoglutaraldehyde (γ, δ dioxovaleric acid).

(d) Utilisation of the aldehyde carbon (derived from the glycine-2-C and therefore the ALA-5-C) as a one-carbon fragment.

(e) The re-establishment of succinate from the four carbon residue.

The importance of this cycle in haem-purine relationships is self-evident. ALA has been shown to be an obligatory precursor of haem. Not only does the succinate-glycine cycle provide an alternative metabolic pathway for ALA, but this pathway maybe a significant if not the main source of those "one-carbon fragments" incorporated into carbons in position 2 and 8 (the

ureidocarbons) of the purine nucleus. Evidence for the existence of this cycle and the extent to which it operates, will therefore be considered in detail.

(a) Reactions of Cycle.

(i) Synthesis of ALA.

The first two reactions of the cycle, the condensation of succinyl coenzyme A and glycine to form ALA and CO_2, have already been discussed in detail.

(ii) Transamination of ALA.

The trans- or deamination of ALA in-vivo, would lead to the incorporation of the ALA nitrogen into aminoacids, urea, ammonia, etc. As the deamination of the aminomethyl group of PBG in the synthesis of uroporphyrin would have the same effect, the observations by Berlin et al (24) on the labelling of nitrogenous compounds by ALA-N^{15}, do not necessarily provide evidence for the occurrence of this step in-vivo.

The product of the oxidative deamination or transamination of ALA would be α ketoglutaraldehyde (γ, δ dioxovaleric acid). The specific transaminase catalysing this reaction has been isolated in corynebacterium diphtheriae by Bagdasarian (12), in Rhodopseudomonas spheroides by Neuberger and Turner (322) and in mammalian tissues by Kowalski et al

(228,229). The enzyme has been named ALA transaminase (12) or γ,δ dioxovalerate (α ketoglutaraldehyde) transaminase (322) according to the direction of the reaction catalysed in the particular system.

Bagdasarian (12) showed that cell-free extracts of Corynebacterium diphtheriae were able to catalyse the transamination of ALA with α ketoglutarate or pyruvic acid as the amino-acceptors, glutamine and alanine being formed respectively.

Neuberger and Turner (322) purified the transaminase twenty-five fold from extracts of Rhodopseudomonas spheroides and found that in their system, the transaminase had the following properties:-

(a) Equilibrium favoured production of ALA from α ketoglutaraldehyde.

(b) The most efficient amino-group donor was alanine although other aminoacids e.g. δ aminovaleric acid were effective with the purified enzyme. Serine, glutamine, glutamic acid, taurine, asparagine, aspartate, lysine and cysteine were all neglible as amino-donors.

(c) The enzyme was found to be a sulphydryl protein inhibited by iodoacetate etc.

(d) Common to other transaminases, pyridoxal phosphate was a necessary coenzyme. In this instance, it was tightly bound to the enzyme and

adding extra pyridoxal phosphate did not enhance the reaction. As with the activation of glycine, inhibition was noted with such compounds as penicillamine, homocysteine, cysteine, etc.

(e) Inhibition was also effected by a wide variety of α ketocarboxylic acids, ketoaldehydes, dicarboxylic acids and other dioxocompounds. The following compounds inhibited, by the percentage of control activity shown in parenthesis, when pre-incubated with the enzyme at a concentration equimolar with the substrate, α ketoglutaraldehyde: methylglocal (91%), glyoxylic acid (86%), pyruvic acid (56%) oxaloacetic acid (21%) and α ketoglutaric acid, only 5%. The measurement of transaminase activity, using ALA as the aminogroup donor and pyruvic acid as the acceptor, was hindered by the strong inhibitory effect of the latter.

Thus a transaminase capable of transferring the amino-group of ALA with the production of α ketoglutaraldehyde has been isolated in micro-organisms and mammalian tissue. Too much cannot be inferred from these in-vitro experiments. For example, the findings of Neuberger et al (332) implied that the

equilibrium favoured the production of ALA from α ketoglutaraldehyde. One knows that this is not the way ALA is synthesized in-vivo and Nemeth et al (319) showed conclusively in the intact pigeon and in a duck red cell system, that no active haem was formed from αketoglutaraldehyde-5-C^{14}.

(iii) Metabolic fate of αketoglutaraldehyde.

Relevant to studies on the succinate-glycine cycle, αketoglutataldehyde can be metabolized in the following ways:-

(a) The terminal aldehyde group (αketoglutaraldehyde-5-C) can be metabolized as an active "one-carbon" fragment.

(b) The aldehyde can be oxidized to a carboxyl group with the formation of α ketoglutaric acid. This can enter the citric acid cycle where, following oxidative decarboxylation, the original aldehyde carbon is lost as carbon dioxide.

(c) Following (a) or (b), the 4 carbon residue can re-enter the citric acid cycle as succinate and be metabolized accordingly.

Inferential evidence of a functioning succinate-glycine cycle would therefore depend on studying "one carbon" atom metabolism, carbon dioxide production and regeneration of succinate following the administra-

tion of such labelled precursors as glycine-2-C^{14}, ALA-C^{14} and α ketoglutaraldehyde-5-C^{14}. However, glycine-2-C^{14} or ALA-C^{14} could label CO_2 or C_1 fragments via several possible metabolic pathways, and these should be briefly considered before interpreting the experimental data at our disposal.

(b) Isotopic labelling of CO_2 and C_1 fragments by Glycine-2-C^{14} or ALA-C^{14}.

Glycine-2-C^{14}.

Several known pathways can account for C^{14} labelling of CO_2 and C_1 fragments by the α carbon of glycine.

(i) The succinate-glycine cycle provides a route whereby the δ carbon of ALA, derived from the glycine-2-C, can be metabolized to CO_2 or utilized as a "one-carbon fragment".

(ii) Glycine and acetyl coenzyme A can condense to form aminoacetoacetic acid, which after spontaneous decarboxylation, yields aminoacetone (91,447,448). After deamination to methylglyoxal, the terminal aldehyde group derived from the glycine-2-C, can be a source of CO_2 or C_1 fragments.

(iii) Glycine can undergo oxidative deamination to form glyoxylic acid. Both liver and kidney are rich in glycine oxidase, the specific flavoprotein enzyme catalysing this reaction. Glyoxylic acid may combine with glutamic acid, and by this route, its carbon may

combine with glutamic acid, and by this route, its carbon may be utilized as a "one-carbon" fragment, or be oxidized to formate (314). Formate can also be the result of oxidative decarboxylation of the glyoxylic acid. By whatever route formate is formed, this may be further utilized as a C_1 fragment or be oxidized to carbon dioxide, catalase being important for the latter. These reactions have been extensively studied in rat liver preparations by Nakada et al (312,313,314), Weinhouse et al (468,469,470) and Dowdle et al (75).

There is another way in which the glycine-2-C can be oxidized to CO_2. Serine, formed from glycine can be further metabolized to pyruvate, or following its decarboxylation to ethanolamine (174) can form glycine. In the latter cycle, the original glycine-2-C becomes the glycine-1-C (carboxyl carbon) which is readily lost as CO_2. This cycle has been considered in detail in Chapter IV, pages 219-222.

ALA-C^{14}.

ALA-1-C^{14} can label CO_2 in two ways. Firstly, in the stepwise decarboxylation of uroporphyrinogen to protoporphyrinogen, $C^{14}O_2$ will be liberated. Secondly, after its participation in the succinate-glycine cycle, succinate-1-C^{14} will be formed which on being metabolized further, will yield isotopic CO_2.

ALA-2, 3 or 4-C^{14} takes no part in the synthesis of the carboxyl groups of the porphyrin molecule and therefore, any $C^{14}O_2$, obtained, will reflect the metabolism of that succinate 2, 3 or 4-C^{14} derived from the succinate-glycine cycle. The quantitity of $C^{14}O_2$ evolved will bear no strict relationship to the amount of succinate re-entering the citric acid cycle as intermediates of the cycle are continuously being utilised in the synthesis of other compounds such as amino-acids, ALA etc. However, any ALA-1-C^{14}, newly synthesised from succinyl coenzyme A (derived either directly from succinate-4-C^{14} or from recycling of succinate 2-3-C^{14}, will yield labelled CO_2 by the routes described in paragraph 1. Thus, while the production of isotopic CO_2 from ALA 2,3 or 4 C^{14} reflects a functioning succinate-glycine cycle, the degree of labelling cannot be used as a measure of the magnitude of this alternative metabolic pathway of ALA.

From our present understanding of porphyrin biosynthesis, it is highly unlikely that ALA-5-C^{14} will lable CO_2 or C_1 fragments during its incorporation into haem. However, in 1955, Schmid and Shemin (cited in ref. 421) observed the formation of isotopic formaldehyde during the enzymatic or chemical synthesis of uroporphyrinogen from porphobilinogen whose aminomethyl group was labelled with C^{14}. This potential source

of CO_2 or "one-carbon units" from ALA-5-C^{14} will be considered in detail later this chapter in that section dealing with the synthesis of uroporphyrinogen. It is sufficient at present to mention that more recent work by Bogorad (37), Granick and Mauzerall (145) and Lockwood and Benson (270) virtually preclude the loss of any methene-bridge carbons at this stage in porphyrin biosynthesis. Thus it can be concluded that any labelled CO_2 or "one-carbon fragments" derived from ALA-5-C^{14} during the synthesis of haem, arise from the alternative pathway by which ALA is metabolized. ALA-5-C^{14} thus provides the most efficient and direct means of not only confirming the existence of a functioning succinate-glycine cycle, but also of measuring its activity.

The validity of the above arguments depends on whether significant amounts of carbon dioxide are evolved in the catabolism of haem-C^{14} to bilirubin-C^{14} or in possible further degradation of the radioactive bile pigment before its excretion. In the cleavage of the protoporphyrin ring to form biliverdin, the methene bridge (derived from glycine-2-C or ALA-5-C) is thought to be oxidized to carbon monoxide and split off from the protoporphyrin ring in this form (146). The amount of carbon monoxide exhaled daily by humans is equivalent to that which would be derived from the

normal breakdown of haemoglobin to bile pigment per day (94,423). However, other sources of this gas may exist and it is well established that 20-30% of bilirubin is not derived from the haem of circulating haemoglobin (151,207,273,498).

The haem of infused haemoglobin is nearly quantitatively passed in the bile as bilirubin (184) suggesting that in normal subjects, any degradation of bile pigment before its excretion is minimal. Humans suffering from the Crigler-Najjar syndrome and Gunn rats do not possess the enzymes necessary for the conjugation and excretion of bilirubin via the liver (11,386). However, both in the human and murine syndromes, the degree of hyperbilirubinaemia remains constant for months or years. Hence alternative pathways of bile pigment metabolism must exist and from studies by Schmid with bilirubin-C^{14} (390) the major fraction of the isotope was excreted in metabolites other than bilirubin. It is unlikely that such alternative pathways are significant in normal subjects(390).

The only way of settling this question however, would be to administer appropriately labelled bilirubin-C^{14} intravenously to normal subjects and to measure the radioactivity of respiratory CO_2. However, from experimental data to be presented, it appears that $C^{14}O_2$ is

is maximally evolved during the synthesis rather than the breakdown of haem (25,78). Thus, the previously expounded arguments appear valid and any isotopic CO_2 evolved following the administration of ALA-5-C^{14} will only reflect the activity of a functioning succinate-glycine cycle.

(c) Experimental evidence for succinate-glycine cycle.

Nemeth, Russel and Shemin (319) showed that after the administration of ALA-5-C^{14} in-vivo to ducks, pigeons and rats, radioactive carbon dioxide was isolated and the C^{14} label appeared in the ureide carbons of purines and in formate. These findings confirmed the presence of an alternative route by which ALA could be metabolized.

On degrading the uric acid molecule excreted by pigeons, Nemeth et al (319) noted the pattern of radioactivity within the purine nucleus. After 72 hours, 48% of the activity resided in the ureide carbons 2 and 8 while 23% was found in C6. This carbon is derived from carbon dioxide and the radioactivity of C6 was found to be in the same order as that of respiratory CO_2. α ketoglutaraldehyde-5-C^{14} produced a similar labelling pattern, 52% of the activity present in C2 and 8 and 19% in C6, seventy two hours after administration. From these findings, Nemeth et al

concluded that both the ALA-5-C and the α ketoglutaraldehyde-5-C were metabolized along the same pathway. As the same authors could not demonstrate the synthesis of haem from α ketoglutaraldehyde-5-C^{14} in-vivo and in-vitro, it was concluded that ALA was first trans - or deaminated to α ketoglutaraldehyde before its carbon was detached.

Following the intraperitoneal injection of labelled precursors to rats, Nemeth et al (319) measured how much activity appeared in urinary formate and in respiratory CO_2 over 6 hours. It is not clear from Nemeth's table and text whether c.p.m. indicate total activity excreted or activity per millimole. The former is assumed but either interpretation would not influence discussion as only ratios are being compared, not absolute values. As the number of millimoles of formate or CO_2 recovered are roughly equal for the three precursors used in Nemeth's experiments, comparisons are valid. After injection of ALA-5-C^{14}, about 37 times more activity appeared in urinary formate than in respiratory CO_2. From previous discussion, one would anticipate that the aldehyde carbon of α ketoglutaraldehyde would be utilized for compounds other than haem in the same manner as the δ carbon of ALA. However, after the administration of α ketoglutaraldehyde-5-C^{14},

relatively more activity appeared in expired CO_2, and urinary formate was only about 3-5 times more active that the $C^{14}O_2$ recovered. These findings conflict with Nemeth's observations on the distribution of activity in uric acid synthesized by the pigeon from the same precursors. One cannot rule out experimental error and the above studies bear repeating.

Studies on the metabolism of the glycine-2-C to CO_2 or C_1 fragments provided interesting information. While glycine-2-C^{14} labelled formate to the same extent as ALA-5-C^{14} far more activity was recovered in respiratory CO_2 from glycine-2-C^{14}. This was anticipated in view of the many pathways available for the oxidation of the C of glycine to CO_2. However, after the intraperitoneal injection of ALA-5-C^{14} or glycine-2-C^{14} to ducks, both haemin and C2 of guanine isolated from red cells were about twice as active when synthesized from ALA-5-C^{14} than from glycine-2-C^{14}. This strongly suggests that ALA was an intermediate in the utilization of the glycine-2-C in this situation. Thus, in contrast

to the participation of other pathways in the production of CO_2 from the glycine-2-C, the succinate-glycine cycle appears to be the only one operative in the incorporation of the α carbon of glycine, as a C_1 fragment, into the purine nucleus.

Nemeth et al (319) also showed that, following the parenteral administration of ALA 1, 4-C^{14} and sodium malonate to rats, highly active succinic acid was recovered from the urine, all activity residing in the carboxyl groups. Thus, in the course of the metabolism of ALA in the rat, not only is haem formed, but some other pathway must be operative whereby the succinate moiety of ALA is regenerated.

Nemeth et al (319) have therefore demonstrated an alternative pathway for ALA, a route by which the δ carbon of ALA can be utilised as a "one-carbon fragment" or oxidized to CO_2, the succinate moiety of ALA being regenerated in the process. It is likely that the above changes follow the de - or transamination of ALA, as α ketoglutaraldehyde-5-C^{14} appears, in some ways at least, to label C_1 fragments (and possibly CO_2) in a pattern similar to that of ALA-5-C^{14}. Thus there is experimental evidence in support of the succinate-glycine cycle, as postulated by Shemin et al (419,420,421).

Scott (402) and Berlin, Neuberger and Scott (25)

studied the metabolism of ALA-1-4-C^{14} in humans and rats. Relevant to the succinate-glycine cycle, a number of interesting observations were made. In rats and man, about 15% of the dose administered was recovered as respiratory CO_2. Considerably more may have been produced and metabolized in the body e.g. in purine synthesis. As mentioned before, with ALA-1-4-C^{14}, $C^{14}O_2$ can arise both from the metabolism of the succinate moiety regenerated via the succinate-glycine cycle or from decarboxylation of the side chains of newly synthesized porphyrin. In the latter pathway, 6/16 of the labelled carbon entering the porphyrin nucleus will be lost as CO_2. If the latter pathway only was operative, at least 40% of the ALA administered would have to be converted to porphyrin to account for 15% of the dose being recovered as $C^{14}O_2$. The sum of the activity in the excreted tetrapyrroles and in circulating haem accounted for 4% of the dose given. However, 20-30% of the activity was retained in the body after 3 days and 25-35% was unaccounted for in urine and stool. Thus over 50% of the administered ALA could have been metabolized as porphyrin without having been detected by the techniques available at the time.

The timing of the $C^{14}O_2$ recovered gave the clue to its origin. Scott (402) showed that after the administration of ALA, PBG appeared in the urine before protoporphyrin was recovered from the bile of rats whose bile ducts had been

accumulated. Berlin et al (25) showed that the peak of pulmonary C^{14} activity preceded by one hour the peak of urinary PBG-C^{14}, i.e. $C^{14}O_2$ was formed, presumably before the synthesis of porphyrins. From these observations, the authors concluded that a significant if not the major portion of respiratory $C^{14}O_2$ recovered arose following the degradation of ALA-1-4-C in the succinate-glycine cycle.

Tschudy et al (443) showed that on incubating liver slices with ALA-4-C^{14}, $C^{14}O_2$ was obtained. Normal and porphyric liver slices showed the same ability to produce isotopic CO_2. On incubating 50μ moles ALA-4-C^{14} with 500 mg of rabbit liver for 3 hours, an average of 0.08 μ moles of $C^{14}O_2$ were recovered. Tschudy et al (443) do not indicate whether 50μ moles of ALA would be sufficient substrate for a whole liver but calculate that, at the same reaction rate, about 4.1μ moles of ALA would be converted by the whole liver of the rabbit to CO_2 i.e. a conversion rate of about 8% of the total ALA present. The only conclusions one can make from their observations is that some succinate must have been produced from ALA-4-C^{14} supporting the existence of a succinate-glycine cycle.

D. FURTHER STAGES IN THE SYNTHESIS OF PROTOPORPHYRIN.

The stages in the conversion of ALA to coproporphyrinogen will not be discussed in detail as this aspect of porphyrin biosynthesis is not directly concerned with the experimental work involved in this thesis.

In the biosynthesis of haem, only 2 reactions require aerobic conditions:

(a) formation of succinyl coenzyme A and

(b) the oxidative decarboxylation of coproporphyrinogen III to protoporphyrin III (9a). These reactions are catalysed by enzymes in mitochondria and such enzymes are therefore confined to the particulate fractions of cell extracts. However, the enzymes involved in the conversion of ALA to coproporphyrinogen are in the soluble part of the cell.

(1) Porphobilinogen.

(a) ALA dehydrase.

The condensation of 2 molecules of ALA to form PBG is catalysed by the enzyme ALA dehydrase (83,120, 121,122,141,384,419). The enzyme has been purified and its activity has been studied in a number of systems including duck erythrocytes (384), chicken erythrocytes (83,86,141,145). It is inhibited by lead (121) and by EDTA (121,145) the latter reversibly by magnesium

ion (145). Inhibition by EDTA suggests that a metal may be essential in this step. Iodice, Richert and Shulman (204) showed that the enzyme contained copper and that in deficiency of this metal, there is reduced ALA dehydrase activity in the livers of rats and the blood of ducklings. Anderson et al (7) noted similar findings in copper deficient ducks. Potassium may be required for its activation in bacterial systems (51). No loss of activity accompanies dialysis from which, Granick (141) concluded that there was no loosely bound coenzyme.

(b) Enzymatic synthesis of porphobilinogen.

Porphobilinogen was first crystallized from the urine of an acute porphyric by Westal (472) and its structure was formulated by Cookson (64). This conformed with the theoretical structure put forward by Shemin (417) and Neuberger (320).

That two molecules of ALA condense to form one molecule of porphobilinogen was demonstrated by Shemin (419) who showed that the molar activity of newly formed porphobilinogen was twice that of the ALA-5-C^{14} from which it was synthesized. Schmid and Shemin (384) provided further evidence when they showed that haem synthesized from ALA-C^{14}, or PBG-C^{14} with twice the molar activity, was equally radioactive. Bresel (84) demonstrated that

practically no active glycine was incorporated into haem when unlabelled PBG was added to the system which meant that very little could bypass the pool. As activity was noted in newly synthesized PBG, this indicated the diluent rather than the inhibitory effect of the added pyrrole. These observations and the findings of Falk (99) and Bogorad (34) confirmed that PBG is a precursor of porphyrin and an intermediate in its biosynthesis.

The reaction catalysed by ALA dehydrase is analogous to a Knorr synthesis in which an aminoketone condenses with a βketoester or an α,γdiketone to form a pyrrole. ALA dehydrase acts specifically on ALA not catalysing any condensation products from other aminoketones (120,121,122, 145). Gibson (122) and later Granick and Mauzerall (145) both felt that ALA dehydrase possibly catalyses the aldol condensation between two carbon atoms and that the ketimine condensation between the carbon and nitrogen atoms may occur spontaneously.

(2) Uroporphyrinogen.

(a) Enzymes - PBG deaminase and uroporphyrinogen isomerase

The enzymatic synthesis of uroporphyrinogen from PBG with the liberation of ammonia has not been fully elucidated. When PBG is incubated with extracts derived from Chlorella (34) or with red cell haemolysates (40,145,

269), only porphyrins of type III isomer are formed. However, if the enzyme system is preheated to a temperature of 55° to 65° C (depending on the system) for 15 minutes, only type I porphyrins are isolated. Neuberger and Heath (186,198) showed that suspension of frozen and thawed Rhodopseudomonas spheroides as well as the acetone dried powders from them condense PBG to uroporphyrinogen III and coproporphyrinogen III whereas the cell-free supernatants, after centrifugation, and the heated acetone-dried powders formed isomer I only. Bogorad (35,36) showed that the aqueous extract of spinach leaf acetone powder could catalyse the disappearance of PBG with the appearance of uroporphyrin I. In 1958, Bogorad (36,37) succeeded in preparing two enzyme fractions, PBG deaminase and uroporphyrinogen isomerase from spinach leaf tissue and wheat germ respectively, and showed that the former acts before the isomerase.

From the above studies, the following observations have been made. It appears that two enzymes are necessary for the biosynthesis of uroporphyrinogen III from PBG. The one, PBG deaminase, is heat stable and after deamination of the aminomethyl group of PBG with the liberation of ammonia, it is able to catalyse the condensation of four molecules of PBG to uroporphyrinogen I.

The second enzyme, uroporphyrinogen isomerase, has no effect on PBG when incubated alone with it; nor is it able to convert uroporphyrinogen I alone, or with PBG, to uroporphyrinogen III (37,39). Both these enzymes together, however, synthesise uroporphyrinogen III.

Inhibition of PBG deaminase has been observed with silver, mercury and formaldehyde (36). The latter, a possible by-product of the deaminase reaction (389,421) may provide a regulatory mechanism whereby the synthesis of uroporphyrinogen III is promoted.

(b) Enzymatic synthesis of uroporphyrinogen molecule.

No one has elucidated the sequence of events by which a porphyrin of a particular isomer is synthesized from individual pyrrole units. Several theories have been advanced and only a few will be briefly discussed.

Schmid and Shemin (cited in ref. 421) found that by heating under acid conditions or by enzymatic conversion of PBG to uroporphyrinogen in cell-free extracts, formaldehyde was formed. This was established by heating or incubating PBG labelled with C^{14} in the aminomethyl group. This is a highly important finding for at least two reasons. Firstly, it could provide a mechanism whereby the glycine-2 carbon or the ALA-5-C could be converted to C1 fragments or be oxidised to carbon dioxide. Secondly, as four PBG molecules, in condensing to form porphyrin,

would use all four carbons of their aminomethyl groups as methene bridges, no carbon atoms should be available for liberation as formaldehyde. To explain the radioactive formaldehyde detected above, synthesis of the porphyrin molecule must result in wastage of pyrroles stripped of their aminomethyl groups.

Shemin (419,420,421) attempted to explain the above along the following lines. Three molecules of PBG could condense to form a tripyrrylmethane compound which could then split up into a dipyrrylmethane and a monopyrrole. In the formation of the tripyrrylmethane, one aminomethyl chain would be lost, presumably as ammonia and formaldehyde. The structure of the dipyrrylmethane would be determined by the site of splitting and various combinations of such fragments could give rise to porphyrins of both isomers.

However, subsequent work by Bogorad (37), Granick and Mauzerall (145) has shown that more than 80% of the PBG metabolized by an avian red cell preparation can be recovered as porphyrin. Further, Shemin's hypothesis is incompatible with the report of Dresel and Falk (86) that about 90% of PBG in their avian red cell system could be accounted for as porphyrin. Formaldehyde, as reaction product, is not mentioned by any of the above

authors.

For Shemin's hypothesis to be tenable, one pyrrole unit should accumulate for every four used i.e. the pyrrole level should not drop below 20% in the enzyme system. Bogorad (37) by his techniques, could not detect any pyrrole remains at the completion of the reaction although he admitted that polymerization of the residual pyrroles or failure to react with his re-agent could account for their not having been detected. Bogorad (36) confirmed his earlier work (35) that approximately one mole of ammonia was released for every mole pf PBG consumed. This deamination of PBG by PBG deaminase is probably a major route by which the ALA nitrogen enters amide groups of proteins, amino acid and urea, as observed by Berlin et al (24) in their experiments with N^{15} labelled ALA. The other possible route is after de- or trans-amination of ALA to form αketoglutaraldehyde, a step in the succinate-glycine cycle.

To explain the above phenomena, many theories have been put forward. Bogorad (37) felt that it was reasonable to assume that the isomerase requires two substrates for its action. One of these is PBG and the other would probably be some product of the action of PBG deaminase on PBG, a di- - or tripyrryl. The nature of this second substrate was speculative.

Granick and Mauzerall (145) felt that the deaminase condensed PBG molecules to form polypyrrylmethanes by the elimination of ammonia. The isomerase, by an unkown mechanism, inverted one of three of thePBG molecules to give isomer III sequence.

Lockwood and Benson (270) modified the mechanism put forward by Bullock, Johnson, Markham and Shaw (50) in their conception of the chemical synthesis of uroporphyrin III rather than I in acid solution. Lockwood and Benson suggested that after the initial condensation between two aminomethyl groups of PBG with the liberation of ammonia, the activated - CH_2 group formed, migrated via the nitrogen atom of the condensations of PBG, with the loss of ammonia, forming a linear polypyrrolic chain. Isomerase acted on the linear tetrapyrrole forming uroporphyrinogen III. If the isomerase did not act and a longer polypyrrolic chain was formed, ring closure would give rise to one molecule of uroporphyrinogen III followed by an indefinite number of molecules of uroporphyrinogen I.

(3) Porphyrins or Porphyrinogens as intermediates.

Although Falk et al (99) and Salomon et al (371) using a chick haemolysate and rabbit bone marrow suspension respectively, reported the synthesis of radioactive haem from C^{14} labelled uroporphyrin III, many authors

have subsequently found this porphyrin ineffective as a substrate (35,84,134,186,198,288,399). Neve et al (323) using a haemolysed duck red cell system, demonstrated that the incorporation of Fe^{59} into haem was markedly stimulated by the addition of uroporphyrinogen III but not uroporphyrin III. Neare and Heath (198) showed that colourless uroporphyinogen was the first substance formed from PBG by an extract of Rhodopseudomonas spheroides and that the porphyrins which ultimately appeared, almost certainly arose from auto-oxidation (361). They also showed (198) that suitably prepared uroporphyrinogen III was readily transformed enzymatically to coproporphyrinogen III. Additional studies by Bogorad (35,38) and Mauzerall and Granick (289) have established that the biosynthesis of protoporphyrin from PBG proceeds over a series of porphyinogens, the colourless reduced porphyrins containing six additional hydrogen atoms.

The oxidation of uroporphyrinogen to uroporphyrin proceeds in a step-wise manner (289) but as the amount of porphyrin excreted in the urine is extremely small (when compared with haem turnover), the fraction of hexahydroporphyrin that is oxidised and escapes from the biosynthesis path must be insignificant. Exclusion of light and the presence of reducing agents such as

glutathione and cysteine are probably important in maintaining the porphyrins in a reduced state (289). Failure of cell reducing systems may account for the accumulation and escape of porphyrins from red cells in symptomatic porphyria (187).

(4) Coproporphyrinogen.

Work by Mauzerall and Granick (289), Bogorad (38) and Hoare and Heath (194) have established that the four acetic acid side chains of uroporphyrinogen I or III are converted to methyl groups through the action of uroporphyrinogen decarboxylase. This reaction occurs in a step-wise and random manner so that intermediate prophyrinogens with seven, six and five carboxyl groups are isolated from the biological systems (100, 289,412).

While the enzyme is specific in that it will not act on ALA, PBG or uroporphyrin, it acts on all four isomers of uroporphyrinogen (194). However, it acts 2-3 times faster on uroporphyrinogen III than it does on uroporphyinogen I (289). As expected, reduced glutathione and cysteine enhance the reaction whereas oxygen inhibits it (289). The enzyme is also inhibited by mercury, copper and manganese (38,289).

Of the coproporphyrinogens formed, only isomer III is metabolized further. This now leaves the soluble portion of the cell and enters the mitochondria where

oxygen is required for its further metabolism.

E. PROTOPORPHYRIN III (9a).

Protoporphyrin III (9a) is the key porphyrin in both animals and plants as from this, haem and chlorophyll are derived. It is formed from coproporphyrinogen III (142,143,346) and its formation involves two stages:-

(1) The oxidative decarboxylation of two propionic side chains to vinyl groups to form protoporphyrinogen.

(2) The oxidation of the protoporphyrinogen to protoporphyrin III (9a), the precursor of haem.

Reaction (1) is catalyzed by the enzyme coproporphyrinogen oxidative decarboxylase (346) also termed coproporphyringen oxidase by Sano and Granick (375). For the purposes of simplicity, this enzyme is often referred to as coprogenase.

Reaction (2) is thought to be catalyzed by a separate enzyme protoporphyrinogen oxidase (346). Evidence for the two enzymes is presumptive, however, as neither has been isolated in the absence of the other (346).

Protoporphyrin in nature is always of the type III isomer and will, in the text to follow, be referred to by the more familiar designation, protoporphyrin 9.

(1) Enzyme Systems.

While the conversion of ALA to coproporphyrinogen takes place in the soluble portion of the cell, its further metabolism occurs in the cell mitochondria. Evidence for this rests on the following observations:

(a) Chlorella enzyme preparations can synthesize coproporphyrinogen III but not protoporphyrin 9 from ALA. If rat mitochondria are added to the system, protoporphyrin 9 is formed. Rat mitochondria themselves do not metabolise ALA (34).

(b) Liver mitochondria enhance the conversion of ALA to protoporphyrin 9 in the haemolysate of human red cells (36) and in soluble chicken red cell preparations(374).

(c) In avian erythrocytes, the cellular particles, presumably mitochondria, are required for the first and last steps of protoporphyrin 9 synthesis (374).

(d) The intracellular distribution of coprogenase was shown by Sano and Granick (375) virtually to be confined to the mitochondrial elements (about 80%), while the nucleus and supernatant fractions of the cell accounted for about 0.3% of the total activity. The remainder lay in the fluffy layer and microsomes of the preparation.

The enzyme system has been obtained in soluble form from liver mitochondria by Sano et al (374,375) and Porra (343). This system has been purified about twenty

times from beef liver mitochondria (375).

(2) Enzymatic Synthesis of Protoporphyrin 9.

(a) Oxidative decarboxylation of coproporphyrinogen III.

The high degree of substrate specificity of coprogenase for coproporphyrinogen III accounts for the absence in nature of protoporphyrin or haem corresponding to isomer I. In-vitro experiments have confirmed that coprogenase does not act on coproporphyrinogen I or II, coproporphyrin I or II and a wide variety of other porphyrinogens (346,375). However, Porra and Jones (346) demonstrated that their ox liver mitochondrial system could convert coproporphyrinogen IV to an undefined isomer of protoporphyrin and deuteroporphyrinogen-IX-4-propionic acid into 4-monovinyldeuteroporphyrin. The biological importance of these observations is not certain.

Coprogenase catalyses the removal of two hydrogen atoms and a carboxyl group from each of the two propionic acid side chains of rings A and B of the porphyrin nucleus with the formation of two vinyl groups (375). Both these reactions appear to occur simultaneously (375). The failure by Porra and Falk (346) to replace molecular oxygen with alternative electron acceptors confirmed the view of Sano and Granick (375) that oxygen was essential for the action of coprogenase in the mammalian liver.

(b) Oxidation of protoporphyrinogen 9.

The product of the above reaction is protoporphyrinogen 9. Its presence was confirmed spectrophotometrically by Porra and Falk (346), but even at their first readings, much of the protoporphyrinogen was oxidized, indicating that mitochondrial extracts contain protoporphyrinogenoxidase. Gibson et al (126) felt that the oxidation of protoporphyrinogen could occur non-enzymatically in the presence of such reducing substances as reduced glutathione (GSH), important in maintining iron in its ferrous state within the mitochondrion, the enzymic oxidation of protoporphyrinogen seems likely. This is supported by the following observations:-

(i) In the same system forming protoporphyrin 9, coproporphyrinogen was not oxidized, even by the addition of oxidizing agents (346).

(ii) Mauzerall and Granick (289) had, in 1958, reported the stability of porphyrinogens in the dark at a neutral pH.

(iii) Sano and Granick (375) showed that when protoporphyrinogen was incubated in the dark at 38°C with serum albumin at a neutral pH, auto-oxidation was inhibited almost completely.

(3) Coprogenase.

This enzyme is widely distributed in mammalian tissues.

It is mainly found in the liver although bone marrow, kidney and spleen are also rich in the enzyme (375). Its distribution appears to be related to haem turnover by the particular organ. Drabkin (80,82) found a rapid turnover of cytochrome in the liver (8 days) and less rapid in the kidney. Bone marrow, the major site of haemoglobin production, also has high activity. Heart muscle, with a slow myoglobin turnover has low activity but even this is three to five times more than striated muscle.

The high specificity of coprogenase has already been mentioned. The enzyme is inhibited by o-phenanthroline and α-α1- dipyridyl. Other metal chelators do not inhibit but enhance the yield of protoporphyrin (375) Sulphydryl re-agents are without appreciable effect.

(4) Protein-bound protoporphyrin.

Protein-bound porphyrins may be intermediates in haem synthesis. Eriksen (96) distinguished between "free" and "bound" forms of protoporphyrin and felt that the latter was the immediate precursor of haem. Porra et al (346) cited the work of Sugita who detected a protein-bound protoporphyrin complex, a possible intermediate in haem formation. Sano and Granick (375), observing the reaction between protopophyrinogen and mercaptoethanol or cysteine, raised the possibility that cytochrome C may be formed by the interaction of

the - SH groups of apocytochrome C peptide with the vinyl groups of protoporphyrinogen.

Porra and Falk(343) felt that the porphyrins were linked covalently to the protein, possibly by a thiother linkage. Acid hydrolysis of the protein-bound porphyrins formed during incubation of the mitochondrial extract with coproporphyringen III, yielded copro-porphyrin and a monovinyl-dicarboxylic porphyrin.

F. THE HAEM MOIETY.

The final step in the synthesis of haem involves the incorporation of ferrous iron into protoporphyrin to form haem. No iron compounds of uroporphyrin and coproporphyrin are known to occur biologically. Although this reaction can occur in the absence of enzymes, it is much more rapid in the presence of tissue preparations.

(1) Enzyme systems.

Biologically, the reaction is catalysed by the enzyme ferrochelatase (iron-protoporphyrin chelating enzyme). This enzyme is located in mitochondria (326, 374) and has been isolated in soluble form from mammalian liver mitochondria (238,344,345) and avian-erythrocytes (135,214,232,268,301,398). Human reticulocytes (135,268), homogenates of splean and marrow (268) and various microorganisms (345) also exhibit ferrochelatase activity.

(2) Substrates.

Porra and Jones (344) showed that in a pig liver system, meso- and protoporphyrins were substrates for ferrochelatase while the corresponding porphyrinogens did not appear to be used. Many workers have demonstrated the role of protoporphyrin as a true intermediate in the biosynthesis of haem (134,135,238,397).

Protoporphyrin in its oxidized form is the end

product in the oxidative decarboxylation of coproporphyrinogen III (see previous section). It is extremely unlikely that cell reducing systems will then reconvert protoporphyrin to its haemhydro form as even powerful reducing agents such as dithionite will not do this in-vitro (187). Moreover, Neikel, Lockwood and Rimington (187) in their studies on the non-enzymic formation of haem, showed that ferrous iron readily co-ordinates with porphyrins but not porphyrinogens.

It thus appears that the synthesis of haem from protoporphyrin 9 is the first step in the biosynthesis of this compound where an oxidised porphyrin is the substrate.

(3) Ferrochelatase.

Using a variety of micro-organisms as sources of ferrochelatase and a number of different dicarboxylic acid porphyrins as substrates, Porra and Jones (345) obtained evidence for the existence of several forms of ferrochelatase with different specificities. He concluded that pig liver mitochondria themselves may contain more than one enzyme.

Ferrous but not ferric ions are used by ferrochelatase (344). In pig liver mitochondrial extract, Porra and Jones (344) showed that activity was most

active in vacuo and completely inhibited by aerobic conditions.

Enzyme activity was stimulated by reduced glutathione (GSH), ascorbic and dehydroascorbic acid, ergothioneine homocysteine, histidine, NAD and allylisopropylacetamide (AIA) (135,238,268,326). The stimulatory effect of NAD was not additive when given with other reducing agents and its mode of action was not clear (238). The enhancing effect of GSH and ascorbic acid was probably a reflection of their ability to maintain iron in its ferrous form under conditions not strictly anaerobic (344). Porra et al (344) found no evidence in support of Labbe and Hubbard's conclusions (238) that ferrochelatase was a sulphydryl enzyme.

Citrate, adenosine, inosine, thymidine and B_{12} were not stimulatory. The reaction was inhibited by metal binding agents such as BAL, EDTA and cyanide (238). Bilirubin inhibited, in part at least, by competing with protoporphyrin (237).

C. CONTROL OF HAEM SYNTHESIS.

The mitochondrion is the key intracellular structure in the biosynthesis of the complex haem molecule from such simple substrates as glycine and succinic acid. It is here that succinyl coenzyme A is generated and ALA formed. ALA that is not metabolized via the succinate glycine cycle, must move out of the mitochondrion in order to be converted by the soluble enzymes of the cytoplasm to coproporphyrinogen. This would have to migrate back into the mitochondrion in order to be converted to protoporphyrin and haem. Sano and Granick (375) make the interesting suggestion that such compartmentation of enzymes would permit porphyrin biosynthesis to be controlled in part by the active or passive permeability of the mitochondrial membrane to ALA and coproporphyrinogen III.

Mitochondrial membrane permeability, factors influencing ALA synthetase activity and the extent of the succinate-glycine cycle would seem to be the most important mechanisms by which haem biosynthesis is controlled.

CHAPTER III.

ASPECTS OF HAEM-PROTEIN AND BILIRUBIN METABOLISM.

I. HAEM-PROTEINS.

In its biologically active form, the haem moiety is conjugated to a protein. In most instances, the complex is easily split by simple solvents, e.g. acid-acetone. However, in tightly bound haems, there is a covalent attachment between the haem and protein moieties. This is exemplified in cytochrome C where the cysteine peptides of the protein fraction are linked via thioether bonds to the vinyl chains of the porphyrin (146).

Each haem is a monomer or polymer of a unit comprising one haem per one polypeptide chain (146). Molecular weight therefore depends on the number of units and the length of the polypeptide chain.

Haem-proteins are highly important biological substances, necessary for both the transport and utilisation of oxygen. For the purposes of this thesis, it is necessary to consider their distribution and rates of turnover in mammals, in order to understand more fully the origin of bile pigments.

A. COMMON HAEM PROTEINS IN MAMMALS.

85 to 90% of the total body haem in the vertebrate occurs as haem of haemoglobin (146). The haem of myoglobin comprises a further 10% and less than 1% is present in all the other haem proteins combined (146). The common haem proteins will be briefly considered.

(1) Haemoglobin.

This is a tetramer, each unit containing one haem attached to one polypeptide of about 17,000 molecular weight (146). It is an easily-split complex whose main function is the transport of oxygen from the lungs to the tissues. The iron of the ferrous haem is co-ordinated with six ligands, four to pyrrole nitrogens, one to an imidazole nitrogen of histidine and one to oxygen or water, in oxy- or reduced haemoglobin respectively.

(2) Myoglobin.

This respiratory pigment, present in muscle, is capable of carrying oxygen from the blood to the mitochondria (146). It is an easily-split monomer containing one haem per polypeptide with a molecular weight of 17,000. Rossi-Farrelli et al (366) demonstrated three fractions in human heart muscle, separable by electrophoresis.

(3) Cytochromes.

Cytochromes a, a_3, b, c, c_1 are haem-proteins

localized in mitochondria, essential in the transport of electrons from hydrogen atoms released by dehydrogenase systems to oxygen, which now ionized, combines with protons to form water. Energy is released in a stepwise manner and can be coupled as high energy phosphate bonds. They therefore play an essential part in aerobic biological oxidation.

Cytochromes a, a_3 and b are easily-split haems, not chemically bound to corresponding polypeptides. Cytochrome a_3, or cytochrome oxidase has copper as an essential constituent (68) and its haem nucleus differs from that of protoporphyrin (62,305,339,455). It has a formyl and hydrophobic acid side chain instead of a vinyl and methyl group respectively.

The most extensively studied cytochrome, cytochrome c, is a monomer with a molecular weight of about 13,000. As mentioned earlier, it is a tightly-bound haem, the cysteine containing polypeptide linked covalently with the porphyrin moiety. (146).

4. Catalase.

This widely distributed haem-protein enzyme catalyses the breakdown of hydrogen peroxide into oxygen and water, the iron atom undergoing a change of valence in the reaction. It is a tetramer, each polypeptide-haem unit having a molecular weight of about 55,000 (439).

5. Tryptophan pyrrolase.

Tryptophan pyrrolase is an iron-porphyrin protein catalyzing the oxidation of tryptophan to formyl kynurenine. The reaction has been studied by Tanaka and Knox (438).

B. TURNOVER AND DISTRIBUTION OF HAEM-PROTEINS.

1. General Considerations.

The rate of new haem formation varies both with the haem-protein, the tissue and the varying needs of that tissue.

Of the haem-proteins, liver catalase probably has the most rapid turnover (70,349,383,442), being more rapid than catalases elsewhere (349,442), than liver cytodrome C (81,82), than haemoglobin (13,152,272,412, 461).

Turnover conforms to tissue needs for the haem-protein. The liver synthesizes catalase more rapidly than the kidney (349) or red cells (442) in rats and guinea pigs. Cytochromes are seen in highest concentration in those tissues with high oxygen consumption, e.g. liver and heart more than skin (82).

It is not surprising therefore that haem-protein synthesis responds differently to varying tissue needs. While anaemia or androgens stimulate haemoglobin synthesis (318,450), this has no effect on cytochrome c (450). Hyperthyroidism, by stimulating cell oxidation, increases cell haems more than blood haem (80). In lead poisoning, while haemoglobin synthesis is impaired (85,86,95,210,450), cytochrome c concentration may increase in different tissues (450).

2. Haem-protein activity in different tissues.

By two entirely different approaches, an estimation of haem-protein activity in different tissues has been obtained in experimental animal studies.

Schwartz and Cardinal (401) administered ALA-4-C^{14} intra muscularly to normal dogs and sacrificed them at intervals from half-an-hour to six days after the injection of the isotope. Easily-split and tightly-bound haems were extracted from liver, kidneys, heart, lungs, spleen, brain, muscle, erythrocytes and leucocytes. More than 90% of the total haem-C^{14} activity was in the easily-split fraction. Peak values were obtained within half to two and a half hours when 22.2% and 7.3% of the administered dose was present in the latter haems of liver and kidney respectively. At twenty-nine hours, these values had fallen to 11.8% and 1.5%. Minimal activity and slower turnover was found in other tissues and virtually no activity in the brain. Turnover of tightly-bound haems were slower in all the tissues studied. The obvious disadvantage of the above study was the varying permeability of tissues to ALA which, for example, is known to enter haemopoietic tissue poorly. Liver and kidney, besides being the sites of rapid haem-protein turnover, are also probably more permeable to ALA than other organs. The experiment bears repeating using glycine-2-C^{14} as precursor as this,

by entering all tissues easily, will more accurately reflect the haem-protein turnover in different organs, including bone marrow. Nevertheless, the exceedingly rapid synthesis of tissue haems (peak activity in ½ to 2½ hours) and the relatively rapid fall-off of activity (about 50% in the liver and 75 to 80% in the kidney in 29 hours) emphasise the speed with which liver and kidney haem-proteins turnover.

A more indirect approach of assessing tissue haem-protein activity is to measure coprogenase activity in the various organs. This reflects protoporphyrin synthesis which, in turn, should reflect haem turnover if there is no additional factor impairing the incorporation of iron into the protoporphyrin moiety. Tissue coprogenase activity was measured by Sano and Granick (375) in the guinea pig and rabbit. Liver had the most activity followed by bone marrow, kidney and spleen. While muscle in general had low activity, heart muscle had about three times more enzyme activity than skeletal muscle. There are species differences as well in that while rabbit brain had more activity than that of the guinea pig, the small intestine of the latter had a high concentration of coprogenase compared to the minimal quantities noted in other animals. This emphasizes the dangers of applying experimental findings in animals to that in humans. It is

reasonable, however, to deduce that the main sites of haem-protein synthesis are the liver, marrow and kidney.

3. Turnover of individual haem-proteins.

(a) Haemoglobin.

Using labelled glycine (272,412), lysine-C^{14} (13, 152) and N^{15}-labelled erythrocytes (461), research workers have observed that the concentration of the label in circulating haemoglobin rises rapidly to a plateau of activity which is maintained for about 100 days and then rapidly declines. From their findings, it can be concluded that the haemoglobin of circulating red cells remains outside the general metabolic pools of protein and porphyrin interchange, that red cells are destroyed as a function of their age, not randomly or indiscriminately and that the mean life span of normal red cells is about 120 days. This implies that 1/120 or 0.8% of circulating haem (equivalent to 250 to 300 mg haem in an average 70 kg human) is turned over daily.

Studies on serum bilirubin-C^{14} activity following glycine-2-C^{14} administration by Israels and his group (205,207,498), indicate that not all the haemoglobin synthesized in haemopoietic tissues behaves in the manner just described. A small proportion appears to be lost to the general circulation shortly after it has been made. This wasteful or ineffective

erythropoiesis is not entirely understood. There may be an "infant mortality" among a small number of young erythrocytes, especially in haemolytic or post-haemorrhagic states (41,42,97). The spleen has been shown to have a higher proportion of reticulocytes than circulating blood (21). Do these all mature or are a proportion prematurely destroyed? Bessis et al (26) and Jones (211) have shown that on extrusion of normoblastic nuclei in the marrow, a small amount of cytoplasmic haemoglobin is carried out of the cell as well

Whatever the mechanisms involved, the magnitude of ineffective erythropoiesis is probably less than 10% of the total haemoglobin produced. Thus haemoglobin haem turnover is in the order of about 1% per day.

(b) Myoglobin.

Little is known about myoglobin turnover in man other than that it is very slow (189,442).

(c) Cytochromes.

The synthesis of cytochrome c in various organs has been extensively studied by Drabkin (81,82). After the intraperitoneal administration of glycine-2-C^{14} to the rat, he showed that, in decreasing order of magnitude, labelled cytochrome c appeared in liver, kidney, skeletal muscle and myocardium (82). From the rate of incorporation and the decline of radioactivity in normal and partially hepatectomised rats, Drabkin (81,82) calculated that

about 12.7% of cytochrome c was newly synthesized every day, i.e. a life time of 8 days in rat liver compared to the 120 days of red cell haem in humans. Helwig et al (189) and Theorell et al (442) have also studied cytochrome c turnover in skeletal muscle.

(d) Catalase.

Theorell (442) studied the biosynthesis of liver and red cell catalase by Fe^{59} tracer techniques and showed that in guinea pigs, liver catalase turnover was far more rapid than that of other haem-proteins studied, including red cell catalase.

The kinetics of catalase synthesis and destruction, in-vivo, have been studied by Price et al (349) in rat liver. After the administration of 3 amino, 124 triazole, a substance which irreversibly inactivates catalase, return of enzyme activity was paralled by a corresponding uptake of Fe^{59} into catalase indicating formation of new enzyme. When allyl isopropyl acetamide (AIA), which blocks catalase synthesis, was given, disappearance rate was nearly the same as new catalase synthesis. From their data, they were able to show that rat liver makes more than ½ mg new catalase per gram liver per day.

Sedormid, like AIA, inhibits catalase synthesis without specifically affecting the formation of other haem proteins (393). Following the administration of

sedormid, Schmid and Schwartz (383) observed that in rats, liver catalase activity fell by 90% in 3 days, i.e. a turnover rate of over 30% per day. In rabbits, the fall off in activity was less dramatic, 50% in 3 days and 90% within 6 days of giving sedormid.

de Duve (70) noted that the life span of liver catalase was less than 2 days.

These observations underline the rapidity with which catalase is synthesized and destroyed in the liver. Price et al (349) further showed that rat liver synthesized catalase about 3.5. times more rapidly than rat kidney. It also appears that there are at least 2 catalases in rat liver, the synthesis of one being affected by AIA (349).

II. BILIRUBIN.

The excreted end product of haem degradation is bilirubin. As there are many types of haem-proteins, each with their own metabolic patterns of behaviour, it would be expected that bilirubin is derived from multiple metabolic sources. Quantitative analysis of the pattern of labelled stercobilin excretion following isotopic glycine adminstration indicates that in normal human subjects, 70-80% of bile pigment is derived from the breakdown of mature circulating red cells at the end of their life span (151,273). Although direct synthesis of bilirubin from porphyrin precursors has been postulated (459), it seems reasonably certain that the remaining 20-30% of bile pigment originates from haem-proteins other than circulating haemoglobin. Detailed evidence for this will be presented later.

A. CATABOLISM OF HAEM.

Existing knowledge of haem catabolism has largely stemmed from studies on the degradation of haemoglobin As bilirubin, from whatever metabolic source, has the same structure, it is likely that the breakdown of other haem-proteins is essentially similar to that of haemoglobin.

(1) End Products Excreted.

(a) Bilirubin.

By far the most important final product in the catabolism of haem is bilirubin. In physiological states, there is strong evidence that, for practical purposes, this is the only end product excreted. In 1939, Hawkins and Johnson (184) reported the recovery of 90-100% of the injected haemoglobin solutions as bilirubin in bile fistula dogs. In 1962, Ostrow et al (332) published results of elegant studies in similar experimental animals following the injection of sensitised red cells and haemoglobin solutions labelled with C^{14}, Fe^{59} or Cr^{51}. They showed that plasma was cleared from activity within 22 hours. Where small quantities of haemoglobin in solution, calculated not to exceed the haemoglobin binding capacity of rat plasma, were administered, recovery of bilirubin-C^{14} in the bile of 4 of 5 animals studied approached values expected if all

the injected haemoglobin were quantitatively converted to bilirubin.

(b) Alternate excretory products.

When haemoglobin-C^{14}, in solution or in sensitised red cells was administered I.V. to the bile fistula dogs in doses larger than just described, Ostrow et al (332) observed that 20-45% of the activity injected was not recovered in bile or urine as bilirubin-C^{14} despite the complete clearance of plasma-C^{14} activity in 22 hours. As in similar circumstances, injected bilirubin-C^{14} was virtually all recovered in the bile 4 hours after its administration, alternate excretory products derived from haem or from bilirubin must have been formed within the reticuloendothelial system (R.E.S.) The identity of these compounds has not been established. In all experiments, the greatest percentage conversion to bilirubin was observed after the administration of the smallest dose of haemoglobin in solution. The authors (332) felt that under this set of conditions, the pigment was removed from the circulation as a complex with haptoglobin. Their conclusions were that the metabolic disposition of intracorpuscular haemoglobin and of unbound plasma haemoglobin may differ from that of the haemoglobin-haptoglobin complex.

Whether the observations of Ostrow et al reflect an alternate pathway of haem catabolism or further degradation of bilirubin in the R.E.S. is not known. It has been well established however, that alternate routes of bilirubin metabolism do exist in conditions where bilirubin is deprived of its normal excretory channels. In children with biliary atresia (55) or with congenital unconjugated hyperbilirubinaemia due to the absence of glucuronyl, transferase in the liver, i.e. the Crigler-Najjar syndrome (390), the degree of jaundice may remain constant over months despite the continuous breakdown of haem-proteins in the body. Thus alternate pathways of bile pigment metabolism and disposition must exist in order to remove bilirubin at the same rate at which it is formed. Cameron and his co-workers (55) have shown that in congenital biliary atresia, following the administration of bilirubin-C^{14} I.V., 44-70% of the dose is excreted in the urine and 0-5% in the stool. As only about 60% of the urinary activity is accounted for as bilirubin, a significant fraction must be excreted in the form of diazo-negative breakdown products. In congenital unconjugated hyperbilirubinaemia (Crigler-Najjar syndrome), Schmid (403) has shown that injected bilirubin-C^{14} is handled in a slightly different manner. In a child

studied, he showed that 82% of the injected dose was recovered in the stool and only 7% in the urine. No activity was excreted as bilirubin, urobilin or mesobilifuscin. Most of the C^{14} label was present as water soluble, diazo negative bilirubin metabolites.

(c) Summary.

Infused solutions of haemoglobin can be quantitatively recovered as bilirubin in bile fistula dogs (184,332). Where the load of infused haemoglobin to animals is high (332) or where the normal excretion of bilirubin is precluded by biliary atresia (55) or congenital inability to conjugate bilirubin (390), alternative handling of haem (332) and bilirubin (55,390) has been demonstrated. While such alternate pathways may exist in the normal, these are probably of minor or no functional significance.

(2) Sites of Haemoglobin Degradation.

The catabolism of haemoglobin has been studied more extensively than that of other haem-proteins. Studies in haemolytic states (478) and experimentally (332) indicate that the major sites of destruction of aged or sensitized red cells are in the reticuloendothelial cells of the liver or spleen.

Time relationships of red cell destruction and bilirubin formation have been studied in bile fistula dogs after the infusion of labelled sensitized red

cells (332). Ostrow et al (332) demonstrated that 50% of the infused red cells were removed from the circulation in 15 to 70 minutes. Constant rate of bilirubin excretion in the bile was not achieved until about 1 hour after the onset of sequestration. The mean interval between sequestration of half the injected red cells and the appearance of half the bile pigment was approximately 3 to 5 hours. In comparison, after the I.V. infusion of protein bound unconjugated bilirubin-C^{14}, labelled pigment in bile appeared within 3 minutes, 50% was excreted in 20 minutes, 86% in 1 hour and virtually all in 4 hours. Thus, there is a delay of a few hours within the reticuloendolthelial system between lysis of red cells and release of comparable amounts of bile pigment into the circulation.

Ostrow et al (332) further observed that widely differing doses of haemoglobin were converted to bilirubin at similar rates and that the mechanisms for bilirubin formation was not saturated by a load of haem pigment 50 times more than the physiological turnover of rat RBC.

(3) Intermediates in the Formation of Bilirubin.

Following the degradation of haemoglobin, iron is almost completely reutilized in the formation of new iron-containing compounds. Globin, before re-use, is probably broken down into its constituent aminoacids. The protoporphyrin moiety is lost to the body and undergoes cleavage at its methyne bridge to form, ultimately, biliverdin. This is reduced to bilirubin and excreted as such.

The nature and sequence of chemical events in the formation of bilirubin have not yet been fully elucidated. It has been shown that, in-vitro, protoporphyrin per se is not a precursor of bilirubin (219,315,497) and that the presence of attached iron is essential before the porphyrin ring is opened (146,306). What is not known is whether cleavage occurs before or after the separation of globin from the iron-protoporphyrin complex. In the former, Lemberg et al have proposed the choleglobins as intermediates while haematin is thought to be the intermediate in the latter.

(a) Haematin.

Gray (153) and Watson (458) feel that the initial step is the splitting off of globin. Haematin (ferrihaem hydroxide) appears to be an intermediate in this scheme. Evidence for this occuring physiologically is scanty as in health, the conversion of haemoglobin to bilirubin

takes place within the reticulo endothelial cell and the recovery of intermediates is therefore difficult. In disease states, however, following intravascular haemolysis, haematin has been isolated in plasma bound to albumin, viz. methaemalbumin. Both in-vivo and in-vitro. Kench et al (219), London (276) and Pass et al (337) have demonstrated the conversion of haematin to bile pigments. More recently, Snyder and Schmid (425) injected rats with C^{14} haematin and observed that conversion to bilirubin was achieved with an efficiency similar to that with comparable amounts of haemoglobin-C^{14} (332). At autopsy, only the liver exhibited significant amounts of C^{14} activity indicating the possible exclusive importance of this organ in forming bilirubin in haemolytic states. Similar results have been obtained with labelled haemoglobin.

Although haematin per se can be degraded to bilirubin in-vitro (219), it may still be bound to a protein in-vivo. Kench et al using an ascorbic acid system, showed that ferric haemoproteins such as methaemoglobin and methaemalbumin were as suitable as bilirubin precursors as haemoglobin. Haem-proteins, in fact, were more effective than haematin alone and coupling of free haematin with protein improved the

yield of bile pigment. The protein effect was non-specific in that enhancement of the reaction was not related to the type of protein or the mode of its linkage to the iron-protoporphyrin complex (219).

In summary, the above observations do not prove that haematin is a true intermediate in the conversion of haemoglobin to bile pigment. They do, however, indicate that in contrast to Lemberg's concept (256), the ferriprotoporphyrin ring can be cleaved in-vivo and in-vitro without being attached to native globin. Thus there is the possibility that splitting of haem and globin may be the first degradative reaction in haemoglobin catabolism with an iron-containing porphyrin as an intermediate in the formation of bilirubin. (425)

(b) Choleglobins and verdohaemoglobin.

Lemberg et al (252,253,254,255,256) studied coupled oxidation of haemoglobin and ascorbic acid in-vitro and obtained evidence for the existence of a series of green bile-pigment-iron globin complexes viz. choleglobins and verdohaemoglobin, which on treatment with acetic acid yielded biliverdin, globin and free iron. Their postulate was that cleavage of the porphyrin ring occurred while the haem group was still attached to the

globin moiety. These views have been extended more recently, by Mills et al (299) and Kaziro et al (218) who have suggested protective mechanisms within circulating erythrocytes preventing the excessive formation of choleglobin. While the demonstration of choleglobin-like compounds in red cells is technically difficult, Schmid (388) quotes the papers of Lemberg et al, Gajdos et al and Kiese et al who demonstrated small amounts of choleglobin-like pigment in red cells of normal and phenylhydrazine treated rabbits and the stored erythrocytes of humans.

(c) Intermediates in in-vitro enzyme systems.

The identification of the intermediates in the conversion of haemoglobin to bilirubin in-vivo will only be complete once the individual enzymatic steps in the process have been elucidated. Recently, two enzyme systems (306,315,479,480) have been described and from these studies, newer concepts of haemoglobin catabolism have emerged. While evaluation of their physiological role is not possible at present, the data is of tremendous interest and potential and will be discussed more fully in the following section.

(4) The Enzymatic Conversion of Haem to Bilirubin.

In the catabolism of haem, cleavage of the protoporphyrin-iron complex is brought about by the oxidation of the α methyne carbon, probably to a carbon monoxide (146,480

The amount of carbon monoxide exhaled in a human per day is equivalent to that expected from the normal breakdown of haemoglobin to bile pigment (94,423). Following scission of the haem moiety, loss of iron results in an open chain blue green tetrapyrrole, biliverdin, still in its ring form. This is enzymatically reduced to bilirubin, an orange yellow substance, by the addition of 2 hydrogen atoms at the double bond of the γ methyne bridge. It is at this stage that the bilirubin is released from the R.E.S. into plasma although the exact mechanisms by which this occurs is not known.

The specificity of the site of fission of the haem moiety pointed to an enzyme catalyzed reaction. Recently, enzyme systems have been isolated from beef and guinea pig liver by Nakajima et al (315) and from the haemophagous organ of dog placenta by Wise et al (479,480). The enzymes involved have been characterized and the individual reactions partly elucidated.

(a) Beef and guinea pig liver enzyme systems.

Using this system, Nakajima et al (306,315) showed that the metabolic pathway from pyridine haemochromogen and the haemoglobin-haptoglobin complex to a precursor of biliverdin, was catalyzed by an enzyme haem α methenyl oxygenase. The hydrolysis of the precursor

formed to biliverdin was catalyzed by a second enzyme, haem α methenyl formylase (401).

Haem α methenyl oxygenase activity was present predominantly in the liver and kidney while it was virtually absent from the spleen and bone marrow. The enzyme was isolated from the supernatent fraction of liver homogenate and has been purified from acetone powder of beef liver. Nakajima et al (315) found that NADPH, ferrous iron and an activator were necessary for enzyme activity. The activator, which was present in and could be released from nuclei, was not a haem compound. The reaction proceeded only under aerobic conditions and oxidation occurred without intervention of hydrogen peroxide. The reaction was not inhibited by a large excess of catalase but was by metal chelating agents and sulphydryl inhibitors.

Substrate specificity of the enzyme was of interest. Conversion of free haemoglobin in this system failed to yield positive results. In view of the quantitative yield of bile pigments from haemoglobin in-vivo, these results appeared anomalous. However, by the addition of serum to haemoglobin before incubation, rate of enzymatic conversion to bilirubin was markedly enhanced. The factor in serum appeared to be identical to haptoglobin. The biological role of haptoglobin in the enzymatic

conversion of haemoglobin in-vivo, was supported by the observations of Murray et al (310) on the metabolic disposition of Fe^{59} - haemoglobin and Fe^{59}-haemoglobin-haptoglobin complex in rabbits. These authors inferred that in normal human subjects, any plasma haemoglobin arising from intravascular haemolysis would be disposed of following combination with haptoglobin. Only when the haemoglobin-binding capacity of serum was exceeded, would other disposal pathways become operative. Nakajima et al (315) further demonstrated that several haemochromes including pyridine haemochromogen and histidine haemochromogen were active as substrate whereas alkaline haematin and protoporphyrin IX were quite inert. These authors (315) concluded that neither free haematin nor protoporphyrin IX were intermediates in the enzymatic conversion of haemoglobin to bile pigments.

In a later paper, Nakajima (316) put forward the individual enzymic steps, with formulae of intermediates, in the oxidation and cleavage of the α methyne carbon. For the reaction process to be initiated, the protoporphyrin complex had to have a chelated ferric iron. Thereafter, there was stepwise oxidation of the

α methyne bridge and, ultimately, cleavage occurred with the formation of a formyl radical at the site where the ring was opened. This compound, compound IV in his scheme, was isolated, crystallised and shown to be a single, pure substance exhibiting positive aldehyde reactions and positive colour reactions for bile pigments. Nakajima was able to hydrolyse this intermediate into stoichiometric amounts of biliverdin, formaldehyde and iron.

(b) Cell-free enzyme system from haemophagous organ of dog placenta.

Wise and Drabkin (479,480) have recently developed the above enzyme system which in many ways differs from the beef and guinea pig liver system of Nakajima (306,315). The system was capable of producing 18 to 23 mg biliverdin C^{14}/hour/g haemophagous tissue with haemoglobin-C^{14} as substrate and 21 to 79 from haemin-C^{14}. Pyridine haemochromogen was a relatively poor substrate.

In contrast to the liver system, Wise and Drabkin (479,480) found the enzyme activity in the fast sedimenting fraction (479) and localised it to light mitochondria (480). For maximal activity, enzyme required ATP, NAD, NADP, nicotinamide and boiled cell sap.

The production of $C^{14}O$ simultaneously with biliverdin was also demonstrated.

Thus 2 distinct enzyme systems with different substrate specificities and cellular localisation of enzymes have been developed, both capable of producing biliverdin from haem substrates. It is too early at present to judge the physiological implications of the data obtained.

(5) Direct synthesis of Bilirubin from Haem Precursors.

While it is accepted that bilirubin is the excreted end product of haem catabolism, some workers have suggested that a portion of bile pigment is anabolic in origin. Watson (459) has considered the possibility of direct synthesis of bilirubin from the linear tetra pyrrylmethane (TPM) precursor of uroporphyrinogen. However, there are important objections to this idea. According to recent concepts of uroporphyrinogen III formation (37,50,145,270), the order of pyrrole rings in the linear TPM would be that of a porphyrin ring cleaved at the γ rather than the α methyne bridge i.e. any compound formed from TPM would have a structure different to that of bilirubin.

The configuration of bilirubin, with its vinyl, methyl, propionyl and acetyl side chains clearly point to protoporphyrin IX as an obligatory precursor. The question to be resolved, however, is whether haem is an

obligatory precursor or whether some bilirubin can be formed directly from free protoporphyrin or from a protoporphyrin-protein complex. London et al (277) and Ibrahim et al (203) observed that after the I.V. injection of highly active protoporphyrin-C^{14} to dogs, stercobilin of low activity could be isolated from the stool, and bilirubin-C^{14} from bile in their respective experiments. Incorporation to haemoglobin protoporphyrin was negligible (203). This may merely mean that a small fraction of the administered protoporphyrin was first converted to haem before being broken down to bile pigment. Kench et al (219) using an in-vitro ascorbic acid system, and Nakajima et al (306,315), Wise et al (479,480) and Yamaguchi et al (cited 17), using in-vitro enzyme systems, have all similarly noted that while a variety of haem compounds were converted to bilirubin, free protoporphyrin IX never yielded bile pigment. Nakajima (306) also found that protoporphyrin had to be chelated to ferric iron before enzymic degradation could commence.

Thus to date, no evidence exists in support of direct synthesis of bilirubin from free protoporphyrin or its precursors. It appears, on available data, that haem is an obligatory precursor of bile pigment.

B. METABOLIC SOURCES OF BILE PIGMENT.

(1) General Considerations.

Bilirubin is continuously produced from the breakdown of haemoglobin and other haem proteins in the body. The distribution of total body haem has already been discussed. In the vertebrate, 85-90% is present as haemoglobin while 10% can be accounted for as myoglobin and less than 1% by the remaining haem proteins (146). However, to equate the excreted bilirubin with the haem catabolised, it is necessary to consider not only the absolute amounts of the various haem proteins in the body, but also their sizes and respective life times.

Eppinger (cited in ref. 146,459) in 1920, reported the excretion of 300 to 370 mg of bilirubin per day in two patients with complete external biliary fistulas. Although the spectrophotometric method he employed is not entirely acceptable (459), the data above is of value. As haem of infused haemoglobin is very nearly quantitatively converted to bilirubin (184,332), and as about 0.8% of circulating haemoglobin is turned over daily (13,152,272,412,461), one can calculate that in an average 70kg man, about 220 to 260 mg bilirubin will arise from the destruction of aged red cells. In other words, 20-30% of bilirubin is not derived from circulating haem.

With the advent of tracer techniques, the role of the various haem proteins in bilirubin formation has been more accurately assessed. London et al (273) and Gray et al (151) showed that following the administration of glycine-N^{15} to normal humans, the N^{15} label appeared in faecal stercobilin in two distinct peaks of activity. The smaller, initial peak occurred during the first week while the second larger peak fell between the 80th and 140th day following glycine administration at a time when the senescent labelled red cells were being maximally destroyed. That the second peak of activity arose from circulating haem was clearly confirmed by Watson-James et al (461) who studied faecal stercobilin-N^{15} activity following the infusion of N^{15}-labelled erythrocytes to normal man.

Quantitative analysis of the pattern of labelled stercobilin excretion suggests that 70 to 80% of activity is derived from the breakdown of aged red cells (151, 273). 10 to 20% is excreted during the first week and comprises the early labelled fraction (151,273), while the remaining 5 to 10% appears in the stool between the early and late peaks of activity. This latter fraction probably reflects the catabolism of slowly turning over haem proteins, e.g. myoglobin, although low grade random destruction of labelled erythrocytes may serve as an

additional source to this fraction (393).

The initial peak of N^{15} labelled stercobilin occurs at a time when the isotopic activity in circulating haemoglobin is still rising. This fraction is often referred to as "early-labelled" or "shunt" bilirubin as it is not derived from circulating erythrocytes. The nature of this fraction will be discussed in detail in the following section.

(2) "Early-labelled" or "Shunt" Bilirubin.

The origin of "shunt" bilirubin is not clear. Theoretically, as suggested by Gray et al (153,155), it might be formed (a) by direct synthesis from glycine and active succinate, although this is most unlikely, (b) by degradation of haem proteins other than haemoglobin, (c) by degradation of red cells of very short life span which never reach the circulation, (d) degradation of newly-formed haem during the nucleated stages of maturation of the red cell or (e) degradation of haem in excess of that required for haemoglobin synthesis.

Our knowledge of what comprises early-labelled bilirubin has stemmed largely from studies in physiological and pathological states on the incorporation of the isotopic label in stercobilin, bile bilirubin and serum bilirubin in humans and bile fistula dogs following

the administration of labelled glycine and ALA. These experimental studies will be briefly reviewed.

(a) Isotopic experimental studies.

(i) Stercobilin-N^{15} activity.

London et al (273) and Gray et al (151) established that approximately 10 to 20% of the total stercobilin-N^{15} activity excreted was recoverable within about one week of administering glycine-N^{15}. Although early-labelled stercobilin was clearly derived from sources other than circulating haemoglobin, the observations that this fraction was increased in various disorders of haempoiesis such as pernicious anaemia (274), congenital porphyria (151,275) and thalassaemia (162), suggested that it was in some way related to red cell formation. This concept was supported by the findings of Gray and Scott (154) following haemorrhage. However, Watson-James et al (462) demonstrated a similar increase in patients with erythroid aplasia suggesting that this fraction may have arisen, in part, from a non-erythroid source.

(ii) Bile-fistula dogs.

Bile fistula dogs given glycine-2-C^{14} I.V. were used by Israels et al (206, 207) to quantitate and time the appearance of early-labelled bilirubin under varying states of erythropoiesis. In normal dogs, about 2.5% of

the dose appeared in circulating haem and 0.10% in the early-labelled bilirubin excreted. After venesection circulating haem took up 4% and bilirubin 0.2% of the injected counts. Thus, while the activity of "shunt" bilirubin formed in post-haemorrhagic states was twice that of normal dogs, in both circumstances, the early-labelled bile pigment activity was 4 to 5% that of circulating haem.

The pattern of bile pigment activity was interesting. Activity reached a peak within 24 hours and then fell to a plateau at 48 hours which remained until the 5th day following glycine-2-C^{14} administration (206). This was entirely different to the pattern observed in dogs given 700 r total body irradiation with resultant marrow aplasia. These animals, while incorporating less than 0.3% of the injected dose into circulating haem, still excreted 0.1 to 0.25% of the dose as "shunt" bilirubin. Activity reached an early peak at 4 hours and fell sharply, no secondary plateau occuring (206).

Israels et al (207) next studied the effects of chemically induced porphyria on the excretory pattern of bile pigment activity. Following Sedormid feeding to a point where bile became strongly fluorescent, dogs were given I.V. glycine-2-C^{14}, and bilirubin and

protoporphyrin were isolated from the bile. Radioactivity appeared rapidly in the excreted bilirubin with a peak activity at the first observation at 3 hours as compared with 12 to 24 hours in the normal dog. Peak protoporphyrin activity was also present at this time and exceeded that of bilirubin. As Sedormid porphyria is primarily a disease of liver with no demonstrable changes in bone marrow, Israels et al suggested that this was evidence for the first component of "shunt" bilirubin being hepatic in origin.

Ibrahim, Schwartz and Watson (201,202,203,400) have also performed extensive and elegant studies on bile-fistula dogs. Following the administration of glycine-2-C^{14} they correlated total daily excretion of bile bilirubin C^{14} with total haemoglobin protoporphyrin-C^{14} activity. They showed that non-erythropoietic component was limited to the first 2 days and the erythropoietic component was more prolonged. The total non-erythropoietic activity excreted was 5 to 8 times 10^5 dpm/millicurie glycine given. The erythropoietic fraction was more variable depending on the state of haemopoiesis in the particular animal. Total excretion varied between less than 1 to 38 times 10^5 dpm/millicurie glycine. In the normal dog, about 2/3 of early-labelled bilirubin was erythropoitic in origin (400).

Following the administration of ALA-C^{14} and protoporphyrin-C^{14}, 20% of the dose was excreted as bilirubin-C^{14} in the bile (400). Of this, 99% was non-erythropoietic in origin and a total of 4,500 x 10^5 dpm/millicurie was excreted (400). Peak activity occurred earlier than after glycine-2-C^{14} at about 3 to 6 hours (201,202,408) and 94% of the activity was excreted in 48 hours. The specific activity of bilirubin at its peak was 22 x 10^6 dpm/mg bilirubin/millicurie ALA-C^{14} administered (202). The pattern of bile pigment activity excreted following the administration of protoporphyrin-C^{14} was almost identical to that following ALA-C^{14} (203) while in the same animals, incorporation to haemoglobin protoporphyrin was negligible.

It can be calculated from the data of Ibrahim et al that about 0.1% of the total dose of glycine-2-C^{14} administered, is excreted as early-labelled bilirubin. This figure is similar to the one observed by Israels et al (206) in their experiments. In comparison, 20% of the ALA-C^{14} is excreted as "shunt" bilirubin despite minimal incorporation of the label into red cell haem.

In summary, following the administration of isotopic glycine, early-labelled bilirubin comprises at

least two components, the first appearing within hours even in the absence of active erythropoiesis (206) and the second accentuated by increased marrow activity following blood loss and absent in post-irradiation aplastic anaemia (206). This second peak is clearly related to active erythropoiesis and confirms that glycine is an efficient red cell haem precursor in-vivo. Following ALA-C^{14} or protoporphyrin-C^{14} administration (201,202,203,400), the second peak is absent demonstrating how poor these substances are in-vivo as haemoglobin precursors. However, the activity excreted as the early peak after ALA-C^{14} is about 500 to 900 times more than that following glycine-2-C^{14}. Thus, ALA-C^{14} is a much more efficient non-haemoglobin haem precursor than glycine-2-C^{14}. One can infer that in those organs e.g. the liver, where non-erythropoietic haem turnover is rapid, ALA and glycine readily enter the cells and are available for porphyrin synthesis whereas the marrow readily utilises glycine but is relatively impermeable to ALA.

(iii) Plasma bilirubin-C^{14} activity.

Techniques whereby plasma bilirubin-C^{14} activity can be measured directly, have provided more accurate information as to the origins of early-labelled bilirubin. ALA-2-4-C^{14} I.V. (205,207,498), ALA-5-C^{14} orally (78) and glycine-2-C^{14} I.V. (205,207,498), this thesis, have all

been used as bilirubin precursors. Following the I.V. administration of glycine-2-C^{14} to normal human subjects, radioactivity was detectable in plasma bilirubin within 6 hours and exhibited two distinct peaks of activity, the first within 12 to 24 hours and the second at 3 to 5 days (205,207,498)(this thesis). The second phase probably began at day one, the initial part of its production being overlapped by the first component.

When ALA-C^{14} (orally or I.V.) was the bilirubin precursor, the C^{14} label appeared in plasma bilirubin within 30 minutes, reached a peak of activity within 90 minutes and then fell away rapidly (78,205,207,498). There was no second peak.

These findings confirmed the composite nature of "shunt" bilirubin and established that there were at least two distinct and separable components, each one almost certainly representing individual turnover of completely different haem-proteins. The possible origins of these fractions will be discussed in detail in the following section.

(b) <u>Sites of origin of early-labelled bilirubin.</u>

Although haem proteins are widely distributed in the body, the liver, marrow and to a lesser extent, the kidney appear to be the principal sites of haem-

protein synthesis (375). It would follow, therefore, that a major portion of early-labelled bilirubin is derived from haem-protein turnover in these organs. Information regarding the origins of "shunt" bilirubin is scanty and largely inferential but as more is known about the second component, it is convenient to consider this fraction first.

(i) The second fraction (peak).

The second peak of early-labelled bilirubin occurs 3 to 5 days after glycine-2-C^{14} administration corresponding in time to the maximum increment in circulating haem activity i.e. with the release of maximally labelled red cells from the marrow (207,498)(this thesis). This fraction is absent in human subjects (78,207,498) and bile fistula dogs (201,202,400) receiving ALA-C^{14} where minimal incorporation of the label into circulating red cells occurs (78,201,202,207,400,498). In bile fistula dogs with post-irradiation aplastic marrows, the secondary plateau of activity in the excreted bile pigment (analogous to the second plasma peak) is also absent (206). The second component of early-labelled bilirubin is clearly related, therefore, to bone marrow erythropoietic activity.

Israels and his group (207,498) studied the ratio of activity between haem and globin of circulating haemoglobin in various pathological states. They

showed that in patients with a large secondary peak such as those with pernicious anaemia and shunt hyperbilirubinaemia, haem: globin ratios did not differ from those of normal controls. They felt that this was strong evidence that the bilirubin was derived from preformed haemoglobin rather than from red cell haem or its precursors.

The observation that the second peak coincides with the emergence of maximally labelled erythrocytes from the marrow not only suggests that preformed haemoglobin is the bilirubin precursor, but that the haemoglobin is in some way released from late normoblasts or newly formed red cells. Although the mechanisms underlying ineffective erythropoiesis have already been alluded to in an earlier section, they are particularly pertinent to the present discussion. To recapitulate, Beesis et al (26) and Jones (211) noted that during the maturation of the normoblast, some cytoplasmic haemoglobin accompanied the nucleus on its extrusion from the cell. An "infant mortality" among young erythrocytes, especially in haemolytic or post-haemorrhagic states, has been suggested by a number of workers (41,42,97). The spleen has been shown to have a higher proportion of reticulocytes than circulating blood (21) suggesting either premature destruction or rapid maturation of these cells. While the relevance of these observations is purely speculative, all the observed facts are ..... P.T.O.

consistent with the evidence that the second component of early-labelled bilirubin is derived from preformed haemoglobin of late normoblasts or newly formed red cells.

(ii) The 1st fraction (early peak).

The site of origin of the early peak of shunt bilirubin is not altogether clear. Following the administration of glycine-2-C^{14}, the C^{14} label appears in plasma bilirubin in 90 minutes rising to a maximum at between 12 and 24 hours (205,207,498) a time when there is no activity in circulating haem. In the irradiated bile fistula dog with marrow aplasia, not only is the first peak still present, but it occurs earlier and is of greater magnitude than in normal dogs (206). Where ALA-C^{14} is the precursor both of serum bilirubin-C^{14} in humans (78,205,207,498) and of bile bilirubin-C^{14} in bile fistula dogs (201, 202,400), the early component occurs much sooner and is many hundred times greater than following glycine-2-C^{14} despite virtually no activity in red cell haem (25,78, 201,202,207,400,498). The full implications have already been discussed. It is sufficient to say that the first fraction of "shunt bilirubin" is clearly independent of erythropoieses and must be derived, in the main, from haem-proteins other than haemoglobin.

The major organs of porphyrin synthesis are the marrow and the liver (375) and to a much lesser extent, the kidney. By inference, the liver is the main site of non-haemoglobin haem-protein synthesis. There is a certain amount of experimental evidence in support of this inference.

The experimental observations of Schwartz and Cardinal (401) have already been discussed at length. To reiterate, following the I.M. injection of ALA-4-C^{14} to dogs, peak values of haem-C^{14} activity were obtained within 1/2 to 2 hours when 22.2% and 7.3% of the administered dose was present in the easily-split haems of liver and kidney respectively. There was a rapid fall-off of activity over the following 29 hours. What is the time between breakdown of tissue haem-C^{14} and the appearance of the label in plasma bilirubin? Ostrow et al (332) showed that a constant rate of bilirubin-C^{14} excretion in the bile was observed as soon as an hour after the onset of sequestration of infused labelled red cells. This time lag represented liberation of haemoglobin from the red cells, entry into the R.E.S., degradation to bilirubin, and excretion in the bile. One can infer from these findings, that the interval between the breakdown of liver and kidney haem-C^{14} and the appearance of bilirubin-C^{14} in the plasma must be in the order of well under an hour. .....P.T.O.

probably minutes. The work of Schwartz and Cardinal (401) thus provides direct evidence that, in dogs, the turnover of hepatic and renal haem-C^{14} is rapid enough to account for the early peak of "shunt" bilirubin and that activity in the liver is significantly more than in the kidney.

Israels et al (207) showed that in bile-fistula dogs rendered porphyric with Sedormid, the first peak of early-labelled bilirubin occurred very much sooner, following glycine-2-C^{14}, than in normal controls. As Sedormid primarily affects porphyrin synthesis in the liver, these observations are in favour of the first peak being hepatic in origin.

The elegant studies on bile-fistula dogs by Schwartz, Ibrahim and Watson (400) have added further important information. They made the following points. 20% of the ALA-C^{14} administered was excreted within a few days in the bile as bilirubin-C^{14}. Of this, 99% was derived from non-erythropoietic haem. Peak activities occurred in 3 to 6 hours. Under similar experimental conditions, animals were sacrificed at varying intervals following ALA-C^{14} administration and non-haemoglobin haem compounds were extracted from liver and other tissue They showed that liver haems alone contained more C^{14} at 3 to 83 hours than would be necessary to account

for all the bilirubin-C^{14} excreted in parallel experiments on comparable injected dogs. Disappearance of C^{14} liver haems closely paralleled the excretion of bilirubin during the same intervals. The authors concluded that a major portion of liver haem-compounds are intermediates in the synthesis of bilirubin and constitute the bulk of the non-erythropoietic component of "shunt" bilirubin.

The excretion of bile pigment in bile fistula rats was studied by Robinson, Owen, Flock and Schmid (364). Following the infusion of glycine-2-C^{14} I.V., the rate of C^{14} bilirubin excretion in the bile was maximal after 1½ hours and then fell rapidly. In these animals, the rapidity of the process made it unlikely that the labelled bile pigment resulted from the degradation of red cell haem in the bonemarrow. Direct hepatic synthesis was more likely.

Robinson et al (364) then carried out isolated rat liver perfusion experiments in which surviving rat livers were perfused with glycine-2-C^{14}. They noted remarkably rapid labelling of bile bilirubin, the rate and magnitude of which were comparable to those of the early labelled pigment in the intact animal. It is therefore possible that direct synthesis of bilirubin by the liver alone may account entirely for the early component of shunt bilirubin.

Both by inference and experimental evidence, the liver appears to be the principal organ producing that component of early-labelled bilirubin derived from non-haemoglobin haem. It is not known whether hepatic haems, haem-proteins or both are the immediate bilirubin precursors. Data on haem-protein turnover is scanty and has already been reviewed. While about 12.7% of cytochrome c is newly synthesized by the liver every day 81,82), liver catalase is the most rapidly turning over haem-protein in the body (442). The exceedingly short life span of a few days (70,237,383) means that a 1/3 to a 1/2 of liver catalase may be newly synthesised and destroyed each day. This rate of turnover is compatible with the early appearance of the first peak of shunt bilirubin.

(c) Relevance to present thesis.

South African genetic porphyria and symptomatic porphyria are diseases characterized by disordered porphyrin metabolism in the liver. How this affects haem and haem-protein turnover is important in our understanding of the biochemical defects involved and the mechanisms responsible for the acute symptoms in the inherited forms of the disease. The technical difficulties in estimating the turn-over of liver haem are considerable when dealing with the minute amounts of

this organ obtained by conventional biopsy. Thus, by studying the effect of human porphyria on the first, non-erythropoietic component of early-labelled bilirubin, valid indirect information may be obtained regarding liver haem turnover in this group of diseases.

C. TRANSPORT OF BILIRUBIN IN SERUM.

Burnstine and Schmid (52) have shown that, at physiological pH, only small amounts of unconjugated bilirubin are soluble in protein-free aqueous solutions. In plasma, virtually all bilirubin is bound to protein (118) and albumin plays a major if not the sole role in this binding (118,223,460). Some authors have suggested that, in addition, specific bilirubin carrying proteins may exist in α or β globulins (14, 146,150,286,311). However, Klatskin et al (223) could not demonstrate the role of globulins in bilirubin-protein binding.

Because of the difficulties of measuring and identifying the small quantitities of bilirubin normally present in serum, these studies were performed in icteric sera (14,150,223,311,460). Ostrow and Schmid (333) obviated the above difficulties by studying how small quantities of added bilirubin-C^{14} of high specific activity were bound to various serum proteins. Using continuous flow electrophoresis, these authors were able to show that in both human and murine sera, where bilirubin concentrations were within or close to physiological range, bilirubin was bound solely to albumin.

In man, one mole of albumin is capable of binding 2 moles of bilirubin in such tight association that it

becomes virtually non-dialysable (14,286,311,460). In other words, 1 gram of albumin can bind tightly 16 mg of bile pigment. Below this concentration, there is very little free plasma bilirubin; above this level, Ostrow and Schmid (333) found significant amounts of the label in the dialysate as diazonegative water soluble pigment derivatives. It has been claimed that certain organic anions such as salicylates, sulphonamides, bile acids and fatty acids may compete with bilirubin for common binding sites on albumin and thus may substantially alter the pigment-binding capacity of plasma (262). The binding of conjugated bilirubin to albumin is less well understood and the binding forces are different (262).

The functional significance of serum bilirubin-albumin binding is more than one of transport of bile pigment from the R.E.S. to the liver. It is also a major factor in regulating the distribution of bilirubin between blood, extravascular fluids and other organs. In man, bilirubin and albumin have a similar compartmental distribution (391). Another important function may be in governing the rate of pigment removal from the blood by the liver. In foetal blood, dissociation of albumin and bilirubin has been shown to take place at the placenta (379). In human studies, bilirubin disappears

from plasma (467) far more rapidly than albumin (15). However, the measured uptake of albumin by the liver may only appear slower than that of bilirubin as this may represent the net result of more rapid bidirectional fluxes of the protein (333). For these reasons, it cannot be decided at present whether bilirubin is dissociated from its binding site on extra-cellular albumin before entering liver cells or whether the albumin-pigment complex reaches the intracellular space intact with subsequent rapid return of the de-pigmented albumin to the extracellular fluid (333). The former seems more likely (393).

D. CONJUGATION AND EXCRETION OF BILIRUBIN.

Bilirubin flux into the liver cell depends primarily on concentration gradient across the cell membrane. This may be accomplished by an acceptor substance in the cell with a high affinity for bilirubin or by conjugation of the pigment immediately after its transfer into the cell (393).

(1) Conjugation of Bilirubin.

This aspect of bilirubin metabolism will be dealt with briefly as it does not directly concern work done in this thesis.

Bilirubin is excreted in the bile mainly as the conjugated water-soluble glucuronide (28,387,393). As it is water-soluble, it exhibits a "direct" action with diazo reagent as opposed to unconjugated bilirubin which must first be "solubilized" by the addition of a suitable solvent, e.g. alcohol. Glucuronic acid is attached to the carboxyl groups of the propionic acid side chains. The conjugate, therefore, can be a mono- or diglucuronide. In man and animals, however, recent observations suggest that the little monoglucuronide isolated from conjugated bilirubin is not a pure substance but a labile equimolar complex of bilirubin and bilirubin diglucuronide (159,466).

The source of the glucuronide group is the nucleotide uridine-diphosphate glucuronic acid (UDPGA) formed by the dehydrogenation of uridine diphosphate glucose (393). This reaction is catalyzed by a soluble liver cell dehydrogenase system (88,433).

The biosynthesis of bilirubin glucuronide has been studied with liver slices (249), homogenates (163,249) and microsomal preparations (385). By these and other studies, it has been established that the enzyme system, glucuronyl transferase, which catalyses the reaction, is localized to the liver microsomes (88,385,424,433), probably in the smooth portion of the endoplasmic reticulum (252).

In summary, unconjugated bilirubin after entering the liver cell, becomes attached to or enters liver microsomes. Here conjugation with glucuronic acid is catalyzed and the bilirubin glucuronide is excreted via the biliary duct system to the intestine.

(2) Fate of Bilirubin in the Intestine.

(a) Reduction to stercobilin.

The major portion of conjugated bile pigment excreted undergoes stepwise reduction by bacterial enzyme systems, mainly in the large bowel. Thus mesobilirubin, mesobilirubinogen and stercobilinogen are formed, the latter readily autooxidized to stercobilin.

Part of the stercobilinogen may be absorbed and re-excreted by the liver, thus constituting the entero-hepatic circulation. When the load of absorbed stercobilinogen presented to the liver is large, as in haemolysis, or when there is liver failure, some may be excreted in the urine as urobilinogen.

Although 300 to 350 mg bilirubin are excreted daily, only about 140 mg can be accounted for as faecal stercobilin and 5 to 20 mg as faecal bilirubin (378). The reasons for this discrepancy are not clear. During the enterohepatic circulation of stercobilinogen, part may be destroyed by the liver (459) or following its re-excretion to the duodenum. Experimental techniques may be inadequate in quantitatively recovering all ~~the~~ all the stercobilin present in the stool. It has already been noted that in diseases where there is inability by the body to excrete bilirubin via the normal channels (55,390), bile pigment may be excreted in the form of diazonegative breakdown products. It is possible that following normal excretion, some bilirubin may be degraded in a similar manner in the intestine. Although Gilbertson et al (129) and other workers (388) have isolated and identified dipyrrolic compounds belonging to the group of bilifuscin, mesobilifuscin and pentdyopent, it is by no means established that faecal dipyrrols are derived from bilirubin (129).

These compounds are thought to be mainly anabolic in origin (459).

(b) Absorption of bilirubin.

It is only recently that with the development of methods for the biosynthesis and crystallization of bilirubin-C^{14}, the enteral absorption of unaltered bile pigment has been studied by Lester et al (258, 259,260,261) in humans and experimental animals. Rats were given free and conjugated bilirubin-C^{14} via duodenal tube. After the infusion of the unconjugated bilirubin-C^{14} into the duodenum, 8 to 18% of the administered dose could be recovered from freshly excreted bile as crystalline bilirubin-C^{14} while with bilirubin glucuronide, corresponding values were 7 to 20% (260). However, during the first 4 hours following the administration of unconjugated bilirubin-C^{14}, biliary excretion of radioactivity was 2 to 5 times more rapid than when the conjugate was given. In the former case, unconjugated bilirubin could be crystallized from the portal vein while in the latter absorption of the conjugate was too slow to perform comparable studies. These findings suggest that free bilirubin is absorbed as such while the conjugate is first hydrolyzed in the rat before absorption. Using isolated segments of intestine with intact blood supply, Lester (260) was able to show that the radioactive label was absorbed

from the whole length of the small bowel and also the large intestine.

Similar experiments were performed on Gunn rats and a child suffering from the Crigler-Najar syndrome. The Gunn rat is a mutant strain known to lack hepatic glucuronyl transferase. It therefore retains all unconjugated bilirubin but is able to excrete exogenous conjugate (386). The Crigler-Najjar syndrome is characterized by congenital, familial, non-haemolytic, unconjugated hyperbilirubinaemia presumed to be due to an enzymic defect comparable to that in Gunn rats (11). Unconjugated and conjugated bilirubin C^{14} were administered into the duodenum and 24 hours later, bilirubin was crystallized from the plasma. In the rats studied, irrespective of the nature of bilirubin administered, 6 to 34% of the dose was retained in the plasma, all as unconjugated bilirubin-C^{14} while no conjugated pigment was excreted in the bile (260). In the human study, with unconjugated labelled pigment, 80% of the dose was absorbed and retained as unconjugated bilirubin while, with the conjugate, virtually no bilirubin-C^{14} activity appeared in the serum.

In studies on two human adults with external biliary drainage and normal liver function (260), 15%

of duodenally administered unconjugated bilirubin was absorbed and excreted as conjugated bilirubin-C^{14} while less than 2% of the dose was present in the bile of the patient receiving the conjugated pigment (260).

In summary, the following important points have emerged from the experimental findings of Lester et al. In humans and rats, unconjugated bilirubin is absorbed intact and unaltered, and can reach the systemic circulation in appreciable quantities if the liver is incapable of clearing the portal blood. However, the conjugate remains unabsorbed unless hydrolysed. While rat intestine can hydrolyse and thus absorb bilirubin glucuronide, human intestine cannot. As physiologically bilirubin is excreted in its conjugated form, one can infer that there is no functioning enterohepatic circulation of bilirubin of any significance in man.

This observation is of particular relevance to the present thesis. It can now be assumed that, following the administration of glycine-2-C^{14} or ALA-C^{14} to humans, serum bilirubin-C^{14} activity will be an index of endogenous bile pigment production only and will not be affected by the negligible intestinal absorption of bilirubin-C^{14} excreted in the bile. Thus the significance of two discrete peaks of early-labelled bilirubin activity following isotopic glycine, remains unaltered.

B. THE BILIRUBIN POOL.

In man, bilirubin and albumin have a similar compartmental distribution (391). Thus bilirubin is widely distributed in intravascular and extravascular fluids and various organs. Takeda and Reeve (434) studied the distribution of albumin in healthy men with the aid of autologous I^{131} albumin. They showed that the ratio of distribution between the extravascular and intravascular spaces was about 1.4:1. One would expect a similar ratio between the extravascular and intravascular bilirubin pools.

Infused bilirubin-C^{14} is so rapidly cleared in normals that an equilibrium between endogenous and exogenous pigment is not attained. Schmid (390) administered bilirubin-C^{14} to a 4½ year old boy with unconjugated hyperbilirubinaemia due to congenital absence of glucuronyl transferase in his liver (Crigler-Najjar syndrome) and therefore inability to conjufage and excrete bilirubin normally. Schmid (390) showed that, following the injection, there was a rapid fall of activity in the blood followed by a slower phase of mixing. From 30 hours, specific activity declined logarithmically indicating complete equilibrium within the miscible pool. In this 20 kg child, plasma pool was 280 mg and the total miscible pool 568 mg. The total pool was 28.4 mg/kg and the extravascular/intravas-

cular pool ratio was 1:1, comparing closely with the 1.4:1 ration obtained by Takeda et al for albumin.

Cameron et al (55) performed similar studies on infants with conjugated hyperbilirubinaemia due to biliary atresia. There was a rapid decline in serum radioactivity (mixing of the isotope in the miscible pool) extending over 48 hours. This was followed by a second, more gradual, linear decline. In three children studied, average total ratio was 7.2:1. This figure was at variance with the ratios obtained above. Undoubtedly, there was minor cirrhosis with some alteration in albumin distribution, but in none of the cases was the disturbance gross enough to account for the disproportionately large extravascular distribution of bilirubin. A more likely explanations is that, in the human subject, unconjugated bilirubin is more completely bound to albumin than conjugated bilirubin and this may account for the discrepancy between children with biliary atresia (conjugated hyperbilirubinaemia) and the child with Crigler-Najjar syndrome (unconjugated hyperbilirubinaemia). The less complete binding of the conjugated bilirubin by albumin may allow the pigment to ~~leak off~~ escape and bind to tissue proteins or lipids (55).

Thus, by inference, in a non-icteric human subject,

once the intravascular bilirubin pool has been calcula
it is likely that the total miscible pool is in the or
of 2 to 2½ times larger.

CHAPTER IV.

THE METABOLISM OF PURINES AND URIC ACID.

A. THE BIOLOGICAL CHEMISTRY OF URIC ACID IN MAN.

(1) General Considerations.

Uric acid (2,6,8 - trioxypurine) is the fully oxidised ultimate intact purine product of the biological oxidation of purines, further oxidation resulting in the disruption of the purine ring. It is merely a metabolic waste product with no perceptible physiological function.

In the uricotelic vertebrates e.g. birds and reptiles, the enzymes necessary for the biosynthesis of urea are lacking and uric acid nitrogen may constitute over 90% of total urinary nitrogen (171). The low solubility of uric acid allows nitrogen to be excreted via the cloaca in solid form, thus conserving water.

The mammal, however, is ureotelic, i.e. can make urea. In most mammals, any uric acid passing through the glomerulus is reabsorbed and converted by uricase (chiefly in the liver) to readily soluble allantoin. Man and other higher primates differ in that, although ureotelic, they have no uricase and excrete oxypurines as uric acid (171).

Uric acid is a divalent weak organic acid due to the ionisation of the hydrogen atoms at positions 9 (pka = 5.75) and 3 (pka = 10.3) of the molecule (405). The hydrogen atoms at positions 1 and 7 do not ionise significantly (405). As free uric acid is less soluble than the monovalent urate ion, the pH of the biological fluid determines many of the characters of urate in the human body.

(2) Urate in Biological Fluids.

(a) Serum.

In blood, urate is not bound to protein but occurs mainly in free form (71,405). This was shown by plasma electrophoresis where neither the uric acid originally in the plasma nor that added to it migrated with protein (451). Virtually all plasma urate is readily ultra-filtrable (499). At the plasma pH of 7.4, the acid is mainly present as the monoalkali salt. Since the principal cation of extra-cellular fluids is sodium, the solubility properties of uric acid in the body will be predominantly those of monosodium urate and, as expected, gouty tophaceous deposits are largely composed of this salt (171,405). In serum with 0.13 molar sodium content, saturation should occur at a urate concentration of 6.4 mg per 100 ml (405). The presence of much higher values in hyperuricaemic

states may be due to the tendency of sodium urate to form supersaturated solutions or to an unexplained enhancement of urate solubility by serum constituents (405).

The serum urate concentration for the normal adult male is 5.1 ± S.D. 0.93 mg per 100 ml. when determined by the enzymatic spectrophotometric procedure (156). Values obtained with the colorimetric determination of Archibald average 0.23 mg per 100 ml less than duplicate determinations by the enzymatic spectrophotometric procedure (266). The distribution of 940 non-gouty normals and 60 gouty males was studied by Seegmiller et al (404,405) and it was found that there was a significant overlap between the two distribution curves. Because of this overlapping, it was felt by the authors that a value of 7 mg per 100 ml i.e. 2 standard deviations above the mean, should be regarded as the upper limit of normal. By the same token, values below 3.2 mg per 100 ml should be regarded as abnormally low and this has been born out by Seegmiller's distribution pattern where it appears that less than 2% have urate concentrations below this figure. Gutman et al (164) used a normal ~~in men~~ of 6.5 mg per 100 ml. in men and ~~in women~~, 5.5 mg per 100 ml. in women.

(b) Urine.

At the lower pH levels encountered along the urinary tract, a substantial portion of urate ions are converted to free uric acid. Whilst the solubility of monosodium urate is 118 mg per 100 ml water, that of free uric acid is only 6.5 mg per 100 ml water. Deposits of urate in the urinary tract will consist primarily of uric acid rather than monosodium urate (cf. tophi) (171,405).

Monosodium urate occurs as the monohydrate forming crystals in a monoclinic or triclinic system, characteristically needle or bar shaped. If precipitation is rapid, amorphous fine crystals produced. Free uric acid crystallizes in an orthorhombic crystal system forming rhombic plates in pure solutions (405).

On a low purine diet, an adult man excretes about 200 to 500 mg. uric acid per day in his urine. An ordinary meal containing purines is followed by an appreciable rise in urinary uric acid excretion and clearance with little effect on serum urate levels (171,405). Only with larger amounts of added purine in the diet does the serum urate level rise. Thus while serum urate is an accurate reflection of the miscible uric acid pool in the body, daily urinary excretion of uric acid in addition promptly reflects changes in dietary purine. These observations and the

fact that man can develop normally on a diet devoid of purines indicate the presence of a de novo biosynthetic pathway of purine synthesis from non-purine precursors.

(c) Other body fluids.

In normal man, the serum concentration of uric acid is about twice that calculated to be present if the total body pool of urate were uniformly distributed in the body water (16). Thus urate is not uniformly distributed among several body water compartments. Assuming extracellular fluid to be in equilibrium with plasma urate, some intracellular compartments must have relatively little urate. Gudzent (cited ref. 171) found very little urate in some tissues, e.g. fat, while red cell urate was found by Jorgensen et al (213) to be about half that of plasma. Seegmiller (405) in discussing this aspect points out the correlation between low C.S.F. urate and the rarity of tophaceous deposits in the central nervous system. Low concentrations of urate have been observed in sweat and the urate concentration of saliva is substantially lower than that of plasma (405).

B. BIOSYNTHESIS OF THE PURINE RING.

(1) General considerations.

It is only in the last ten to fifteen years that research workers have elucidated the individual reactions in the biosynthesis of the purine nucleus. Recent excellent reviews include those of Wyngaarden (496), Gutman (171), Seegmiller (405), Watts (464) and Buchanan et al (47,48). The investigations indicate that the purine ring is elaborated stepwise in a complex series of sequential enzymatic reactions from the following small molecular weight precursors:- glutamine, glycine, aspartic acid, formyl derivatives of tetrahydrofolic acid and carbon dioxide, each donating specific nitrogen or carbon atoms.

The initial intermediate is 5 phosphoribosyl-1-pyrophosphate (PP-riboseP) an important intermediate in the metabolism of purine nucleotides. The ribose-phosphate moiety is retained throughout the biosynthetic pathway of purines, intermediates thus occuring as ribonucleotides. The first complete purine to be formed in the sequence is inosinic acid (hypoxanthine ribonucleotide) which is therefore the primary nucleotide from which all other purines, including uric acid, are subsequently elaborated.

The overall reaction can be summarized by the following equation:- $2NH_4^+$ + glycine + aspartate + 2 active C-1 fragments + HCO_3^- + ribose-5-phosphate + ATP = inosinic acid (inosine monophosphate or IMP) + fumarate + 9 H_2O. It can be seen that of the 9 atoms comprising the purine nucleus, 6 are derived directly from amino acids, viz. glutamine amide (2), glycine (3) and aspartate, 2 from active fragments of which serine is an important donor and one from carbon dioxide derived in part from amino acid carbon. Thus the biosynthesis of purines is, to a large extent, dependent upon amino acid metabolism (171).

(2) 5 - phosphoribosyl-1-pyrophosphate (PP-ribose-P).

There are two main pathways of nucleotide biosynthesis in the human and PP-ribose-P is a key precursor in both.

(a) De novo biosynthesis implies step-wise elaboration of the purine ring from small molecular weight precursors. The first and rate-limiting step is the irreversible condensation of PP-ribose-P and glutamine.

(b) "Salvage pathways" are those in which PP-ribose-P combines directly with formed purine bases in the direct synthesis of purine ribonucleotides by a condensation reaction, inorganic pyrophosphate being liberated. (101,283).

PP-ribose-P is formed by the transfer of the terminal pyrophosphate group of ATP to carbon 1 of ribose-5-phosphate (227,357):

Ribose-5-phosphate + ATP _____ PP-ribose-P + AMP.

Ribose-5-phosphate can arise from three possible pathways (496), viz.

(a) as an intermediate in the 6 phosphogluconic oxidation of glucose (direct oxidative pathway of glycolysis),

(b) as a product of non-oxidative cleavage of fructose-6-phosphate,

(c) as a late product of the metabolism of glucose-phosphate via uronic acid pathway.

The phosphogluconic acid pathway is the main source of ribose-5-phosphate in those tissues with an active direct oxidative pathway for glucose e.g. liver (32) and bone marrow (33). The fructose cleavage pathway is important in tissues where Embden-Meyerhof pathway of glycolysis is dominant, e.g. skeletal muscle (32). Both pathways are operative in man (192) the uronic acid cycle being of minimal physiological significance.

(3) <u>The enzymatic synthesis of the purine nucleus.</u>

The de novo biosynthesis of the parent purine nucleotide inosine-5-monophosphate (IMP, inosinic acid,

hypoxanthine ribonucleotide) involves at least 10 enzymatically catalyzed reactions. These will be briefly reviewed with a little more emphasis on the incorporation of C4 and 5 (derived from glycine) and C 2 & 8 (from C-1 fragments), those carbons whose activity following the administration of glycine-2-C^{14} has been studied in this thesis.

(a) Reaction 1, formation of N-9.

The first specific reaction of purine biosynthesis is an irreversible reaction in which the amide group of glutamine displaces the pyrophosphate group of α 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P), with simultaneous inversion of configuration to yield β-5-phosphoribosyl-1-amine (137,139,176). The β linkage is characteristic of the glycosidic bond of all known ribonucleotides. The glutamine amide nitrogen contributes N-9 of the evolving purine nucleus. The reaction is as follows:-

α-PP-ribose-P+glutamine+H_2O ——→

β-5-phosphoribosyl-1-amine + glutamic acid + pyrophosphate (PPi).

Being the first irreversible step, it is potentially important in the regulation of purine synthesis. The enzyme catalyzing the reaction, glutamine phosphoribosylpyrophosphate amidotransferase, is the site of a

feed back control by certain adenine and guanine ribonucleotides (60,325). Both Caskey (60) and Wierlich (325) using pigeon liver and aerobacter aerogenes as source of the purified enzyme, showed that inhibition was strictly competitive with PP-ribose-P and mixed competitive-non-competitive against glutamine. Kinetic evidence suggests (60) that the enzyme has separate binding sites for 6 aminopurine and 6 hydroxy-purine ribonucleotide inhibition and there is an increased inhibition when adenine and guanine nucleotides are both present (60,325). Adenosine monophosphate (AMP), guanosine triphosphate (GTP) and inosine monophosphate (IMP) showed less inhibition while no effect was obtained with adenosine triphos-phate (ATP), adenine alone and cytosine monophosphate (CMP) (325).

(b) Reaction 2, formation of C 4,5,N7.

The next step in the synthesis of the purine ring is the reaction between glycine and phosphoribosyla-mine to form glycinamide ribonucleotide (GAR). ATP is involved in this reaction (138,139,175,177) which is as follows:-

β-5-phosphoribosyl-1-amine + glycine + ATP $\rightleftharpoons$ β-glycinamide ribonucleotide (GAR) + ADP + P

The reaction is catalysed by a specific kina-

synthetase, glycinamide ribonucleotide kinosynthetase. By this reaction, the glycine molecule is incorporated in-toto- contributing C4, C5 and N7 of the evolving purine structure. Hartman et al (177) have studied the reaction with P^{32}-labelled ATP. From their data, the authors concluded that the reaction is reverible and that all the substrates interact at the enzyme surface without formation of new covalent linked compounds as intermediates.

(c) Reaction 3, formation of C8.

The ureido carbons (C2 and C8) of the purine nucleus are derived from active carbon fragments. The metabolism of C-1 units and its relationship to purine biosynthesis will be discussed in more detail later this chapter under the section dealing with the incorporation of labelled glycine into the purine nucleus (see page 222-225).

In reaction 3, β-glycinamide ribonucleotide (GAR) receives a one-carbon fragment from an active formyl tetrahydrofolic acid (THFA) derivative (138,175,178, 456,457). Hartman and Buchanan(178) using a highly purified transformylase system free from amidohydrolase activity were able to show that N^5, N^{10} anhydroformyl THFA rather than N^{10} formyl THFA was the immediate formyl donor. The formylation of GAR is catalised by the enzyme glycinamide ribonucleotide transformylase

with the production of α-N-formylglycinamide ribonucleotide (FGAR) according to the following reaction:-

GAR + N^5, N^{10} anhydroformyl THFA + H_2O $\rightleftharpoons$ FGAR + THFA + H^+ .

(d) Reaction 4, formation N-3

Amidization now occurs, the FGAR compound receiving the amide group of glutamine to form the corresponding amidine compound (264,293). In this way, the N-3 atom of the purine nucleus is incorporated.

FGAR + glutamine + ATP + H_2O $\rightleftharpoons$ α-N formylglycinamidine ribonucleotide (FGAM) + glutamic acid + ADP + Pi.

(e) Reaction 5, closure of the imidazole ring.

Enzymatic ring closure occurs to complete the imidazole ring (263). This 5-membered imidazole ring will form one of the two fused heterocyclic rings of the purine nucleus. ATP is required for the reaction which is:-

FGAM + ATP ——— 5 aminoimidazole ribonucleotide(AIR)+ ADP + Pi.

(f) Reaction 6, formation of C6.

AIR now receives a carboxyl group at C-4 by a CO_2 fixation reaction catalysed by AIR carboxylase (282) probably involving biotin as a coenzyme(303). In this way C-6 is formed.

AIR + HCO_3^- = 5 amino 4 imidazole carboxylic acid ribonucleotide (C-AIR)

(g) Reaction 7, formation N-1.

The carboxyl group serves as a point of condensation with aspartic acid through an amide linkage, ATP being necessary (282). In this reaction, 5 amino-4 imidazole-n-succinocarboxamide ribonucleotide (SAICAR) is formed, the nitrogen atoms of aspartic acid forming the eventual N-1 of the evolving structure.

C-AIR + Aspartic acid + ATP $\rightleftharpoons$ SAICAR + ADP + Pi.

(h) Reaction 8, hydrolysis of SAICAR.

By hydrolysis, the succinic acid residue is split off, yielding 5-amino-4 imidazole carboxamide ribonucleotide (AICAR), a compound lacking only C-2 for a complete purine ring.

SAICAR $\rightleftharpoons$ Fumaric acid + AICAR.

(i) Reaction 9, incorporation C-2.

AICAR receives a "single-carbon" fragment from active formyl THFA (102,103,178) probably N^{10}-formyl THFA (178). The reaction is catalysed by the enzyme AICAR transformylase (102,103,178). When 5-amino-4 imidazole carboxamide itself is given, it is first converted to its ribonucleotide, AICAR by a nucleotide pyrophosphorylase before being metabolized further (101). For details re "one-carbon" atom metabolism and its relationship to purine synthesis, refer to section on the incorporation of labelled glycine into purines later this chapter - pages 222-225.

AICAR + N^{10} formyl THFA $\rightleftharpoons$ formamido-4-imidazole-carboxamide ribonucleotide (formyl AICAR) + THFA.

(j) Reaction 10, closure of the ring.

Ring closure completes the synthesis of the purine nucleus, inosine-5-monophosphate (IMP) or inosinic acid being formed.

C. METABOLISM OF PURINE NUCLEOTIDES.

(1) General considerations.

Purine nucleotides are compounds composed of a purine base linked via D-ribose or 2-deoxy-D ribose to a phosphate moiety. Various nucleotides differ according to their nitrogenous base or to their sugar bridge. Thus there are ribonucleotides and deoxyribonucleotides.

(a) The nucleotide pool has two potential sources:-

(i) Newly synthesized nucleotides.

(ii) Those arising from the catabolism of nucleoproteins and nucleic acids. The latter may be endogenous in origin or arise from dietary purines. Aspects of their metabolism will be discussed later.

(b) The nucleotides in the pool also have two potential fates:-

(i) Cleavage, with formation of nucleosides and ultimately purine bases.

(ii) Reincorporation into nucleic acids.

(c) Following cleavage, purine bases and their nucleosides may be:

(i) "Salvaged" and reincorporated into nucleotides, or

(ii) "lost", oxidised and excreted as uric acid.

Aspects of the above will be discussed in the following sections.

(2) The Synthesis of Purine Nucleotides.

There are two main pathways in the biosynthesis of purine nucleotides namely,

(a) de novo synthesis from small molecular weight precursors of the parent nucleotide, inosine-5-monophosphate (IMP) and its subsequent conversion to other purine nucleotides, and

(b) Salvage pathways whereby purine nucleotides are synthesised from preformed nucleotides or purine bases, these arising either from dietary purines or from previous cleavage of purine nucleotides. These pathways "salvage" purine compounds from oxidation into uric acid. Two types of reactions are operative:-

(i) condensation of a free purine base with PP-ribose-P or

(ii) phosphorylation of a purine nucleoside catalysed by a nucleoside kinase.

(a) de Novo synthesis of purine nucleotides.

IMP is the parent purine nucleotide from which by a variety of interconversions, other metabolically important purines are formed.

(i) Adenosine-5-monophosphate (AMP, Adenylic acid).

The conversion of IMP to AMP (2,3,107,267,297,298) occurs in 2 steps:-

(a) IMP + Aspartic acid + GTP ⟶ adenylo succinic acid (AMP-S) + GDP + Pi. Energy derived from GTP. This reaction is analagous to reaction 7 in the formation of the purine nucleus and is catalyzed by adenylo-succinate synthetase.

(b) Cleavage of AMP-S yields AMP + fumaric acid and is catalyzed by the enzyme adenylo-succinase. This reaction is freely reversible and is analagous to reaction 8 in purine synthesis.

AMP-S ⟷ AMP + fumarate.

(ii) Guanosine monophosphate (GMP, Guanilic acid).

The formation of this nucleotide also takes place in 2 steps (2,241,284):

(a) The first is the irreversible oxidation of IMP to Xanthosine-5-monophosphate (XMP, xanthylic acid) with NAD as the hydrogen acceptor.

IMP + NAD^+ + H_2O ⟶ XMP + $NADH^+$ + H^+ .

This is catalyzed by inosine-5-phosphate dehydrogenase

(b) In the second reaction, xanthosine-5-phosphate aminase catalyzes the transfer of the amide nitrogen of glutamine to carbon 2 of the purine moiety of XMP, thus forming GMP.

XMP + Glutamine + ATP + H_2O ⟶

GMP + glutamic acid + AMP^2 + PPi.

(b) Salvage pathways of purine nucleotide synthesis.

Two pathways exist for the regeneration of purine

nucleotides from purine nucleosides and free purine bases.

(i) Condensation of free purine base with PP-ribose-P.

PP-ribose-P may react directly with free purine bases to form the corresponding ribonucleotides along the following lines:

Purine base + PP-ribose-P ⟶ Base-ribose-phosphate + PPi.

The reaction is catalyzed by purine ribonucleotide pyrophosphorylases of which two different types have been identified. One acts on adenine (101) and the other on hypoxanthine, guanine and 6-mercaptopurine (283,348). These enzymes are widely distributed (496). The reaction is strongly inhibited by ATP (369,465).

(ii) Phosphorylation of a purine nucleoside.

Purine bases may first be converted to a ribonucleoside phosphorylase (106,216,224). This may subsequently be phosphorylated to the ribonucleotide by the action of the appropriate kinase (58,140,226). These reactions may be summarized as follows:-

Purine + ribose-1-phosphate ⟶ ribonucleoside + Pi

Ribonucleoside + ATP ⟶ ribonucleotide + ADP

Nucleoside phosphorylase are widely distributed in mammalian tissue especially liver (224) and erythrocytes (373).

(c) The biological importance of "salvage" as opposed to "de novo" Pathways of Nucleotide synthesis.

Salvage pathways are of considerable biological importance. Firstly, the salvage pathways are more economical with regard to conserving energy than the de novo route. Only one high energy bond is used in the form of PP-ribose-P or ATP in nucleotide synthesis as opposed to de novo biosynthesis where a minimum of 6 high energy molecules are required (496).

Secondly, not only may many tissues use salvage pathways preferentially (140) but some tissues may be entirely dependent upon a supply of preformed purine bases or nucleosides from the liver or diet (365) for purine nucleotide synthesis.

Goldthwait (140) showed that in heart muscle extracts, synthesis of purines via the de novo pathway was less efficient than via salvage route. Of the latter, phosphorylation of nucleosides seemed more efficient than the direct condensation of purine base with PP-ribose-P. Skeletal muscle has been shown to be able to utilise adenine and guanine in-vitro (140) as has liver and intestine in-vivo (20,45).

Abrams et al (3) showed that in-vitro, rabbit bone marrow extracts could convert C^{14}-inosine and C^{14} IMP

to adenine and guanine compounds. Hypoxanthine-8-C^{14} not utilized in this in-vitro system. Lajtha et al (242) reviewed in-vitro experiments on synthesis of purine by rabbit and human bone marrow. Formate-C^{14} was utilized inefficiently for de novo synthesis of purines but efficiently labelled thymine in DNA. When liver extracts were added to these systems, the ability to synthesize purines from formate was restored. Lajtha et al (242) set out to evaluate the role of the liver (as a supplier of preformed purine bases and nucleosides) in bone marrow purine synthesis in-vivo. Rabbits were bled to ensure reactive marrow. Control and hepatectomized rabbits studied. Effects of trauma etc. were allowed for by a suitable variety of controls. 5 to 10 min. post-op, sodium formate-C^{14} was given I.V. Rabbits were killed 90 min. later and femoral and tibial bone marrow were examined. Results showed that where liver was intact (controls), rabbits could readily synthesize thymine and adenine in the bone marrow. However, in hepatectomized rats, while incorporation into thymine still occurred (at a lower level), incorporation into bone marrow adenine was virtually nil. The authors considered three mechanisms to explain these observations. The liver may have provided an enzyme with which bone marrow could synthesize purines de novo. This enzyme would

have to have had a life span of minutes to explain results as formate-C^{14} given 5 to 10 minutes post-operatively. On the other hand, the liver may have provided an advanced purine precursor, e.g. AICAR Again this was unlikely as bone marrow has been shown to incorporate AICAR poorly compared to adenine-to purine or formate-C^{14} to thymine. The final possibility was that the liver provided a readily utilizable supply of preformed purine bases or nucleoside-C^{14} which were incorporated into nucleotide by bone marrow salvage pathways. This seemed the most likely mechanism.

From their findings, Lajtha et al (242) have concluded that in mammals, the liver is the main source of purine nucleotide precursors for bone marrow cells.

In confirmation with above findings, Lowy et al (280) have shown that while mature human red cells can utilise purine bases and nucleosides for ATP and GTP formation, they cannot incorporate sodium formate into IMP, nor can they convert IMP to AMP. This again emphasizes the dependence of haemopoietic tissues on salvage pathways for purine nucleotide biosynthesis.

(3) <u>Relevance of Salvage Pathways of Nucleotide synthesis to Thesis.</u>

The above findings stress how in some tissues

salvage pathways may be more important than de novo routes in the biosynthesis of purine nucleotides in peripheral tissues (140). It appears that haemopoietic tissue is completely dependent upon preformed purine bases and nucleosides for nucleotide synthesis (3,242,282). Lajtha (242) has provided excellent evidence that the liver de novo biosynthetic pathway is virtually the entire endogenous source of preformed purines for utilisation by peripheral tissues.

In this thesis, an attempt has been made to study purine synthesis in porphyria - a disorder of hepatic enzymic function. If liver purine synthesis is impaired in hepatic porphyria, the metabolism of purines in many other tissues will be materially affected. Thus, purine bases available for urie acid production from any tissues other than the liver will drop, thus magnifying any hepatic defect. Finally, this thesis attempts to not only include pick up gross changes in the quantity of urie acid production, but also the more subtle changes in the composition of the purine nucleus as shown by the disposition of C^{14} activity within the urie acid molecule following glycine-2-C^{14} administration. At first glance, it would appear that if the liver synthesised an abnormal

molecule, other tissues would not and any defect may be masked by normal purine metabolism elsewhere. However, any labelled purine nucleotide synthesised in peripheral tissues may be derived largely from purine moieties formed de novo from glycine by the liver. Thus, by studying the disposition of radioactivity within urinary uric acid-C^{14} one may get a shrewd idea of how the liver is handling glycine-2-C^{14} in hepatic forms of human porphyria.

D. REGULATION OF PURINE NUCLEOTIDE SYNTHESIS.

Factors influencing the rate of purine synthesis may assert their effect on the de novo synthesis of IMP or on its subsequent conversion to AMP and GMP.

(1) De novo biosynthesis of IMP.

The irreversible formation of phosphoribosyl amine from glutamine and PP-ribose-P is the first reaction in the biosynthesis of the purine nucleus. Phosphoribosylamine is the first specific purine precursor and no branching of the sequence pathway occurs prior to the synthesis of IMP. For reasons enumerated by Wyngaarden (496), it is generally believed that this first reaction is the rate-limiting step in purine biosynthesis. Factors influencing the synthesis of IMP will therefore include the availability of substrates glutamine and PP-ribose-P and the level of glutamine-PP-ribose-P amidotransferase, the enzyme catalysing the reaction.

(a) Substrates.

(i) PP-ribose-P.

The biosynthesis of PP-ribose-P from ribose-5-phosphate and ATP has already been discussed. There are so many pathways involved in the metabolism of glucose that it is highly unlikely that any derangement of glucose metabolism will account for an excess or

deficiency of PP-ribose-P available for purine synthesis. High carbohydrate diets are incapable of eliciting a a perceptible increase in uric acid excretion (171). However, the inhibitory effect of purine and pyrimidine bases on de novo purine synthesis may be due to diversion of PP-ribose-P from the first reaction into salvage pathways of nucleotide synthesis. This has been demonstrated in bacterial (193) and pigeon liver (491) systems. The action of aminoimidazole carboxamide (403) and orotic acid may be similar in mode of inhibition.

(ii) L-Glutamine.

This compound is often regarded as a storage form of ammonia (291) and is an important route of disposal of this product of amino acid metabolism. It is formed from α ketoglutaric acid via the following two reactions:-

α ketoglutarate + NH_3 + $NADH^+$ + H^+ $\rightleftharpoons$ L glutamate + H_2O + NAD.

L glutamate + NH_3 + ATP $\longrightarrow$ glutamine + H_2O + Pi + ADP.

The reversible fixation of ammonia by α ketoglutarate is catalysed by glutamic acid dehydrogenase. The irreversible formation of glutamine is catalysed by glutamine synthetase, an enzyme found in high concentration in liver (429) cerebral cortex (409) and in

kidneys of some animals, e.g. rat (356) liver, brain and kidney of all species are rich in glutamine (454).

The formation of glutamine from ∝ketoglutaric acid affords yet another link between purine and porphyrin metabolism. One of the sources of ∝ketoglutaric acid is the Krebs cycle where it is the precursor of succinic acid; another is ∝keteglutaraldehyde, an intermediate in the succinate-glycine cycle (see chapter on porphyrin metabolism pgs 56-57). Any block in the deamination of ALA will not only cause an increase in production of ∝ ketoglutaral-dehyde which, both as a formyl donor and a glutamine precursor, plays a part in the de novo synthesis of purines. Further, any increased utilisation of succinyl coenzyme A in porphyria may shunt its immediate precursor ∝ketoglutarate away from glutamine synthesis. By these mechanisms, increased formation of porphyrins in human porphyria may decrease substrates available for purine biosynthesis.

The importance of glutamine and purine metabolism relative to gout was stressed by Gutman et al (171). In uric acid degradation experiments following glycine-N^{15}, they showed that in primary gout, from the first day, higher ratio of N^{15} (9+3+1) to total uric acid-N^{15} were found when compared with the normal controls

studied (168). With more complete degradation (169,170), they were able to show that only those nitrogen atoms derived from glutamine via. N3+9 were disproportionately enriched. One possible explanation was that glutamine was deviated from another pathway to that concerned with de novo purine synthesis. In these experiments (169,170), it was shown that in gout, there was a substantial decrease in urinary $N^{15}H_3$ expressed as percentage N^{15} dose, when compared with normal controls, these changes being observed in the absence of renal disease. This may explain why in primary gout, urine tends to be more acid than in normals. The presence of normal amounts of glutamine in the blood of gouty subjects (Orentha, Segal cited by Seegmiller (406)) indicates that impairment of renal production of NH_3 in gout is the effect rather than the cause of increased purine synthesis in this disease.

(b) Glutamine phosphoribosylpyrophosphate (PP-ribose-P) amidotransferase.

This enzyme has been studied in pigeon, chicken and rat livers (60, 176, 179) and in bacteria (325).

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Hierlich et al (325) and Cashey et al (60) showed that the enzyme was inhibited by its ultimate products, the ribonucleotides of adenine and guanine. With high levels of either AMP or GMP, complete inhibition was observed (325). ADP, GTP and IMP were less effective inhibitors and ATP, cytosine monophosphate and adenine had no effect on enzyme activity. The enzyme was shown to possess separate but interrelated sites for the binding of adenine and guanine ribonucleotides (325). This concentration of low levels of AMP and GMP were much more effective in inhibiting the enzyme than the same concentrations of either nucleotides alone. Thus there is a concerted feedback control of purine biosynthesis, maximal when intracellular concentrations of AMP and GMP are optimal. A deficiency of either ribonucleotide would tend to promote de novo synthesis of purines.

(2) Biosynthesis of AMP and GMP.

AMP and GMP can be synthesized de novo from IMP or via salvage pathways from the purine base plus PP-ribose-P, (see earlier section this chapter). The activity of either route is influenced by certain factors.

(a) De novo biosynthesis of AMP and GMP.

The synthesis of AMP from IMP involves a two step reaction, the first of which is irreversible and

requires GTP as a source of energy (267). This reaction is inhibited by AMP (495). The synthesis of GMP from IMP is also a two-stage process and the first irreversible step is inhibited by GMP. The second, also irreversible, requires ATP as a source of energy.

Thus GTP and ATP each are necessary for the synthesis of the other nucleotide (435) and GMP and AMP each controls its own biosynthesis. As Stetten (432) describes it, there exists a homeostasis whereby not the abundance but the ratio of abundances of a pair of products tends to remain constant. It must be remembered as well that the combined concentrations of AMP and GMP, by negative feed-back on the first step of de novo purine synthesis, regulate production rate of IMP.

(b) Salvage pathways of AMP and GMP.

The salvage pathways whereby adenine and guanine react directly with PP-ribose-P to form corresponding nucleotides are strongly inhibited by ATP(369,465). In other words when there is enough ATP, de novo synthesis of GMP will proceed adequately via XMP. Conversely, when there is a lack of ATP, salvage pathways will be relatively more important in nucleotide synthesis.

By all these mechanisms, intracellular levels of adenyl and guanyl nucleotides are kept at an optimum and the surplus of IMP, AMP, XMP and GMP, deviated directly to uric acid formation via the "shunt pathway" is at a minimum. Any disruption of this complex self regulation would permit an excess of purine nucleotides, nucleosides and bases to be available for uric acid production via this route.

E. ASPECTS OF NUCLEIC ACID AND NUCLEOPROTEIN METABOLISM.

The subject of nucleoprotein and nucleic acid metabolism does not fall directly into the scope of this thesis. The subject is fully dealt with in standard text books of biochemistry and only a few facets dealing with structure, situation in cell, function and turnover will be dealt with in this section. This brief summary has been included to aid later discussion.

(1) Structure of nucleoproteins and nucleic acids.

Nucleoproteins are conjugated proteins characterized by the presence of a non-protein prosthetic group viz. nucleic acid and one or more molecules of a simple protein, usually a basic protein such as protamine or histone.

Nucleic acids are polynucleotides, each individual nucleotide being composed of a purine or pyrimidine base attached to a sugar by a glycosidic linkage. The sugar is combined with phosphoric acid. Nucleotides are incorporated into nucleic acids by the action of polymerases (171). The two purine bases found in nucleic acids are adenine and guanine and three pyrimidine bases have been isolated, cytosine (2-oxy-6-amino-pyrimidine), thymine (2,6, dioxy-methyl pyrimidine) and uracil (2,6,-dioxypyrimidine).

Nucleic acid is named ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) according to the nature of their sugar bridges. While adenine and guanine are common to both these forms, of the pyrimidine bases, thymine is only present in DNA and uracil only in RNA. Cytosine is present in both.

Nitrogenous bases, purine and pyrimidine are attached to carbon 1 of the sugar. Linkage between mononucleotides is effected by a phosphodiester group attached to carbon 3 of the sugar bridge of one nucleotide and carbon 5 of the next. In DNA, 3,5, linkage is characteristic; in RNA, this linkage predominates although the 2,3 linkages may also be present.

Studies on the physicochemistry of DNA suggest that the nucleic acid is composed of 2 polynucleotide polymers present in the form of a double helix. The two chains are linked by hydrogen bonds between pairs of bases, in DNA adenine to thymine and guanine to cytosine.

(2) Intracellular location and function of nucleic acids.

(a) Deoxyribonucleic acids (DNA).

DNA is found in nuclei, confined to chromosomes. Each gene appears to be a nucleic acid molecule composed of some 10,000 nucleotide units, the sequence of which represents the genetic message (174). In cell division

it is proposed that the double helix unravels and each of the two strands serves as a template for the synthesis of another complementary chain, the two resulting nucleic acids being identical to the original parent pair. By its very nature, new DNA synthesis will only occur with cell division and turnover will reflect mitotic activity of the particular tissue. In proliferating tissues, e.g. haemopoietic tissue, DNA turnover will therefore be more rapid than in liver, muscle etc.

(b) Ribonucleic acids (RNA).

Ribonucleic acids are important in the synthesis of proteins. RNA is thought to be formed in the nucleus following the synthesis and subsequent unravelling a DNA/RNA hybrid helix. The synthesis of the resultant RNA, a virtual mirror image of DNA, is catalysed by RNA polymerase (174) and is designated messenger RNA. This migrates to the cytoplasm of the cell. Electron microscopy has revealed a network of membranous tubules and saccular vesicles in the cytoplasm, the endoplasmic reticulum. Attached to the membranous tubules are numerous dense spherical granules which contain about 80% of the RNA of the cell and are thus called ribosomes. These are the sites of protein synthesis within the cell.

In the cytoplasm are RNA molecules of relatively low molecular weight, approximately 30,000 containing

only 30 to 100 nitrogenous bases (174). These, called soluble RNA (S-RNA) or transfer RNA attach themselves to activated amino acids and form a complex with messenger RNA in the ribosome. The alignment of the amino acids in a particular sequence is dictated by the "code" of the messenger RNA template. Messenger RNA is a long polynucleotide with more than 1500 purine and pyrimide bases (174).

Cellular proteins e.g. enzymes, are in a constant state of flux, adapting every moment to the immediate cellular requirements. One would expect therefore, that the "message" from the nucleus for the synthesis of a particular enzyme would, by the very dynamic state of the cell, be short-lived and that the turnover of messenger RNA would be accordingly rapid.

(c) Nucleotides.

Nucleotides are fundamental to cellular respiration and life. In the mitochondria, biological oxidation involves the transfer of hydrogen and electrons through a series of enzymes including the purine nucleotides, nicotinamide adenine dinucleotide (NAD), nicotinimide adenine dinucleotide phosphate (NADP) and the flavin adenine dinucleotides (FAD). The cytochromes (which are haem proteins) finally transfer the electrons to oxygen. The energy liberated during the process would largely be lost as heat were it not for the process of oxidative

phosphorlytion which couples oxidation with the phosphorylation of nucleotides 90% of which are adenine nucleotides (AMP and ADP). GTP is another example of a purine nucleotide (guanine) trapping energy. These phosphorylated nucleotides provide the energy for many fundamental reactions in cellular metabolism.

(3) Turnover Rate of Nucleic acids and Nucleotides.

(a) Liver nucleic acid turnover.

Furst (108) and Loeb (271) have both studied nucleic acid turnover in the liver with the aid of isotopic precursors. Both have come to the conclusion that the ½ life of RNA is a matter of 5 (609)to 10 (108) days whilst that of DNA is very much longer (108). However, on critical analysis of their results, their observations are not entirely conclusive and their data does not exclude the possibility of perhaps even the greater fraction of RNA turning over in hours.

Furst et al (108) administered adenine-N^{15} to rats and sacrificed them after 5 days. They showed that the greatest uptake of N^{15} was in the liver, again emphasising the important role of this organ in purine synthesis. Furthermore, in the non-growing liver, whilst the incorporation of adenine-N^{15} into RNA was about 21.2% that in DNA was only about 0.29% i.e. 1.2% as extensive as in RNA. However, following partial hepatectomy,

incorporation values were 22.7% and 16.3% respectively. Thus, only DNA synthesis was affected and the amount incorporated rose from 1.2% to about 72% of that of RNA, reflecting rapid liver regeneration. In similar experiments with partially hepatectomised animals, after giving adenine-N^{15} for 5 days, authors then fed non-isotopic adenine and sacrificed animals after 27 days. Whilst the percentage incorporation into RNA at 5 days dropped to only 15% of the original value at the end of the experiment, that of DNA fell only slightly, again emphasizing the slower turnover of the latter. Assuming that the loss of N^{15} followed a first order response, the authors calculated that, allowing for newly growing liver, the half-life of RNA was about 8 to 10 days whilst that of DNA was considerably longer.

There are two objections to this conclusion. Firstly, data was obtained from two values, namely the percentage incorporation at 5 days and again at 27 days. There may well have been an extremely rapid fall off in in the first hours after stopping adenine-N^{15}, indicating a rapidly turning over component of RNA. Secondly, adenine is a poor precursor to use for detecting a rapidly turning over nucleic acid pool. As opposed to a single sharp "pulse" of activity, as occurs after labelled glycine, work by Wyngaarden et al (492,493)

on urinary adenine-C^{14} and uric acid-C^{14} activity following glycine-1-C^{14} (492) and adenine-8-C^{14} (493) shows that adenine-C^{14} activity remains high for many days following administration of the isotope probably due to the absence of adenine deaminase activity in mammalian tissue (493), with subsequent difficulty in oxidation of this purine base into uric acid. A slowly turning over adenine pool would mean constant reincorporation and recycling of the isotope into newly synthesized nucleic acids thus masking an early and sharp decline in N^{15} incorporation which would result from rapidly turning over RNA.

Loeb et al (271) injected a pyrimidine precursor, orotic acid-6-C^{14} intraperitoneally into rats and sacrificed them at 4 day intervals from 3 to 21 days. They isolated ribosomal RNA from the rat liver and showed that the specific activity decreased logarithmically at a rate corresponding to a half-life of about 5 days (121 hours). Addition of similar quantities of unlabelled orotic acid in addition to above in some animals did not alter results indicating that the orotic acid-C^{14} was given in tracer amounts. The authors interpreted the logarithmic descent as indicating that a molecule of ribosomal RNA has no fixed life span but was destroyed randomly regardless of age.

In appraising the work of Loeb et al (271) it is perhaps pertinent to refer briefly to the observations of Gutman et al (170), Seegmiller et al (404) and Dowdle et al (76,77) on the incorporation of isotopic glycine into urinary hippuric acid (76,77,170,404) and respiratory CO_2 (76,77). These authors showed that there was a sharp peak of activity all within the first 24 hours, very little being excreted thereafter. This reflected two aspects, the rapid turnover of glycine and the rapid turnover of the labelled products studied, namely hippuric acid and CO_2. Analysing excretion logarithmically, Dowdle et al (76,77) were able to express the rate of decline of specific activity excreted in respiratory $C^{14}O_2$ in a curve comprising 3 components, the first with a half-life of 2.6 hours, possibly rapid mixing of glycine, the second with a half-life of 20 to 25 hours, corresponding in time to the discrete peak of urinary hippuric acid-C^{14} excreted during the first 24 hours of the experiment and a third, slow component accounting for excretion of the remaining activity, with a half-life of about 190 to 260 hours. This latter component decreased logarithmically in a linear manner.

Loeb's observations (271) of a linear logarithmic descent in RNA-C^{14} activity with a half-life of about 21 hours following orotic acid-C^{14} could be compared with component III of the decay curves of respiratory $C^{14}O_2$ and urinary .....P.T.O.

hippuric acid-C^{14} excreted after glycine-2-C^{14} (76,77), the great bulk of activity having already been excreted much earlier. The experimental data of Loeb et al (271) fail in that all observations are made 3 days after giving orotic acid-C^{14}. It would be worthwhile repeating Loeb et al's experiments using glycine-C^{14}, ALA-5-C^{14} and other precursors, sacrificing animals, e.g. at 6-hourly intervals (or less) for the first 24 hours, and daily thereafter. One may find that the bulk of RNA is rapidly, extensively but only briefly labelled.

Goldthwait (140) quoted the Ph.D. thesis of Spilman, who injected formate-C^{14} to dogs which were sacrificed 90 minutes thereafter. Acid soluble nucleotides were isolated and counted (cpm/2mg purine) and results were as follows:- G.I.T. 680, heart 467, kidney 289, liver 34, urinary uric acid 3,150. Knowing that the liver is a major organ in both purine synthesis and uric acid production and observing high specific activity in urinary uric acid, the low activity detected in liver nucleotides must indicate a tremendously rapid turnover of liver nucleotides, in the order of minutes, as opposed to turnover in other organs. Whether nucleotides concerned were newly-formed (IMP, AMP, GMP), whether they were those nucleotides involved with cellular respiration

(e.g. ATP, GTP, NAD, NADP) or whether they were derived from rapidly turning over RNA, cannot be determined on available data.

Staehelin et al (431) injected P^{32}-labelled phosphate to animals and showed that an RNA fraction of high specific activity had become associated with purified ribosomes 30 minutes after injecting the phosphorus. During incubation, to effect incorporation of aminoacids, a large portion of this rapidly labelled fraction was degraded into acid soluble products whereas the total bulk of ribosomal RNA remained stable. From rapid synthesis of RNA, it must be inferred that breakdown occurs at the same rate so as to maintain steady state conditions.

The direct measurement of RNA degradation has become possible with the use of Actinomycin. This is a substance which combines specifically with DNA- both in-vivo and in-vitro, thus blocking DNA-dependent RNA synthesis (431). Actinomycin C_3 (431) or actinomycin C, the latter identical to actinomycin D (611) have both been used to measure the rate of RNA turnover in bacterial systems (265) and rat liver (431).

Levinthal et al (265) administered Actinomycin D to suspensions of B subtilis and concluded from their studies that messenger RNA functioned for an average of

10 to 20 times in protein synthesis. Labelling messenger RNA with uracil-C^{14} (265) these authors noted that after addition of Actinomycin D, radioactivity decayed exponentially, the mean decay time being 2 minutes. This extremely rapid rate of turnover may have been peculiar to the rapidly changing demands of the bacteria but data served to indicate how rapidly RNA can be synthesized and degraded.

More applicable to human physiology are the interesting observations of Stahelin et al (431) who have studied the breakdown of rat liver ergosomes in vivo after Actinomycin inhibition of messenger RNA synthesis. Ribosomes isolated from rat liver occur predominantly in the form of aggregates (ergosomes) which on physicochemical studies correspond to multiples of 73S particles held together by messenger RNA. In its functionally active form, aggregates comprise at least five 73S particles. If messenger RNA is degraded by ribonuclease, progressive fragmentation occurs to smaller aggregates e.g. $(73S)_4$, $(73S)_2$ etc. Therefore, messenger RNA is responsible for the structural integrity of ergosomes. If there were rapid turnover of messenger RNA, one would expect to find that inhibition of RNA synthesis would result in breakdown of ergosomes into 73S particles.

In control rats, Staehelin et al (431) showed that

60 to 70% of ribosomes were present in particle size (73S)5 and contemplated that the remainder were broken down to smaller aggregates during isolation procedures. Injection of actinomycin in doses sufficient to inhibit nes RNA synthesis resulted in a conspicuous rise in 73S and $(73S)_2$ aggregates after only 4 hours, and by 8 hours most of the ergosomes had disintegrated to 73S monomers or (73S) dimers. When analyzed, these results indicated that 50 to 80% of the ribosomal messenger RNA was degraded within 4 to 8 hours of injecting actinomycin in doses adequate to inhibit RNA synthesis completely. As actinomycin has no effect on the structural and functional integrity of ergosomes in-vitro-, the rate of disintegration of ribosomal aggregates appears a valid measure of the rate of degradation of messenger RNA, which in turn should reflect its turnover. In all the experiments, there was a residual fraction of about 20% of the original nucleoprotein that resisted breakdown and remained biologically active for prolonged periods even after the administration of high doses of actinomycin.

In summary, it appears that in mammalian liver, the bulk of ribosomal messenger RNA turns over rapidly in hours with a smaller but significant portion of longer half-life. It may be this fraction whose radioactive decay has been measured by Furst et al (108) and Loeb et al

(271). There is now experimental evidence that uric acid of high specific activity, isolated from the urine within 24 to 48 hours of administering an isotope purine precursor, may not reflect rapid cleavage of newly synthesized nucleotides but may in part be derived from rapidly turning over RNA in the liver.

(b) Haemopoietic nucleic acid turnover.

The complexity of leucocyte dynamics has been discussed at length by Craddock et al (67). It appears that the volume of maturing granulocytes myeloid tissue, the "marrow granulocyte reserve (MGR)" is about 20 to 25 times that of circulating granulocytes. Once the mature granulocyte enters the circulation, in-vitro DNA labelling experiments have shown that they circulate for variable but brief periods with an average circulation time of 4 to 8 hours. On leaving vessels to enter tissue spaces, they do not appear to re-enter the circulation. Time relationships of in-vivo dynamics are not known and authors feel that in-vitro labelling may traumatize cells altering the true picture.

Craddock et al (67) administered labelled adenine and formate to normal humans and followed the appearance of the label in the DNA of circulating granulocytes. Because of the short circulation time of these cells,

activity in the blood will reflect activity in those cells just leaving the marrow. It was observed that there was a delay of about 5 to 6 days before the isotope was detected in peripheral cells. As only blast cells divide and therefore incorporate the label into newly synthesized DNA, this hold-up must reflect maturation of the promyelocyte to granulocytes and the passage of the latter into the circulation. Activity in circulating granulocytes rose rapidly for the first few days thereafter, and then fell, the average "life-span" of isotopic DNA in the blood being about 10 to 13 days after administering the labelled precursor. How long isotopic DNA-labelled granulocytes remained viable in tissue spaces after leaving the circulation was not assessed.

Hamilton (172) studied nucleic acid turnover of leucocytes in chronic leukaemia following the I.V. infusion of adenine-8-C^{14}. RNA and DNA was separated from cells up to 670 days later and activity in individual purines was determined. In granulocytic cells, adenine-C^{14} was incorporated equally into DNA and RNA and there was extensive transformation of adenine to guanine. Decline in specific activity of circulating granulocytic nucleic acid was initially rapid with a half-life of 9 days. Thereafter, there was a second component of decline with a somewhat longer half-time representing a relatively insignificant part of the total. Most activity had

disappeared after about 140 days.

With respect to lymphocytes (172), Hamilton showed that isotopic adenine labelled DNA less than RNA and that the decline in circulating nucleic acid activity could be expressed in two components, a rapid component with a half-life of 18 days and a slower one, with a half-life of about 300 days. It was not determined whether the lymphocytes lived longer or whether they had the ability to reutilise specifically the nucleic acids of their precursors. There is morphologic evidence for phagocytosis of lymphocytes by reticulum cells in lymph nodes.

(4) Nucleic acid Catabolism.

(a) Endogenous nucleic acids.

Enzymatic hydrolysis of the polynucleotide chains is brought about by various nucleases (191) resulting mainly in oligonucleotides. These are further cleaved by phosphodiesterases to yield mononucleotides. These are then acted upon by a series of enzymes liberating free purine bases. AMP may be deaminated to IMP, ammonia being liberated. Dephosphorylation of nucleotide can be catalysed by non-specific phosphatases (190) and specific 5'-nucleotidases (190), e.g. AMP ⟶ adenosine + phosphate (Pi). Conversion of liberated nucleosides to

purine bases is catalysed by nucleoside phosphorylases (216) which convert the nucleoside into its free base and sugar derivative. This reaction occurs as follows:- Adenosine or Inosine + Pi ——— Adenine or Hypoxanthine + ribose-1-phosphate. Adenosine may first be converted to inosine by the enzyme adenosine deaminase (215). In mammalian tissue, free adenine is not oxidatively deaminated directly to hypoxanthine (394) whilst guanine is readily acted upon by its specific deaminase, xanthine being formed in the process. This may explain the facility with which guanine is oxidized to uric acid in contrast to the tardy removal of adenine in humans (492, 493).

(b) Ingested nucleoproteins.

Following the action of proteolytic enzymes, nucleases and phosphodiesterases in the bowel, nucleotides are liberated from dietary nucleo-proteins. These are usually hydrolysed to nucleosides by nucleotidases and phosphatases. Nucleosides may be absorbed intact or may be further cleaved phosphorylitically to yield free base which is then absorbed.

The absorption of free purine bases and nucleosides may be followed by reincorporation into nucleotides via salvage pathways, or oxidation and excretion as uric acid. Wilson et al (475) fed labelled yeast nucleic acid to three human males and observed the pattern of excretion of isotopic urinary uric acid. The conversion to and excretion as uric acid was so rapid that isotope excretion in the urine was similar to that observed after the I.V. administration of labelled uric acid. From these findings, Wilson et al (475) concluded that the absorbed purine moieties were converted to uric acid largely by direct routes without prior incorporation into body nucleic acids. The small intestine mucosa of man is rich in nucleoside phosphorylase and xanthine oxidase (496). Thus, uric acid may be directly synthesized in intestinal mucosa from dietary nucleoprotein, some of which may be absorbed and some of which may further be catabolized by bowel bacteria.

F. FORMATION OF URIC ACID.

(1) Uric acid precursors.

The immediate precursors of uric acid are hypoxanthine and xanthine, two oxypurines which are further oxidised by xanthine oxidase to uric acid. The end products of nucleoside cleavage are adenine, guanine, hypoxanthine and xanthine. Purine nucleosides phosphorylase acts most readily on inosine and guanosine (199,216,224), and the major bases generated are therefore guanine and hypoxanthine. Further, guanine readily undergoes oxidative deamination by guanase to form xanthine (496) whereas mammalian tissues lack adenine deaminase (394) and adenine is not readily converted to hypoxanthine. While adenine, guanine and hypoxanthine appear to be derived solely from their corresponding nucleoside, xanthine can be formed,

(a) directly following cleavage of its nucleoside xanthosine,

(b) following deamination of guanine by guanase, and

(c) oxidation of hypoxanthine by xanthine oxidase which in turn oxidizes the xanthine produced to uric acid (22).

Following the administration of a labelled small molecule purine precursor such as glycine or formate (see later this chapter), the isotope passes promptly into urinary uric acid often reaching peak values in the first

day or two. Thereafter, the specific activity of excreted uric acid remains high for many days, this plateau reflecting turnover of tissue nucleic acids. In order to explain prompt enrichment of urinary uric acid followed by a plateau of activity, it is thought that uric acid is not only derived from tissue nucleoprotein and nucleic acid turnover but that "shunt pathways" exist whereby purine precursors are converted directly to uric acid without the prior intervention of nucleic acid purines as intermediates. It is necessary to briefly consider these pathways.

(2) Shunt pathways of uric acid formation.

The excretion of highly-labelled uric acid 24 to 48 hours after administration of an isotopic precursor can theoretically result from:

(a) rapid cleavage of nucleotides IMP, GMP, and AMP before their incorporation into more complex purine compounds, and

(b) rapid turnover of nucleoproteins, nucleic acids and more complex nucleotides, the latter including ATP, GTP, NAD, FAD, etc.

The rate of turnover of liver RNA and nucleotides involved in biological oxidation has already been dismissed (see earlier this chapter) and there is no evidence at present excluding the possibility of these

tissue compounds turning over rapidly enough to contribute to early-labelled uric acid.

Most authors however, favour pathway (a) as being the most likely route and Gutman et al (171) and Dyngaarden (496) looked upon this shunt pathway as essentially an overflow bypass for the elimination of surplus inosinic acid and other ribonucleotides generated in excess of biological needs.

An ordinary meal containing purines is followed by an appreciable rise in urinary uric acid excretion, often without accompanying increase in plasma urate (171). This suggests rapid conversion of dietary purines to uric acid and prompt clearance of the latter. Only after large amounts, e.g. 4 gm, of ingested RNA, does serum urate rise (171). As man can subsist without ill effects on a diet devoid of purines, the rate of de novo synthesis of purines is adequate to meet all biological needs. Next to the ingestion of purines the most prolific exogenous source of uric acid is a high protein diet (171).

Wilson et al (475) studied the way the body deals with ingested nucleic acid by giving three males each 1 gm. of yeast nucleic acid whose purine and pyrimidine moieties had been labelled with N^{15}. They showed that the label was excreted in urinary uric acid rapidly and in a linear manner when expressed logarith-

mically, with turnover rates similar to those determined separately following I.V. isotopic uric acid (475). This indicated conclusively that in the three males studied, the dietary nucleic acids were converted directly into uric acid with negligible prior incorporation into body purines. The presence of xanthine oxidase in intestinal mucosa (93) may aid in the prompt formation of uric acid from dietary purines.

Adenine containing compounds, for reasons discussed, are poor uric acid precursors. Most hypoxanthine and xanthine oxypurine bases are derived from cleavage of nucleotides IMP and GMP respectively. Whereas the former may be the main operative shunt pathway dealing with an endogenous overproduction of purine nucleotides, the conversion of GMP to xanthine may be more important when uric acid is derived from the degradation of nucleic acids and those nucleotides involved in cellular reactions (e.g.GTP). Thus pattern of isotopic purine base excretion following the administration of labelled precursors would indicate the major shunt pathway operative in health and certain disease states.

In two normal subjects receiving glycine-1-C^{14}, Wyngaarden (492) showed that there was prompt and striking labelling of urinary hypoxanthine in excess of that of other purine bases. The specific activity curves of

of hypoxanthine and uric acid bore a precursor-product relationship indicating that in normals, the major shunt pathway involved cleavage of IMP. However, early labelling (within 24 hours) was also observed with adenine, 7-methylguanine and xanthine suggesting that in part, cleavage of other nucleotides also contributed to early-labelling of uric acid. After the first day, specific activity of all the urinary purine bases dropped to a plateau of activity (492), hypoxanthine-C^{14} now being the least active. The persistence of activity in excreted adenine, guanine and xanthine was also observed with urinary uric acid-C^{14}, this plateau almost certainly representing cleavage of nucleotides derived from the degradation of nucleic acids.

In primary gout, the pattern was entirely different. As in normals, the C^{14}-label appeared rapidly in the various purine bases (492), but in contrast to normals, initial hypoxanthine labelling values were never higher than those of corresponding uric acid samples. Another important difference was the striking initial labelling of 7-methylguanine which now appeared the main precursor of "shunt" uric acid. These findings are highly significant. 7-methyl-guanine is a minor constituent of soluble and ribosomal RNA of E coli (200) and of DNA and

RNA rat liver (285). Transfer (or soluble) RNA, involved in transfer of activated aminoacids from the cytoplasm to messenger RNA in the ribosomes, have within them methylated purines (376). On available evidence, one cannot say whether the hyperincorporation of the C^{14} label into urinary 7-methylguanine in gout reflects liberation of guanine nucleotides from RNA with exceedingly rapid turnover or whether this signifies excessive cleavage of the nucleotide before incorporation into nucleic acids.

In a case of myeloid metaplasia receiving glycine-1-C^{14}, the very high specific activities of guanine and adenine as compared to hypoxanthine, suggested that direct pathways involving nucleotides of guanine and adenine were quantitatively greater than usual

To recapitulate, isotope data in normals indicate that cleavage of IMP constitutes a major pathway for direct synthesis of uric acid. GMP and XMP also contribute to early-labelling of uric acid and in hyper-uricaemic states where there is over-production of purines or increased turnover of nucleic acids, cleavage of guanine mononucleotides may be the dominant shunt pathway. Whether cleavage occurs before or after its incorporation into nucleic acids and more complex nucleotides e.g.GTP, is not known (492).

(3) Xanthine oxidase.

By whatever route uric acid is produced, xanthine is the immediate precursor. The aerobic dehydrogenation of hypoxanthine and xanthine to uric acid is catalyzed by the same enzyme, xanthine oxidase (22).

Xanthine oxidase is a flavoprotein, molecular weight about 300,000 containing 2 molecules of flavine, 8 atoms of iron and 2 atoms of molybdenum per molecule (464). These "co-factors" are concerned with electron flow which occurs when the enzyme operates. The enzyme has a wide substrate specificity. It oxidizes other purines besides xanthine and hypoxanthine, purine analogues, e.g. 6-mercaptopurine, pteridines e.g. leucopterin and aldehydes e.g. formation of oxalate from glyoxylate (464).

In man, xanthine oxidase is found in high activity only in liver and small intestinal mucosa (93) and in the liver, the enzyme is in the soluble fraction of the cells (464). Low activity may be present in the bone marrow(496). Other tissues, including kidney, spleen, heart, lung, muscle and circulating blood cells lack detectable xanthine oxidase (87,304).

Because of the restricted distribution of xanthine oxidase and view of its great activity in the liver, uric acid synthesis especially from endogenous purine metabolism, may largely be an hepatic process in man. It is presumed that purine degradation products of other tissues are

transported to the liver for futher oxidation although in normal plasma, only 0.1 to 0.3 mg xanthine and hypoxanthine are present per 100 ml (212,407) while no other purine precursors are detectable.

These observations are particularly relevant to the present study. One of the aims of this thesis is to investigate purine metabolism in acute porphyria, a disease of disordered hepatic porphyrin metabolism. Should this in some way interfere with hepatic xanthine oxidase activity, there will be a significant fall in uric acid production, a rise in the excretion of other oxypurines without a direct effect on production and utilisation of purines in the body.

(4) Uric acid ribonucleoside.

The discovery of uric acid ribonucleosides in beef erythrocytes (69) and liver (98) has led to suggestions that this is a precursor of uric acid in man. It is felt however, that the riboside is formed after uric acid production by condensation with PP-ribose-P (181,182) and therefore is not an alternate pathway of uric acid production.

G. MISCIBLE URIC ACID POOL AND ITS TURNOVER.

The first use of isotopically labelled uric acid to evaluate pool size and rate of new uric acid production was described by Benedict et al (16), using uric acid-1-3 N^{15} in the form of the soluble lithium salt. In principle, isotopic uric acid is injected I.V. and the specific activity of uric acid isolated serially from the urine over several days is determined. When the logarithm of isotopic activity is plotted as a function of time, a straight line is obtained. Extrapolation of this line to zero time will give the theoretical activity of isotope in the body at the moment of mixing. The miscible pool of uric acid is defined as that quantity of uric acid in the body of the recipient by which the injected uric acid is promptly diluted. The quantity of uric acid present in the miscible pool is easily calculated by knowing the total dose of isotope activity injected and the concentration of isotope in the uric acid of the body at the time of mixing (29,486,487).

After mixing has occurred, a decline in concentration of isotope in uric acid occurs because of continuous dilution of the labelled body urate pool by newly synthesized non-labelled urate. The slope of the decline is therefore a measure of the daily rate of urate turnover. Thus, multiplication of the slope by the value of pool

size provides numerical value for the total amount of uric acid synthesized daily (16,29,53,119,404,486).

This method, however, involves a number of assumptions including prompt and complete mixing of the labelled with unlabelled uric acid in all its compartments, a steady state in which uric acid elimination equals production over period of study, that infused uric acid behaves like endogenous uric acid in metabolic disposition and other considerations (29,405,496). It has already been pointed out (see Section A, page 148) that urate is not uniformly distributed among several body water compartments (16,171,213). In normal and mildly gouty subjects where all the miscible pool urate is in solution, these assumptions are largely valid. In gouty patients with tophaceous deposits, however, labelled urate of the miscible pool slowly exchanges with unlabelled urate of the solid phase. Thus, the rate of change of isotope concentration in the soluble phase is multifactorial and is not, therefore measure of synthesis of new urate.

The uric acid pool of normal man averages about 1200 mg with a range of 866 to 1,587 mg (16,17,29,53,119,404, 427,486,487). In one female studied, the pool size was 650 mg (487). About 60% (range 45 to 85%) of the uric acid pool is replaced daily by freshly synthesized uric acid. The turnover of uric acid averages 695 mg per day

with values ranging from 513 to 1108 mg/day. In each case quantity of uric acid entering the pool exceeds urinary loss by 100 to 260 mg per day. In gouty subjects, miscible pool is usually about 2,000 to 4,000 mg in patients without tophi (16,29,404) and may reach 18,000 to 31,000 mg in severe tophaceous gout (17). This figure is probably only a very small fraction of total body uric acid as only the peripheral layers of tophi are readily exchangeable with urate in solution.

Following the infusion of isotopic uric acid, recoveries of the isotope in urinary uric acid over the following 7 to 14 days range from 55 to 95% of the administered dose (54,119,429,486,487). By this time, virtually all isotopic uric acid has been eliminated from the body. The average of 14 studies was found by Wyngaarden to be 75.6% (191). Thus, an average of about a quarter of the total uric acid eliminated daily is disposed of via extrarenal channels.

These results emphasize an important difficulty in interpreting data put forward in this thesis. With widely differing extrarenal disposal values (more so in renal disease) from patient to patient, comparison of the incorporation of glycine-2-C^{14} into uric acid between normal and porphyric subjects loses accuracy when based on urinary uric acid-C^{14} data alone.

Seegmiller et al (404) overcame this difficulty by simultaneously administering isotopic uric acid-N^{15} and glycine-1-C^{14} to 6 normals and 14 gouty subjects. In each case, they calculated the percentage dose uric acid-N^{15} excreted in the urine over 7 days and compared this with the number of mg of uric acid excreted in the urine over 24 hours expressed as a percentage of the daily turnover of uric acid. Values by either method were virtually identical. The authors then calculated the percentage dose of glycine-1-C^{14} excreted as urinary uric acid-C^{14} over the 7 days of the experiment, corrected for the now known extra-renal element and were thus able to accurately assess how much glycine-1-C^{14} was incorporated into uric acid over the course of the experiment.

H. INCORPORATION OF ISOTOPIC GLYCINE INTO URIC ACID.

The incorporation of labelled glycine into urinary uric acid hasafforded opportunity of studying the metabolic derangements of various diseases including porphyria (75,76,77)(this thesis), and gout (18,19,30, 165,168,170,308,404,490). The findings in porphyria will be presented and discussed later. This section will be devoted mainly to findings in normal and some gouty subjects, the latter where information may contribute to the understanding of observations in the normal. This section will be discussed from three view points:-

1) The excretion of the label in urinary acid with relation to time, i.e. the day to day pattern of excretion.
2) The cumulative incorporation of glycine into urinary uric acid over a given time period. Values will be expressed as percentage dose excreted in seven days.
3) Disposition of radioactivity within the uric acid molecule. Under this section, turnover and possible recycling of glycine, active "1-carbon" unit metabolism and its relationship to purine biosynthesis, glycine as a formyl donor and the degradation of uric acid-C^{14} formed from glycine will be discussed.

(1) Daily pattern of excretion of isotopic uric acid.

(a) Experimental observations.

Noting and correlating the observations of Gutman et al (165,168,170), Wyngaarden et al (490,492), Dowdle et al (76,77), Seegmiller et al (404), Benedict et al (18,19) and others (30,308), it appears that in normal subjects receiving isotopic glycine orally or intravenously the label reaches peak values in urinary uric acid one to three days after administration, activity thereafter falling in a slowly declining plateau of activity. Gutman (165) remarked that if account were taken of the time required for absorption and initial lag in synthesis of uric acid, it was possible that the highest specific activities were reached some time within the first 24 hours. Wyngaarden et al (492) in order to elucidate more clearly the events occurring immediately after the oral administration of glycine-1-C^{14}, collected one 6, one 11 and two 24-hour fractional urine samples after the isotope was given. They showed that uric acid became maximally labelled in the 6 to 17 hour sample despite a normal cumulative incorporation of C^{14} into urinary urate over the 3 days of the experiment. It is of interest to note that in the same experiment, hypoxanthine and adenine enrichment was maximal in the 0 to 6 hour sample whereas xanthine and 7-methylguanine passed through isotope concentration maxima in the 6 to 17 hour period.

It was uniformly observed that after the initial peak in isotope concentration, the label still appeared in urinary uric acid over the ensuing few weeks in a slowly declining plateau of activity. Gutman et al (165) noted that 7 days after administering isotopic glycine, specific activities had fallen to about 60% of the peak values. In porphyria, a normal pattern of excretion was observed (76,77).

In the various forms of gout, a number of different patterns have been recorded. In primary gout, there may be no deviation from normal. However, it is not unusual, especially in those patients who regularly excrete excessive quantities of urinary uric acid, for a more rapid initial rise to an earlier peak of isotopic activity to occur. The peak, usually 2 to 3 times normal in amplitude is generally followed by a sharper decline in activity over the succeeding days levelling off after about a week to values within the normal range (18,19,30, 165,168,170,308,404,490,492). In contrast to normals, Gutman et al (165) found, that after 7 days, the specific activity of isotopic uric acid was only 24% of the peak values observed on the first day.

In gout, secondary to proliferative haemopoietic disorders, an interesting and different pattern of excretion has been reported (248,428,492,501). Wyngaarden

et al (492) gave oral glycine-1-C^{14} to one patient with polycythaemia vera and another with myeloid metaplasia. In both cases, marked early enrichment of urinary uric acid was followed by a secondary phase of incorporation of C^{14} into uric acid, maximal on the 11th to 13th day. This latter or second peak was shown to be a reflection of secondary maxima, occurring also in the various purine bases, mainly adenine and guanine, suggesting that this reflects turnover of haemopoietic nucleic acids. Lastor and Muller (248) and Sorenson (428) reported two cases of gout secondary to myeloid metaplasia (248) and polycythaemia vera (428) respectively where the early peak of prompt enrichment was absent and the specific activity of isotopic urinary uric acid rose slowly and progressively to a maximum 10 to 15 days following glycine administration. Similar observations re a secondary peak were made by Tu et al (501) and Krakoff et al (230) the latter using formate-C^{14} which gives similar incorporation patterns as glycine (see later). Krakoff confirmed two distinct peaks of isotope excretion in chronic myeloid leukaemia, the first at 2 days, the second, 12 to 15 days. In chronic lymphatic leukaemia, there was no deviation from the normal pattern (230).

(b) Interpretation of above findings.

The prompt enrichment of urinary uric acid following the isotopic glycine reflects its rapid synthesis via shunt pathways (see pages 192 and 196). It will be recalled from labelled purine base studies (492) that in normal subjects, cleavage of IMP with release of hypoxanthine seems to be the shunt pathway of major importance. In primary gout, however (492), cleavage of guanine nucleotides appears quantitatively more important, raising the question of whether AMP or GMP had been made in excess of needs or whether in this disease, there was a more rapid turnover of certain ribonucleic acids and functioning nucleotides, e.g. GTP, ATP, etc., particularly in the liver. The absence of significant early labelling in some cases of myeloid metaplasia (248) and polycythaemia (428) may be interpreted as implying poorly functioning shunt pathways of uric acid synthesis.

Why does this occur in some cases where haemopoietic tissue is proliferating in excess of normal? The answer may probably be more apparent after considering the metabolic fate of newly synthesized liver nucleotides. Some are elaborated further in mitochondria, e.g. ATP, GTP, FAD and play an important role in those reactions involving oxidation and energy transfer. Within the

nucleus, a significant portion is incorporated into ribo- and deoxyribonucleic acids whose metabolism has already been discussed (see page 176). Another route of metabolic disposal of liver nucleotides, nucleosides and purine bases involves transport via the blood to those tissues relying partly or fully on salvage pathways for nucleotide and nucleic acid synthesis (3,140,242,280). Haemopoietic tissue has not the wherewithal to synthesise IMP de novo and is completely dependent on preformed purine precursors, mainly from the liver, for the elaboration of nucleotides and nucleic acids (3,242,280). What the liver has not "exported" or utilized itself, represents the surplus of nucleotides available for rapid cleavage and oxidation to uric acid.

In proliferative disorders of haemopoiesis, the demand for liver nucleotides may be so great that there is scarcely a surplus available for prompt cleavage and oxidation to uric acid. This implies that rapidly turning over liver RNA or nucleotides are not in themselves of sufficient magnitude to produce significant early labelling of uric acid, except perhaps in primary gout.

The plateau of activity following the promptly labelled peak of urinary uric acid, is more easily explained. Glycine is a relatively small molecular weight purine precursor that is readily accessible to and

utilisable by all tissues in the body. Whether a particular tissue has de novo purine synthetic pathways or not, following the "pulse" of isotopic glycine activity, all tissues will elaborate isotopic nucleotides, some of which will be incorporated into nucleic acids and nucleoproteins. The difference in turnover between DNA and RNA in one organ, the liver, has already been stressed (108,271). This again differs from DNA and RNA turnover in myeloid (67,172) and lymphoid (172) tissue. Thus throughout the body at different time intervals, dissolution of different polynucleotides will release isotopic mononucleotides, some of which will be reincorporated into nucleic acids and some cleaved, oxidised and excreted as uric acid. This staggering of uric acid production will result in a slowly declining plateau of excretion as has been observed.

When one tissue, e.g. bone marrow, is elaborating nucleic acids to excess, on their degradation, the body will be presented with more than normal load of adenine and guanine nucleotides available for cleavage and subsequent oxidation to uric acid. In normal myeloid tissue, Craddock et al (67) have shown that the "life span" of DNA

is in the order of 10 to 13 days. Hamilton (172) demonstrated that the half-life of DNA within chronic myeloid leukaemic cells was 9 days. It is not surprising, therefore that in proliferative haemopoietic disorders (248,428,492,501), the slowly declining plateau of urinary uric acid activity shows a second peak between the 10th and 15th day after the administration of isotopic glycine and that similar "humps" of activity appear in urinary adenine and guanine (492).

The above discussion serves to emphasize once again the key role played by the liver in purine metabolism. Should hepatic synthesis of purines be impaired in hepatic porphyria, one would expect to see changes in the pattern of excretion of isotope in urinary uric acid following the administration of labelled glycine.

(2) The cumulative incorporation of Isotopic Glycine into Urinary Uric Acid.

(a) Experimental observations.

Only a very small fraction of the administered isotope is recovered as urinary uric acid over a week in normal human subjects receiving labelled glycine orally or I.V. Wyngaarden (496) has summarised and correlated published data and his table shows that in normals, about 0.18% of orally administered glycine-1-C^{14} appears in urinary uric acid in 7 days as opposed to 0.13% following N^{15}-glycine. In gout where less than 590 mg uric acid

are excreted in the urine daily, mean values of incorporation are 0.28 and 0.15% respectively. In this group, the cumulative incorporation of N^{15} was within range in 6 to 7 cases whereas incorporation of C^{14} was excessive in half to two-thirds of the gouty subjects studied. In those gouty subjects excreting more than 590 mg urinary uric acid per day, mean values of 0.75% and 0.35% were observed following glycine-1-C^{14} and glycine-N^{15} respectively. All cases in the latter group incorporated excessive amounts of the isotope over 7 days.

It can be seen that following glycine-1-C^{14}, incorporation of the label is twice that following glycine-N^{15}. In man, the glycine pool is 80 to 90 mg/kg. Whereas glycine-1-C^{14} can be given in tracer amounts of a few mg., the dose of glycine-N^{15} is usually in the region of 50 to 100 mg/kg. Thus, with the latter as a precursor, there will be a 2 to 10 fold dilution of the administered isotope with a similar reduction in the total amount of the label recovered in urinary uric acid (165,490).

(b) Interpretation of findings.

There are limitations as to what one can infer from studies on the cumulative incorporation of isotope glycine into urinary uric acid and these have already been stressed (see page 201). One should always allow for the variable extrarenal elimination of uric acid which may

range from 5 to 45% of the excretion in normals and be much more in renal disease and gout (54,119,487,486,487). Seegmiller et al(404) overcame the difficulty by giving uric acid-N^{15} at the same time as glycine-1-C^{14}. This enabled them to calculate the extrarenal component of excretion in every case and to amend urinary acid incorporation data accordingly.

The main purpose of measuring the incorporation of isotopic glycine into excreted uric acid is to assess what percentage of the administered dose has been converted to uric acid over the course of experimental study. Newly synthesized isotopic uric acid will be immediately diluted in the miscible uric acid pool, the degree of dilution being inversely proportional to the rate of turnover of the pool. Thus, the specific activity of urinary uric acid divided by the fraction of the pool turned over daily will give the value for the concentration of the isotope in the newly synthesized uric acid entering the pool. This value, multiplied by the total amount of uric acid excreted will be a direct measurement of the incorporation of glycine into all uric acid formed during the period of study. Formula-wise, the percentage dose glycine incorporated into uric acid (G inc.) over period of study would equal the number of mg. urinary

urie acid (U^F) plus the number of mg. urie acid eliminated extrarenally (U^{ER}) multiplied by the specific activity of urinary urie acid (dpm/mg UA) divided by the turnover of the miscible urie acid pool (tr).

$$C \text{ inc} = \frac{(U^F + U^{ER}) \times \text{dpm/mg UA}}{tr}$$

All the necessary data is readily attainable if one administers the glycine-C^{14} simultaneously with urie acid-N^{15} so that pool size and turnover, and percentage urie acid eliminated via extrarenal channels can be determined concurrently with glycine-C^{14} incorporation values into urinary urie acid.

Finally, the size and turnover of the glycine pool will in part determine the amount and specific activity of glycine available for purine synthesis. The rate of turnover of glycine has been shown to be more rapid in porphyria (76,77) and is another variable to be borne in mind. Wyngaarden (496) replotted data from Seegmiller et al (404) comparing incorporation values of glycine-1-C^{14} into urie acid (corrected for extra-renal disposal) against turnover of urie acid measured with urie acid-N^{15}. Wyngaarden (496) showed that although positive correlation existed, there was a wide scatter. This may reflect differences in dilution of administered glycine within the different glycine pools of the patients studied.

(3) Disposition of Radioactivity within the Uric Acid Molecule.

Glycine is an important precursor in the de novo synthesis of IMP. The condensation of phosphoribosylamine and glycine to form glycinamide ribonucleotide (GAR) results in the glycine moiety being incorporated in toto into the evolving purine nucleus ultimately accounting for carbon atoms in positions 4 and 5 and nitrogen in position 7 (138,139,175,177). The glycine-2-C readily labels C-1 units (8,9,10,217,319,370,471,476) and is therefore an important source of those active carbon fragments involved in the formylation of GAR (138,178,182,456,457) and AICAR (102,103,178), those reactions responsible for the formation of C8 and C2 respectively. Both the glycine-1-C (490) and the glycine-2-C (75,76,77) label CO_2 via pathways discussed in Chapter 11, page 57 , and theoretically, may account for activity in C6 of the purine moiety. The glycine nitrogen besides labelling N-7 directly, readily, after deamination, finds its way into aspartic acid and glutamine thereby becoming precursors of N-7 and N3 + 9 respectively.

An important consideration in analysing disposition of radioactivity within the purine nucleus is to know how long glycine of high isotope activity remains in the body before its utilisation or excretion. It is also

important to ascertain whether, during this period, the glycine changes in isotopic composition via recycling pathways. All these points need to be known before experimental observations can be presented and adequately discussed.

(a) Turnover of isotopic glycine.

The turnover of labelled glycine is extremely rapid. This has been assessed by measuring the appearance of the isotope in respiratory carbon dioxide (23,76,77,490), urinary urea and ammonia (170) and urinary hippuric acid (76,77,117,196,404), the latter following simultaneously administered sodium benzoate. The products of glycine metabolism measured above are all rapidly formed from this amino acid and rapidly excreted from the body. Thus one can get an accurate idea of how long glycine of high specific activity is available for synthesis of purines, porphyrins, creatine, etc.

The αcarbon of glycine readily labels carbon dioxide by pathways already discussed in detail (Chapter II pg. 57) Dowdle et al (76,77) administered glycine-2-C^{14} I.V. to normal and porphyric human subjects and showed that the isotope appeared in respiratory CO_2 within 5 minutes, reached peak values at 30 minutes and thereafter activity declined rapidly. The sharp peak comprising the bulk

of activity all fell within the first 24 hours and negligible activity was detected after 48 hours. When expressed logarithmically, the rapid excretion of the label could be expressed in a curve with 2 components and the negligible remainder in a third, linear component. Component I has a half-time between 1.5 and 2.6 hours, component II between 18.6 and 27.5 hours and component III between 135 and 300 hours. Similar observations were made by Berlin et al (23) following glycine-2-C^{14}.

The glycine nitrogen, following oxidative deamination, is rapidly excreted in the urine as urea and ammonia. Gutman et al (170) noted that 25% of the dose of glycine-N^{15} was excreted as urinary urea and 2.4% as ammonia in the first 24 hours following administration. Thereafter, activity fell rapidly and after 48 hours, negligible excretion of the isotope was noted. These results are essentially similar to the disposal of the glycine-2-C as carbon dioxide.

Glycine and benzoic acid combine to form hippuric acid, a substance which is rapidly excreted from the body in the urine. Sodium benzoate has thus been used as a trapping agent of the entire glycine moiety and estimates of glycine-C^{14} turnover have been made by this method (76,77,107,117,404). Dowdle et al (76,77) administered sodium benzoate only from the second day following

glycine-2-C^{14} and were able to analyze the logarithmic curve expressing rate of decline of urinary hippuric acid-C^{14} activity into 2 components. The immediate component corresponded to component IIIof the $C^{14}O_2$ curve and was elicitable in 3 of the 5 subjects studied. The slow hippurate component corresponding to component III of CO_2 was similarly linear with a half-time ranging from 146-264 hours. In all experiments there was a fair parallelism between hippuric acid and carbon dioxide curves. Seegmiller et al (404) confirmed the above observations that excretion of the bulk of isotopic hippurate took place within 24 hours with little activity in the urine after 48 to 72 hours. Similar observations were made by Howell et al (196) following the simultaneous administration of glycine-1-C^{14}, αN^{15},

Garfinkel et al (117) used the hippuric acid technique to test the homogenicity of the glycine pool in the first few hours following isotopic glycine administration. Hippurate synthesis has been shown to be a mitochondrial reaction with the addition of glycine to benzoal CO A as the last step. The reaction occurs almost entirely in the liver and kidney. Garfinkel et al (117) injected glycine-C^{14} I.V. into rabbits and rats, sacrificed animals at varying time intervals up to 12 minutes after injection and showed that the specific activity of the glycine moiety of hippuric acid isolated

from liver or kidney was frequently higher than that of the total free glycine isolated from the same organ at the same time. As the specific activity of a product substance would be expected to be the same or lower than that of its precursor, the above findings were interpreted as indicating two or more separate pools of glycine which had not come into equilibrium up to 12 minutes after glycine administration. It would have been interesting for the authors to ascertain how long this compartmentation of glycine existed and whether a fully homogenous pool was ultimately formed. Watts et al (463) felt that hippuric acid glycine was not representative of total body glycine for a period of at least 9 hours after giving labelled glycine.

A concept has thus emerged of a single "pulse" of isotopic activity in the body following the administration of labelled glycine which reaches its peak well within 24 hours, and is of little magnitude after 48 hours. Isotopic uric acid or serum bilirubin therefore represent, respectively, the metabolic products of purine and haem compounds synthesized mainly within 24 hours of giving labelled glycine. During this time, other simple molecules, e.g. ammonia, carbon dioxide, C-1 fragments etc. become increasingly labelled so that the purine moiety formed 1 hour after the administration of

of glycine may have a different intramolecular disposition of radioactivity to the purine ring elaborated 6 hours later. Further, by various pathways, the glycine-2-C may become the glycine-1-C as new glycine is resynthesised from the old. These aspects will be considered in the following sections.

(b) <u>In-toto incorporation of glycine molecule into Purine Ring.</u>

By the formation of glycinamide ribo-nucleotide (GAR) from glycine and phosphoribosylamine (138,139,175,177), the glycine moiety is incorporated in toto into the evolving purine nucleus.

Howell et al (196) studied the incorporation of glycine-N^{15} into N-7 and the incorporation of simultaneously administered glycine-1-C^{14} into C-4 of uric acid. Any disparity would indicate differences in the rates of turnover of the αN and the carboxyl carbon of glycine. This would point to significant activity of a metabolic pathway of glycine synthesis most pertinent to the present discussion. A major fate of glycine is its interconversion with serine. This glycine-serine "shuttle" would not affect the isotopic composition of glycine as the process merely implies the addition or removal of an active one-carbon fragment to and from the glycine moiety. However, serine serves as precursor of glyoxylic acid, a compound readily converted to glycine by glyoxylic-

glutamic transaminase (56,312,468). In the first step of this cycle, namely the decarboxylation of serine to form ethanolamine (174), the original glycine-1-C is lost as CO_2. In the next step i.e. the deamination of ethanolamine to glycolaldehyde (174,317), the glycine nitrogen is removed as ammonia. Glycolaldehyde is then oxidized, to glyoxylic acid (233). Thus, glyoxylic acid arising from glycine-1-C^{14} or αN^{15} via serine would be unlabelled. Glycine can also be deaminated by glycine oxidase to form glyoxylic acid and ammonia (174,355). If this labelled glyoxylic acid were extensively diluted by unlabelled molecules via serine, and if the ammonia-N^{15} from glycine were to find its way rapidly into glutamine, it is theoretically possible for active resynthesis of glycine via serine to result in the reincorporation of N^{15} in excess of C^{14} following the simultaneous administration of glycine-1-C^{14} and glycine-N^{15}. On degrading uric acid C^{14}, N^{15}, the finding of N^{15}-7/C^{14}-4 ratios of more than 1.0 will indicate the presence of a significantly functioning recycling pathway.

This pathway has also a direct bearing on the metabolism of the glycine-2-C. Following the decarboxylation of serine, the glycine-1-C is lost as CO_2. The glycine-2-C is oxidized in the cycle to the carboxyl group of glyoxylic acid and thence to the carboxyl carbon

of resynthesized glycine. Thus, if this cycle operates significantly, glycine-2-C^{14} will progressively give rise to more and more glycine-1-C^{14} thereby labelling C-4 as well as C-5 of the purine moiety.

Howell et al (196) studied the activity of this pathway in a normal subject. After giving an oral mixture of glycine-1-C^{14}, N^{15}, urinary uric acid was isolated and degraded daily for the following 24 days. The percentage dose incorporated into N-7 and C-4 was calculated and the N7-C4 ratios were calculated daily and on a cumulative basis. Hippuric acid was also isolated daily and the N^{15}:C^{14} ratios of the glycine moiety were calculated. Their results showed most conclusively that in the normal subject studied, the N^{15} - 7 to C^{14} - 4 ratios were 1 indicating no recycling of N^{15} into N-7 by secondary pathways.

Gutman et al (168) administered glycine N^{15}, 1-C^{14} to normal and gouty subjects. They showed that in 2 normal subjects the N^{15} - 7 to uric acid-C^{14} (equivalent to C-4) ratios did not significantly deviate from 1 confirming the observations of Howell et al (196). In 2 gouty normoexcretors of uric acid, ratios were 1.0 and 1.3 and in 3 gouty overexcretors, ratios were 1.2 and 1.65, the ratios rising as the experiment progressed.

From these observations, one can conclude that in normal subjects, the composition of each isotopic glycine

molecule in the body does not change during the course of the experiment, indicating that whatever activity there is in the recycling pathway is of little significance. However, in gout, disproportionate enrichment of N-7 was observed. Whether this points to a more active serine-ethanolamine-glyoxylic acid cycle in gout or whether excessive enrichment of glutamine merely shows up a normally active pathway is not certain.

(c) The glycine-2-C and the one-carbon unit pool.

(i) Active "1-carbon" unit metabolism and its relationship to Purine Biosynthesis.

During the process of inosine monophosphate (IMP) biosynthesis, two active carbon fragments at the formate level of oxidation are added to intermediates and eventually become the 2 and 8 carbons of the purine ring. Studies on the two transformylase reactions have already been reviewed earlier this chapter (see pg.154,156). The two enzymes involved, glycinamide ribonucleotide (GAR) transformylase and 5-amino-4-imidazole carboxamide ribonucleotide (AICAR) transformylase catalyze respectively the formylations of GAR (138,175,178,456,457) and AICAR (102, 103,178). These workers have established that the immediate formyl donors are either N^{10} formyl 5,6,7,8 tetrahydrofolic acid (THFA) or N^{5}, N^{10} adhydroformyl THFA. However, in the transformylase enzyme systems employed by Flaks (102,103), Goldthwaite (138),

Hartman (175) and Warren (456,457), it was not possible to determine which of these active formyl derivatives was preferentially used in each reaction. This was due to their rapid interconversion catalyzed by the widely occurring enzyme cyclohydrolase (178,350) and the question was only resolved in 1959 by Hartman et al (178), who were able to prepare highly purified GAR and AICAR transformylases free from cyclohydrolase activity. They were able to show that N^5, N^{10} anhydroformyl THFA was the specific formyl group donor in the GAR transformylase reaction(C8) whereas N^{10} formyl THFA specifically donated its formyl group in the AICAR transformylase reaction (C2).

There are a number of pathways via which active formate becomes incorporated into THFA in a biologically active form. Greenberg, and Jaenicke (158) and Rabinowitz and Pricer (351) have shown that formate may react enzymatically with THFA in the presence of ATP to yield N^{10}-formyl THFA. The hydroxymethyl THFA derivative formed from serine is reversibly converted to an active formyl folic acid, compound by a NADP dependent hydroxymethyl THFA dehydrogenase (158,180,209,329,330). Whilst earlier research workers were able to establish that the active formyl THFA was not the N^5-formyl isomer, they were unable to decide whether N^{10}-formyl THFA or N^5, N^{10} anhydroformyl THFA was the actual product of the reaction as their more crude dehydrogenase systems were contaminated with

cyclohydrolase. However, Osborn and Huennekens (329) were able to purify a dehydrogenase from chicken liver, without cyclohydrolase activity. They were now able to show that when the β-carbon of serine was converted to a formyl compound via hydroxymethyl THFA, the first product formed was N^5, N^{10} anhydroformyl THFA. This compound was also the initial formyl derivative produced in the catabolism of formiminoglycine and formiminoglutamic acid (350), intermediates in the enzymatic degradation of purines and histidine respectively. The biological origin of N^5-formyl THFA is not known (329). Greenberg et al (158) and Warren et al (456,457) have shown that this compound is inactive as a formyl donor in their transformylase enzyme systems. Peters and Greenberg(341) have studied an ATP-dependent conversion of this compound to N^5, N^{10} anhydroformyl THFA.

The active formyl THFA derivatives involved in purine synthesis receive "one-carbon" units from a variety of formyl donors. These include the glycine-2-C (165,188,196,217,319,426, this thesis), ALA-5-C (78,319), formate (188,217,426) and the β-carbon (hydroxymethyl moiety) of serine (92). It has just been shown that unique pathways exist for the synthesis and utilisation of each of the formyl derivatives of THFA. This suggests that a metabolic subdivision of the formate "one-carbon" pool may exist. Thus, formate and

compounds giving rise to formate may be preferentially utilised for position 2 of purines while serine and various formimino compounds may contribute mainly to the N^5, N^{10} anhydroformyl THFA dependent transformylase reactions responsible for the formation of carbon 8. However, it must be remembered that the conditions of these highly purified in-vitro enzyme systems are artificial and not analogous to conditions in-vivo where the widely occurring enzyme, cyclohydrolase will cause rapid interconversion between N^{10} formyl THFA and N^5, N^{10} anhydroformyl THFA. On available experimental evidence, therefore, it is not possible to decide which of the "one-carbon" unit donors is the most important in the formylation of those active THFA derivatives involved in the biosynthesis of the purine nucleus.

(ii) Glycine as a formyl donor.

Three possible pathways whereby the α-carbon of glycine can label one-carbon fragments have already been reviewed in Chapter II, page 97. The glycine-2-C can enter the one-carbon unit pool via the succinate glycine cycle (see Chapter II, pages 51-57), via the aminoacetone cycle (91,447, 448), and following the conversion of glycine to glyoxylic acid (75,312,313,314,468,469,470). The observations by Nemeth et al (319) have suggested that the first of these pathways, the succinate-glycine cycle is the most important one and that in the utilisation of the glycine-2-C as an active carbon fragment, ALA was an ..... P.T.O.

intermediate. Detailed evidence for this has been presented and discussed in Chapter II, pages 64-65.

These observations are fundamental to the basic aims of purine studies presented in this thesis. In this thesis, experiments have been designed to test the hypothesis of Labbe, Talman et al (234,235,437) which was put forward after these authors demonstrate decreased purine synthesis in chick embryos made porphyric with Sedormid. Their hypothesis was as follows (234): "A block in Shemins succinate glycine cycle retarding the entry of the carbon of ALA into the C-1 pool and thence into purines results in an accumulation of this common precursor which is then disposed of via the porphyrin pathway". If this postulate is correct and if Nemeth's observations (319) that the glycine-2-C labels carbons 2 and 8 of the purine nucleus mainly via ALA-5-C is valid, then, in acute human porphyria any decreased synthesis of purines would be reflected in relatively less activity residing in C2 and 8 than in C4 and 5 following the administration of glycine-2-C^{14}.

(d) Degradation of uric acid formed from isotopic Glycine.

(i) Glycine-N^{15}.

The nitrogen atoms of the purine nucleus are derived from glycine (N-7), aspartic acid (N-1) and glutamine (N3+9). Following glycine-N^{15} administration, any

enrichment of aspartic acid or glutamine will be derived from that $N^{15}H_3$ arising from the oxidative deamination of glycine. In the first few hours following labelled glycine administration, one would expect the major portion of N^{15} entering the purine moiety to be derived from glycine. However, over the latter part of the 24 hours in which N^{15} concentration in the body is high, one would expect more and more activity to appear in glutamine and aspartic acid at the expense of glycine. Thus, those purine moieties formed, e.g. 1 hour after glycine-N^{15} administration should have a different disposition of N^{15} activity than purines synthesized for example 12 hours later.

Would these changes be reflected in the disposition of N^{15} activity within the excreted uric acid moiety? The varying distribution of N^{15} in purine precursors may well be reflected within the promptly labelled uric acid molecules formed in the main from IMP via shunt pathways. However, the plateau of isotopic activity in the subsequently excreted uric acid molecules is thought to represent cleavage of nucleotides derived from tissue nucleic acids whose life spans vary considerably. Thus one would expect to find a fairly constant disposition of activity in urinary uric acid except perhaps in those molecules excreted within a few days of giving glycine-N^{15}.

Gutman et al (168,170) studied the intramolecular distribution of radioactivity within uric acid-N^{15} following glycine-N^{15} administration. In one study (168), the authors degraded the uric acid molecule into fractions representing N-7 and N (1+3+9). They showed that N1 + 3 + 9 comprised 23 to 34% of the total molecular N^{15} on day 1 and thereafter, this fraction increased progressively reaching values of 39 to 50% of the total by day 7. Shemin and Rittenberg (413) similarly reported that in a normal subject studied, N1 + 3 + 9 comprised 22.4% of the total molecular-N^{15} activity on day 1 increasing to 35.2% on day 9. Howell et al (196) graphing the percentage dose incorporated into N-7, N-1 and N3+9 showed how over 24 days, the proportion of total N^{15} residing in N-7 gradually and progressively fell while the relative proportions of the isotope derived from N-1 and N3+9 increased slowly with time by similar amounts. The cumulative uric acid-N^{15} activity rose in a linear fashion for the first 2 weeks and thereafter, although the activity continued to rise, the slope became less steep.

These experiments clearly show that the changing pattern of isotopic activity within the uric acid molecule observed over weeks reflects what is happening over 24 to 36 hours in the various purine precursor pools following the administration of glycine-N^{15}.

(ii) Glycine-1-C^{14}.

Glycine-1-C^{14} serves as a specific label of C-4 of the purine nucleus. There is no recycling pathway described which would enable the carboxyl carbon of glycine to re-enter newly synthesized glycine in the α position. The glycine-1-C is readily decarboxylated and excreted from the body as respiratory CO_2 (490). Therefore one would expect glycine-1-C^{14} to label C-6 of the purine moiety as well.

Howell et al (196) and Wyngaarden (490) have both degraded uric acid-C^{14} derived from glycine-1-C in normal subjects (196,490), in subjects with primary gout and gout secondary to myeloid metaplasia (490). In all cases, activity in carbon atoms 4 and 5 comprise virtually 100% of the total C^{14} activity of the molecule. Thus, in human subjects, there is clearly no labelling of C-6 via respiratory CO_2 and the glycine-1-C specifically labels C4 only of the purine moiety. Heinreich et al (188) isolated adenine and guanine from tissue nucleic acids of rats given different labelled precursors. Following glycine-1-C^{14}, about 75% of the total activity in the purine moiety was recovered in C_4 and 5 which on further degradation was shown to reside entirely in C^4. Unfortunately, activity in C^6 was not measured directly.

However, earlier work in pigeons produced slightly different results. Karlsson et al (217) showed that following glycine-1-C^{14}, 97.6% of the total uric acid C^{14} activity was detected in C4 and 5, 2.4% in C6 and 0.0% in C2 and 8. Buchanan et al (46) and Sonne et al (426) administered glycine-1-C^{13} to pigeons and following degradation of uric acid, they showed that 96% of the radioactivity resided in C4 + 5, 4% in C6 and 0.0% in C2+8. The C^{13} atom excess in C6 was 0.11 which was virtually identical to that observed in respiratory CO_2, namely 0.12. The authors (46,426) felt that this supported the role of respiratory CO_2 as a precursor of C6. Similar correlations between respiratory CO_2 and C-6 of uric acid were observed following administration of $C^{13}O_2$, $HC^{13}OOH$, CH_3 C^{13} OOH and other C^{13}-labelled precursors.

The differences noted between human and pigeon experiments may be a matter of degree. Humans are ureotelic, i.e. most of the excreted nitrogen leaves the body as urea. In pigeons however, as with other uricotelic vertebrates, the main route of nitrogen excretion is via uric acid. Thus, enough respiratory CO_2 is deviated into uric acid to be experimentally detectable.

(iii) Glycine-2-C^{14}.

Glycine-2-C^{14} will label C-5 of the purine nucleus directly and C2 + 8 via the active one-carbon unit pool. In this thesis the ratio of activity between C4 +5 and C2 + 8 has been

calculated in degraded urinary uric acid of normal and porphyric human subjects. There is not much data on similar degradation experiments in the literature. Nemeth et al (319) demonstrated labelling of the ureido carbons of guanine by the αC of glycine but only Karlsson et al (217) have fully degraded uric acid isolated from the pigeons after giving glycine-2-C^{14}. They showed that 31% of the activity resided in C2 and 8, 67.2% in C4 and 5 and 1.97% in C6. In the one experiment reported, the C4,5: C2,8 ratio was 2.16.

I. INCORPORATION OF OTHER LABELLED PRECURSORS INTO URIC ACID.

(1) Ammonia-N^{15}.

Gutman et al (168) studied the incorporation of N^{15} H_4 Cl in gouty and normal subjects. The pattern of isotope excretion in the urine was essentially similar to that observed with glycine with an early peak in 2 to 3 days, followed by a plateau of excreted activity. Being a small molecular purine precursor, ammonia presumably enters all cells with facility and uric acid-N^{15} excretory patters are therefore similar to those following glycine-N^{15} for the same reasons.

In two non-gouty controls, percentage incorporation N^{15} after 7 days was 0.053 and 0.077% of the total dose respectively. In gouty normoexcretors, normal incorporation values were obtained whereas in gouty subjects excreting excessive amounts of urinary uric acid, over incorporation to values of 0.283% of the total dose noted after 7 days. Greatest initial activity was in N3+9 via amide nitrogen of glutamine. N 1+3+9 ranged from 69 to 89% of the total uric acid N^{15} activity in all the subjects studied, showing some recycling of the isotope into glycine.

(2) Formate-C^{14}.

The incorporation of C^{14} labelled formate into uric acid has been studied by many research workers (46,49,

188,217,230,426,430,451). Buchanan et al (49), Spilman (430) and Villa et al (451) noted that in normal and gouty human subjects, as with glycine and ammonia, the isotope rapidly enters uric acid and remains in the excreted purine as a plateau of activity for many days after administration. The peak of activity occurred 1 to 3 days after formate-C^{14} had been given.

Krakoff et al (230) noted similar patterns of uric acid-C^{14} excretion in patients with chronic lymphatic leukaemia given sodium formate-C^{14} I.V. However, in chronic myeloid leukaemia, a secondary peak was noted 12 to 15 days after administration similar in timing to those observed in patients with myeloid metaplasia or polycythaemia vera receiving isotopic glycine (248, 428,492,501). Explanations for this phenomenon have already been discussed earlier this chapter, pages 205 to 210. The study by Krakoff et al (230) clearly demonstrates more rapid purine turnover in myeloid as opposed to lymphocytic leukaemia and explains the greater responsiveness of the former disease to purine antimetabolites. Sonne, Buchanan and Delluva (46,426), Karlsson et al (217) and Heinrich et al (88) degraded uric acid formed from isotopic formate. All these workers showed that formate exclusively labelled the ureido carbons, C2 and 8 of the purine moiety, virtually no activity residing in the other carbon atoms. Similar results

have been noted with other isotopic formyl donors such as ALA-5-C^{14} (78) and serine whose hydroxymethyl carbon was labelled (92). In the latter study by Elwyn and Sprinson (92), while serine-C^{14} labelled C2 and 8 predominantly about 10 to 25% of the total molecule's activity resided elsewhere, mainly in C5. These observations are at variance with other published data above and may well indicate activity in the serine-ethanolamine-glyoxylic acid cycle, where serine whose hydroxymethyl carbon was labelled would ultimately give rise to newly synthesized glycine-2-C^{14}, thereby labelling C5 of uric acid as well as C2 and 8.

(3) Isotopic bicarbonate.

Bicarbonate-C^{13} or-C^{14} has been administered to pigeons (46,217,426) or rats (188) and uric acid or guanine has been isolated and degraded. In all studies, bicarbonate labels C-6 maximally incorporation values ranging from 67.5% (46) to 91% (188), the remaining activity residing mainly in C4 and 5.

(4) ALA-5-C^{14}

(a) Pattern of excretion of isotope in urinary uric acid.

The pattern of isotope excretion in urinary uric acid following the administration of ALA-5-C^{14} is most interesting. Dowdle et al (78) gave ALA-5-C^{14}

orally to normal and porphyric human subjects and showed that about 4 to 14% of the dose was excreted as uric acid in 8 days. As with glycine, formate etc., there was prompt labelling of the urinary uric acid. However, this was not followed by a plateau but by a rapid drop in urinary uric acid-C^{14} activity to 35 to 40% of peak values after 4 days. Thereafter the decline in activity persisted, a little more slowly, tending to plateau out by the 7th day at about 15% of initial values.

(b) Interpretation of above findings.

ALA enters certain tissues in-vivo with difficulty. It has already been noted that whereas glycine-2-C^{14} is readily incorporated into marrow and therefore red cells haem (76,77,205,207,496, this thesis), ALA-C^{14} enters haemopoietic tissue poorly in human subjects (78,205,207, 496) and experimental animals (24,201,202,400). However, there is abundant evidence from early-labelled bilirubin-C^{14} (78,116,201,202,207,400) and liver haem-C^{14} studies (116, 400) in humans and experimental animals that ALA-C^{14} is efficiently utilised by liver cells.

Schwartz and Cardinal (401) demonstrated by a neat experiment that following ALA-4-C^{14}, liver and kidney haems were labelled almost to the exclusion of haems in other tissues. Whilst this may merely indicate that these

organs are the sites of maximal haem turnover in the body, it is possible that in addition, ALA enters hepatic and renal cells with greater facility than cells of other tissues.

Bearing in mind the discussion on the derivation of promptly-labelled uric acid (see page 207, this chapter), the presence of an early peak followed by a rapid decline in isotopic urinary uric acid activity after ALA-5-C^{14} (76) must indicate one of the three following possibilities:-

(i) Selective deviation of newly-synthesised IMP-C^{14} to hypoxanthine and uric acid,

(ii) failure by the liver to make isotopic nucleic acid from ALA-5-C^{14}, the uric acid-C^{14} being derived from rapidly turning over nucleotides, e.g. ATP, GTP, GMP, FAD, etc., or

(iii) rapid turnover of the bulk of liver nucleic acids and nucleotides.

There is no physiological reason why IMP should be selectively cleaved before its conversion to AMP and GMP. Therefore either possibility (ii) or (iii) must be operative.

DNA dependent RNA synthesis takes place within the nucleus. It could be argued that whereas ALA enters the liver cell readily, it may not get into the nucleus and

label those nucleotides polymerized into nucleic acids. This presupposes that the nucleotides within the nucleus are made locally and do not enter from the cytoplasm. As this is unlikely, by far the most reasonable explanation is that both liver nucleotides and nucleic acids are readily labelled and in the main, turnover rapidly i.e. possibility number (iii).

There is convincing experimental evidence in support of possibility number (iii). The work by Spilman (vide Goldthwaite) (140) showed that liver nucleotides turned over within 90 minutes rapidly labelling uric acid. Stachelin et al (451) noted that following P^{32} labelled phosphate administration to animals, an RNA fraction of high specific activity associated with ribosomes was detected within 30 minutes. Further, following actinomycin, the majority of ribosomal aggregates (ergosomes), disintegrated within 8 hours, indicating rapid turnover of messenger RNA. These experiments and their implications have been discussed in detail earlier this chapter, pages 184-186. Thus, there is good evidence that in the liver, both nucleotide and the bulk of nucleic acid turnover is rapid enough to account for the promptly labelled uric acid following ALA-5-C^{14} and also, therefore, following ..... P.T.O.

isotopic glycine.

As mentioned before, about 20% of ribosomal RNA remains biologically active for prolonged periods even after the administration of high doses of actinomycine (431). Further, Loeb et al (271) and Furst et al (108) demonstrated a more slowly turning over RNA fraction in the liver with a half-life of 5 to 10 days (see pages 178-/ for details). Moreover, not only may many tissues, e.g. heart (140), use salvage pathways of purine nucleotide synthesis preferentially, but some tissues e.g. bone marrow may be entirely dependent on preformed purine precursors, (242,280), of which the liver is by far the most important source (242). For further discussion, see pages 162-166 of this chapter.

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The tendency for the curve of excretion of uric acid-C^{14} to plateau out at about 15% of initial values 7 days after giving ALA-5-C^{14} indicates that labelling of more slowly turning over nucleic acids both in the liver and other tissues was minimal. The presence of a slowly declining plateau of high activity following early labelled uric acid has been noted persistently with isotopic glycine and other small molecule precursors e.g. formate and ammonia. Thus, one can infer that in most tissues, de novo synthesis of the purine ring is quantitatively more important than salvage pathways in tissue nucleotide and nucleic acid metabolism.

(c) Relevance of above findings to thesis.

The aim of the present study is to observe how purine metabolism is affected in human porphyria by analysing urinary uric acid-C^{14} data following glycine-2-C^{14}. As South African genetic porphyria and symptomatic porphyria are disorders of hepatic porphyrin metabolism, it is likely that any disturbance of purine metabolism will also be localized to the liver.

It has been suggested that uric acid-C^{14} formed after ALA-5-C^{14} may reflect the catabolism of liver nucleotides and nucleic acids. Therefore, ALA-5-C^{14} would be a superior precursor to use in studying porphyria than glycine-2-C^{14} if it can be shown conclusively that the label enters liver nucleic acids as well as nucleotides.

Following ALA-5-C^{14}, activity in urinary acid drops to below 50% of initial values after 3 days (78). By inference, activity in urinary uric acid-C^{14} following glycine-2-C^{14} after 3 days, will be derived mainly from the oxidation of purines made de novo in tissues other than the liver. Thus, data in the first few days of experiments to be presented later this thesis, will provide the information sought for.

(d) Degradation studies.

ALA-5-C^{14}, via the succinate glycine pathway (see Chapter II, pages 59,60), will provide active carbon fragments for purine synthesis thus labelling the ureido carbons 2 and 8 of the purine ring. With labelled formate as the C-1 donor (46,188,217,426), virtually 100% of the total activity within the uric acid molecule resided in carbons in position 2 and 8.

Dowdle (78) confirmed these findings in his degradation experiments following ALA-5-C^{14}; it was also shown that the specific activity of either of the ureido carbons was of a similar order of magnitude at its peak as that of maximally labelled respiratory carbon dioxide. Yet there was negligible activity in C6 of the degraded uric acid-C^{14} molecule.

Nemeth et al (319) found that following the administration of ALA-5-C^{14} to pigeons, the percentage activity residing in C2 and 8 fell from 70 to 50% over the three days of the experiment, C6 becoming progressively more labelled. The specific activity of respiratory CO_2 was similar to that of C6.

The difference in the way in which humans and pigeons label C-6 following glycine-1-C^{14} has already been commented on (see pages 230 this chapter) and presumably similar mechanisms are operative in ALA-5-C^{14} studies.

(5) 5-amino-4-imidazole carboxamide-4-C^{14} (AIC-4-C^{14}).

AIC ribonucleotide (AICAR) is a late intermediate in the de novo synthesis of the purine ring. AIC-C^{13} and AIC-C^{14} have been used as precursors in the study of purine metabolism. The utilisation of AIC involves initial conversion to its ribonucleotide according to the following reaction (101):-

AIC + PP-ribose $\rightleftharpoons$ AIC-ribose 5-P (AICAR) + PPi.

This is followed as in purine biosynthesis by formylation to IMP (102) according to the following reaction:-

AICAR + N_5 N_{10} formyl THFA $\rightleftharpoons$ IMP + THFA.

IMP can be converted to AMP, GMP thereby into other nucleotides and nucleic acids, or it can be cleaved and directly oxidised via hypoxanthine to uric acid.

Seegmiller et al (403) found that in normal man, about 20% of AIC-4-C^{14} was excreted in the urine as isotopic uric acid in 14 days. This compares closely with figures of 4 to 14% in the 8 days following ALA-5-C^{14} (78). Expressing data logarithmically (403,406) they showed biphasic incorporation consisting of prompt and extensive conversion of AIC-C^{14} to uric acid followed by a slower, less direct conversion, occurring after 7 days and thought to reflect relatively slower turnover of tissue purine nucleic acids. Similar observations were

made by Wyngaarden et al (493), using AIC-4-C^{14} in a case of chronic myeloid leukaemia.

These findings are essentially similar to those following ALA-5-C^{14} (28) and almost certainly occur for the same reasons. Isotopic AIC is a relatively large molecule which probably enters the liver more readily than other organs. Another possibility is that isotopic AIC being an immediate precursor of IMP would result in a sudden and excessive production of IMP, the surplus being rapidly oxidised and excreted as uric acid.

(6) Labelled purine base precursors.

A study of the pathways of purine interconversion allows one to predict the fate of administered labelled purine bases. Hypoxanthine is mainly oxidised by xanthine oxidase to uric acid. A little may be re-converted by salvage pathways, to IMP and thereby to other nucleotides and nucleic acids. Thus by devious routes, hypoxanthine may be converted to other urinary purines.

In contrast, as humans lack the enzyme adenine deaminase which in bacterial systems deaminates adenine to hypoxanthine, any uric acid formed would result from the rather devious metabolic route via AMP, IMP, inosine and hypoxanthine. Thus adenine would be predominantly

excreted as such with little conversion to uric acid.

The pattern of isotope excretion in the urine following labelled purine metabolism was interesting and in the main, predictable. Wyngaarden et al (493) administered hypoxanthine-8-C^{14} I.V. to a human subject. The label appeared within 24 hours in high concentration in urinary hypoxanthine-C^{14} and then declined rapidly. A small degree of labelling of other purine bases reflected the incorporation of a minor portion of hypoxanthine into IMP via salvage pathways with subsequent conversion to other nucleotides. Urinary uric acid-C^{14} excretion reflected that of hypoxanthine initially in in that there was prompt labelling followed by a decline in isotope abundance similar to that obtained after the administration of labelled uric acid. This part of isotope excretion represented the metabolic disposition of hypoxanthine rapidly via shunt pathways of uric acid formation underlying the pathways whereby the body eliminates excess purine nucleotides. However, as with AIC-C^{14} and ALA-5-C^{14} there is a tendency for the rapid decline in urinary uric acid-C^{14} to flatten out after about 7 days indicating minimal incorporation of the label into other purine nucleotides and nucleic acids. Expressed logarithmically, the rate of decline of isotope concentration was rapid and linear for about

8 days, before deviating from a straight line.

Administration of adenine-8-C^{14} resulted in an entirely different pattern of incorporation (493). Adenine of high specific activity appeared early and thereafter, activity fell in a slowly declining plateau over the following 7 days. This plateau probably indicated repeated enrichment of the adenine pool via prior incorporation of the label into adenine nucleotides and nucleic acids and turnover of the latter. A portion of the label also appeared in the other urinary purine compounds. As expected, there was relatively little labelling of urinary uric acid, but what activity appeared reached a maximum only on about the 5th to 7th day and declined gradually over the following 7 days. Adenine in part represented the production of uric acid from nucleic acid turnover in the tissues.

This would not represent the complete picture as guanine nucleotides can more readily be converted to xanthine and hence would label uric acid earlier and more extensively than adenine.

(6) Recapitulation.

If labelled uric acid is infused I.V. or given orally, there is an early peak of specific activity

followed by a rapid fall off in the excretion of the isotope in urinary uric acid. The decline of activity is linear when expressed logarithmically, most of the infused dose having been excreted within to 14 days of administration. On the other hand, when small molecular weight isotopic precursors, e.g. glycine, formate, ammonia, are given, the early peak of uric acid activity is followed by a slowly declining plateau ascribed to uric acid produced from the turnover of purine nucleic acids of varying life span in different tissues. The excretion of the isotope in urinary uric acid following larger molecules, e.g. ALA-5-C^{14}, AIC-4-C^{14} lies somewhere in-between with rapid decline in the first week followed by a plateau of low activity. It is thought that these molecules being larger enter certain tissue cells only, mainly the liver. It has been shown that the turnover of liver nucleotides and RNA is in the main rapid enough to account for the excretory patterns of the isotope in urinary uric acid. The low level plateau after the first week may reflect more slowly turning over nucleic acids in the liver and the turnover of these nucleic acids in peripheral tissues synthesized from preformed liver purines via salvage pathways. All the above conclusions have been supported by experimental evidence discussed at length in

the previous two sections.

Degradation of the isotopic uric acid molecule has in the main revealed that the disposition of activity within the purine moiety follows a pattern predictable for the given labelled precursor.

J. EXCRETION OF URIC ACID.

(1) Renal mechanisms.

(a) Sites of uric acid excretion with the Nephron.

Uric acid is a weak divalent organic acid with pka values of 5.75 and 10.30. In urine, there is usually a mixture of uric acid and monourate salts, the proportion varying according to the urinary pH.

Organic acids with the exception of aminoacids and citric acid cycle intermediates, are in the main excreted by proximal tubular secretion (300). It is currently thought that filtered uric acid is virtually completely reabsorbed in the proximal tubules in man and that excreted uric acid is produced almost entirely by a tubular secretion process (166,167).

Tubular secretion has been demonstrated in the rabbit and in some dogs (496) but there is greater inter-species variability in uric acid excretion than with any other organic acid (300) so that few definite analogues can be drawn. Milne (300) has drawn attention to some of the differences between the excretion of uric acid and other organic acids. While it is extremely rare for drugs to enhance the excretion of an organic acid, there a number of uricosuric drugs available. Urate excretion unlike many other organic acids is little affected by urinary pH but the effects

of uricosuric drugs are commonly pH dependent, better in alkaline urine (300). Another difference is that while tubular reabsorption of urate involves active transport, many other organic acids are reabsorbed by passive diffusion without involving intracellular energy (300).

(b) Evidence for tubular secretion of urate.

Evidence for tubular secretion of uric acid in man is both direct and circumstantial. Urate clearance is normally 5 to 10% that of inulin i.e. tubular reabsorption normally occurs (300). Clearances above that of inulin have been obtained in man but under exceptional circumstances (166,347). Four separate factors were found to be necessary (166): (a) reduction of glomerular filtration rate by previous renal disease to 40 to 80 ml per minute, (b) infusion of urate to produce plasma levels of 13 to 22 mg/100 ml, (c) osmotic diuresis by mannitol, and (d) administration of large doses of a uricosuric drug. Urate:Inulin clearances of above 1.10 were obtained in 5 experiments and above 1.20 in two.

There is a case reported by Praeterius and Kirk (347) of a patient who had an extremely low plasma uric acid of 0.2 to 0.6 mg/100 ml with normal excretion of

urinary uric acid. In this case, uric acid clearance values were 28 to 46% more than simultaneously performed inulin clearances. Thus in certain circumstances in man, demonstrable tubular secretion of uric acid does take place.

The dual or paradoxical effects of uricosuric agents such as salicylate, phenylbutazone and probenecid in man (500,503) are best explained by postulating simultaneous tubular reabsorption and secretion of uric acid. In very low dosage, there is reduced urate clearance whereas with high doses, the excretion of uric acid is enhanced. This suggests that both secretion and reabsorption of urate are reduced by uricosuric drugs, inhibition of secretion being more sensitive and solely blocked at low dosage.

Pyrazinoic acid (pyrazinamide) has been shown to inhibit tubular secretion but not reabsorption of urate in the mongrel and Dalmation dog (504). In man, this drug reduces uric acid clearance to 0.6% that of inulin (502) compared to normal values of 5 to 10%. This suggests that the excreted uric acid is the result of proximal tubular secretion.

Finally, urate excretion is poorly correlated with the filtered load of urate suggesting a complex tubular handling involving both tubular secretion and reabsorption.

(c) Abnormalities of Renal Excretion of Urate.

In gout, a number of studies have shown that the clearance of urate when compared with inulin or creatinine is less than in normal subjects (327). In some cases of primary gout, this may be the only abnormality with no overproduction of uric acid (404).

Urate excretion is competitively reduced by hyperlactic acidaemia with increased urinary output of lactic acid (300). Infusions of sodium lactate have been shown to rapidly reduce urinary urate with subsequent hyperuricaemia (295,502). After ingestion of ethanol sufficient to cause mild intoxication, there is an increased production of lactic acid since oxidation of ethanol to acetaldehyde is coupled with pyruvate-lactate oxidative and reductive enzyme systems (473). This may be the reason why alcohol may precipitate gout. Increase in plasma urate due to increased production of lactic acid also occurs in toxaemia of pregnancy (465) and after severe muscular exercise (324). Classical glycogen storage disease due to deficiency of glucose-6-phosphatase is always associated with hyperuricaemia and hyperlacticacidaemia.(197). Gout has occurred in this disease (405).

Hyperuricaemia also occurs in ketoacidosis of starvation (185,257,309,410) and in diabetic ketoacidosis (334). In starvation ketosis (309), decreased urate clearance precedes the first qualitative tests for urinary acetone and acetoacetic acid. The causative agent is more likely to be an increase of urinary hydroxybutyric acid, structurally related to lactic acid and not giving a positive Rotheras reaction. Hyperuricaemia has also been described in hyperparathyroidisin and myxoedema (300) but mechanisms have not yet been explained.

Hypouricaemia not related to uricosuric drugs has been noted in diseases with decreased reabsorption of filtered urate and consequent urate clearance in such generalized proximal tubular disorders as Fanconi syndrome, Wilson's disease and cadmium poisoning (300).

(2) Extrarenal excretion of urate.

In normal man, urinary recoveries of injected unlabelled and labelled urinary uric acid have been shown to be incomplete. Using infused uric acid-N^{15} or uric acid-2-C^{14}, recoveries ranging from 55 to 95% have been reported (53,54,119,404,427,486,487). An average of 14 reported studies was found by Wyngaarden (496) to be 75.6%. Thus about a quarter of uric acid excreted daily is eliminated via extrarenal channels.

Studies of uric acid turnover in normal man have uniformly shown that the quantity of uric acid synthesized daily is larger than that excreted in the urine (427,486). In studies with uric acid-N^{15} (487) or uric acid-C^{14} (427), the fraction of turnover appearing in the urine is essentially the same as the fraction % injected uric acid recovered in the urine.

Following the infusion of uric acid-N^{15} (16), the label appeared in significant concentrations in urinary urea and ammonia. In a normal subject studied (54,486) about 25% of the isotope was found in urinary allantoin, urea and ammonia and in faecal nitrogen. In comparable studies with uric acid-2-C^{14}, a comparable percentage of administered isotope was recoverable in respiratory CO_2, urinary urea, allantoin and allantoic acid and in the faeces (427). Again, about 25% of the injected dose of isotopic uric acid was disposed of extrarenally.

When labelled uric acid was administered orally to normal subjects, only 9 to 11% was absorbed and excreted unchanged in the urine (119,427). With uric acid-2-C^{14}, only 2.4% of the administered dose appeared in urea but 55% was recovered as respiratory $C^{14}O_2$. An additional 16.3% was recovered in the faeces, 83 to 91% within intestinal bacteria. Virtually no isotopic faecal

uric acid was recoverable. By inference, any uric acid excreted in the intestine will largely be degraded, almost certainly by intestinal bacteria.

Extrarenal excretion and degradation of intravenously administered isotopic uric acid was then studied by Sorenson (427) before and after effective intestinal bacteriostasis with antibiotics. He showed that while initially 0.0% of activity was recoverable from the stool as uric-acid-C^{14}, following antibiotic sterilisation of the bowel, a daily average of 186 mg or 26.6% of the total turnover was isolated from the faeces - a figure comprising most of the 30.1% of the administered C^{14} present in the stool. The quantity of C^{14} recovered in various degradation products was reduced from 22.5 to 3.0% after intestinal bacteriostasis.

As the average uric acid pool size is about 1200 mg and as the turnover averages about 700 mg per day, one would expect extrarenal elimination to be in the order of about 175 mg per day. In isotopic studies, the quantity of uric acid entering the pool exceed urinary loss by 100 to 260 mg per day (16,427). Sorenson (427) by investigating uric acid concentrations in accessible intestinal secretions showed that the total uric acid entering the bowel daily via saliva, gastric juice and

bile is about 100 mg. Add to this a similar amount from pancreatic, biliary and intestinal juices and it can be seen that normal man secretes about 200 mg of uric acid into his gut daily, values compatible with the amounts eliminated by extrarenal uricolysis daily.

A trivial amount of uricolysis may occur within tissues of man. Two enzyme systems can destroy uric acid in-vitro, verdoperoxidase (57) and cytochrome-cytochrome oxidase (160). Bien and Zucher (27) have shown that leucocytes, erythrocytes and leucocyte extracts have uricolytic properties. The activity resides mainly in cells of the myeloid series which have verdoperoxidase (4).

In gout, recoveries of injected uric acid-N^{15} (54) or uric acid-C^{14} (278) have ranged from 35 to 54%, significantly lower than normal. Bearing in mind exchange of uric acid with solid tophaceous urate (another extrarenal loss), extrarenal uricolysis is normal or greater than normal in gout (342,427), thus becoming an important compensatory route of elimination of the excess uric acid produced. In normal or gouty subjects with severe renal insufficiency, intestinal uricolysis may be the major process of disposal of uric acid (428).

CHAPTER V.

HAEM-PURINE RELATIONSHIPS IN PORPHYRIA.

The subject of haem-purine relationships and experimental porphyria is a complex one wrought with confusion. Observations by different research workers are often contradictory and results vary considerably according to whether the observations have been made in bacteria, avian embryos, experimental animals or man. It is the purpose of this chapter to review and analyse what has been established up to the present time.

A. BACTERIAL PORPHYRIN SYNTHESIS.

Rhodopseudomonas spheroides is an organism which anaerobically and in the light synthesises porphyrins through the same biochemical mechanisms as in animal cells. Gajdos et al (112) studied the effect of the addition of purine nucleosides and nucleotides on porphyrin synthesis by this organism with glycine and succinic acid as substrates. They showed quite conclusively that under their experimental conditions, purine nucleosides and purine nucleotides exerted a strong inhibitory effect on porphyrin production. ATP was the most effective inhibitor decreasing production by more than 90%, the effect correlating

well with the concentration of the nucleotide in the medium. Inhibition with adenosine, inosine, AMP and ADP was 50 to 70%. As growth of bacteria was not impaired and as there was no increase in bacteriochlorophyll formation to explain reduction in porphyrin production, the authors concluded that any decrease in porphyrin content of samples containing purine derivatives could be attributed to an actual inhibition of synthesis. As porphyrin production from ALA was unimpaired, the authors (112) felt that purine compounds exerted their inhibitory effect at the stage of ALA synthesis.

Kikuchi et al (220,221,422-) studied ALA synthetase activity under varying conditions using particle free extracts of Rhodopseudomonas spheroides and Rhodospirillum rubrum as the source of the enzyme. They showed that ATP was a necessary cofactor in the formation of succinyl coenzyme A from succinic acid and coenzyme A and with the latter two as substrates, ATP thereby stimulating the formation of ALA. The experimental conditions of Gajdos et al were not reproduced and the inhibitory effects of purine compounds were neither confirmed nor discounted.

In summary, Gajdos et al (112) have conclusively shown that purine nucleosides and nucleotides, especially ATP, strongly inhibit porphyrin synthesis from glycine

and α keteglutarate or glycine and succinic acid by suspensions of Rhodoseudomonas spheroides. Purine compounds do not impair porphyrin production from ALA. As in ALA synthetase preparations derived from Rhodopseudomonas spheroides and Rhodospirillum rubrum (220,221,422), ALA is formed from succinyl coenzyme A which requires ATP for its synthesis, it is unlikely that this nucleotide is an inhibitor of bacterial porphyrin synthesis under normal conditions.

B. CHICK EMBRYOS WITH EXPERIMENTAL PORPHYRIA.

Talman et al (234,235,437) induced experimental porphyria in chick embryos with allylisopropylacetamide (AIA). Allantoic fluid, representing secretions from embryonic gut and kidney, was found to have increased amounts of type III porphyrins and, when the induced porphyria was severe enough, porphobilinogen as well. Porphyric embryos were significantly smaller than controls and there was marked impairment of hatching, growth and development of the chicks after Sedormid treatment.

Labbe, Talman and Aldrich (234) showed that chick embryos rendered porphyric excreted significantly less uric acid into allantoic fluid than controls.

This same group of workers (437) then set out to determine whether this decreased excretion of uric acid represented an impairment of production or oxidation of purines or whether it reflected difficulty in getting rid of the oxidised product. They showed that normal chick embryos had no difficulty in oxidizing and excreting administered adenine as uric acid. However, quite surprisingly, the embryo could not handle exogenous guanine or xanthine. The authors did not comment on the singularly selective action of chick embryo xanthine oxidase. Although purine metabolism in uricotelic birds may differ from that of ureotelic mammals,

it is still likely that endogenously produced guanine nucleotides and nucleic acids are ultimately broken down and excreted as uric acid. Thus it is quite conceivable that the experimental techniques of Talman et al (437) do not convey an accurate picture of in-vivo avian purine metabolism.

Whatever the drawbacks of the experimental methods, the results of Talman and her group convincingly showed that porphyric embryos were able to oxidise and excrete the administered adenine as uric acid more efficiently than control embryos. Thus defective uric acid excretion in Sedormid-treated chick embryos has been interpreted as reflecting impaire d purine synthesis.

Talman et al (437) administered glycine-2-C^{14} to normal and porphyric chick embryos and measured the rate of incorporation of the isotope into nucleic acid purines. In all experiments, the peak in C^{14} activity in labelled adenine, guanine and uric acid recovered, occurred significantly earlier in controls than in porphyrics indicating a slower rate of purine turnover in the latter. This again confirmed impaired purine synthesis in those chick embryos made porphyric with ALA or Sedormid.

An unexpected but potentially important observation was the fact that when adenine was given along with Sedormid, the porphyric embryo was significantly less

affected than with the same dose of Sedormid alone with respect to porphyrin excretion, hatchibility, congenital abnormalities, chick growth and embryo weights (437). Gajdos et al (109,111) rendered chick and duck embryos porphyric with AIA and showed that treatment with adenosine monophosphate (AMP) markedly improved the porphyric state both biochemically and clinically.

These convincing results prompted Talman, Labbe and Aldrich (234,437) to incriminate one defect which could cause both the increased synthesis of porphyrins and the decreased production of purines. Work by Shemin et al on the succinate-glycine cycle (see chapter II, pages 51 to 67), showed ALA to be a common precursor of both the porphyrin and purine moieties. These observations led Talman et al (234,437) to reason along the following lines:- A block in the succinate-glycine cycle retarding the entry of the ALA-5-C into the C-1 pool and thence to purines could result in a deficient synthesis of purine nucleosides, nucleotides and nucleic acids which would markedly impair the health of the embryo and the growing chick. With a fall in body purines, a negative feed-back mechanism could operate whereby more succinyl coenzyme A and glycine would condense in an attempt to overcome the

block. The common precursor, ALA, would then accumulate and be disposed of via the porphyric pathway.

The above postulate would thereby account for the observed beneficial effects of administered adenine (234,437) and AMP (109,111) in porphyric avian embryos. The potential implications of this hypothesis are so great that its validity in human porphyria bears exhaustive investigation. This has been one of the major aims of my thesis.

C. EXPERIMENTAL PORPHYRIA IN ANIMALS.

Purine metabolism has been studied in experimental animals rendered porphyric with Sedormid, AIA or hexachlorobenzene (73,74,109,111,235,239,278,279,294). The experimental observations in many ways are quite different to those in chick embryo and are probably more applicable to human porphyria. Purine synthesis in animal porphyria and the effect of administered purine compounds on the disease process will now be considered.

(1) Purine Synthesis in Experimental Porphyria.

The brilliant study by de Matteis et al (74) has established beyond doubt that in animals porphyria there is no evidence of impaired purine synthesis. Rats were made porphyric with AIA, Sedormid and hexachlorobenzene (HCB). They were also given PIA, a substance similar to AIA but without its porphyria-producing qualities. Total and individual liver nucleotides, hepatic levels of RNA and DNA were measured and correlated with the degree of porphyria as assessed by porphyrin and pyrrole excretion and liver porphobilinogen content.

Total adenine and guanine hepatic nucleotide levels as well as the amounts of RNA and DNA in the liver were no different in normal and porphyric rats irrespective of the porphyria-inducing substance administered. However,

in rats rendered porphyric with AIA (and to a lesser extent, Sedormid), there was a fall off in the total ATP levels with a corresponding rise in the AMP and ADP concentrations so that the ratio ATP:AMP fell. Similar but less consistent falls in GTP:GMP ratios were noted. With PIA, although animals were not porphyric, similar observations were made.

Barbiturates reduce intracellular ATP levels either by impairing cellular respiration e.g. oxybarbiturates, or by uncoupling oxidative phosphorylation and activating mitochondrial adenosine triphosphatase, e.g. thiobarbiturates (5). AIA, PIA and barbiturates are similar in action. They may, by this action, interfere with ATP dependent acetylcholine formation and thus play a part in the neurological aspects of acute porphyria (74) but they appear to be unrelated to the increased production of porphyrins. de Matteis (74) noted no relation between porphyria and decreased levels of ATP. While PIA decreased levels of ATP there was no porphyria. HCB produced porphyria but did not interfere with the formation of ATP.

Thus, in summary, in porphyria in rats, purine synthesis of nucleotides and nucleic acids is unimpaired. The fall in ATP with corresponding rise in AMP following AIA porphyria appears to be related to its sedative

action and has no bearing on the porphyric state.

In confirmation of above, de Matteis et al (73) demonstrated no alteration in urinary allantoin excretion in rats with experimental porphyria. Merchante et al (294) also found no significant alteration in the level ofRNA or DNA in livers of Sedormid-treated rats.

In the face of the overwhelming evidence above, the finding by Labbe, Talman and Aldrich (235) of reduced utilization of 4 amino-5-imidazole carboxamide (AIC) by the livers of rabbits with experimental porphyria is hard to explain. The experimental details were not given and one cannot assess the validity of their findings.

(2) <u>The effect of administered purine on experimental Animal Porphyria.</u>

The presence of normal purine metabolism in animal porphyria is an important difference to the experimental findings in chick embryo porphyria. However, many workers have independently observed that as in chick embryos, the administration of purine compounds improves the porphyric state of the experimental animal both from a clinical and biochemical standpoint.

Gajdos et al (112) quoted their earlier in-vitro work which showed that AMP and inosine inhibited porphyrin synthesis from glycine by the reticulocytes or liver

homogenates of rabbits. The same group (109,111) produced porphyria in rats with HCB, an illness characterised clinically by considerable wasting, cutaneous eruptions and neurological symptoms ending in death in 4 to 5 weeks. A group treated with daily subcutaneous injections of AMP showed improvement and regression of the clinical disease and a marked decrease in the amounts of excreted ALA, PBG and uroporphyrin despite continuing with HCB in their diets. Porphyrin levels in the organs of the treated animals were also less than those not receiving AMP. However, this nucleotide had no effect when administered to rabbits with Sedormid-induced porphyria. Gajdos et al also reported improvement in griseofulvin induced rat porphyria (113) and in anaemic, porphyric rabbits poisoned with lead acetate (110) after the administration of AMP.

Lottsfelt, Labbe and Aldrich (279) showed that inosine helped AIA-induced porphyria in rats. By injecting Fe^{59} I.V. twenty-four hours after giving AIA, the workers were able to study the uptake of the isotope into the liver and its incorporation into liver haem.

Lottsfelt et al (278,279) showed that when inosine was administered along with AIA, there was a considerable reduction almost to normal levels of the liver porphyrin content as compared with the grossly elevated porphyrin levels observed with ALA alone.

The results of their Fe^{59} dynamic studies (239,240,278, 279) were most interesting and perhaps explained the mechanisms whereby administered inosine lowered porphyrin excretion in animal porphyria. They showed that porphyric rat livers took up more Fe^{59} than controls and that inosine had no effect in either group on Fe^{59} uptake (279). Most significant was their finding that the incorporation of Fe^{59} into liver haem was increased at all times in the porphyric animal indicating increased turnover of haem in this disease (239,279). This was confirmed by noting increased incorporation of glycine-2-C^{14} (279), acetate-2-C^{14} (239) and succinate 2,3-C^{14} (239) into liver haem in AIA induced rat porphyria.

The crucial finding was the observation that when inosine was given, haem synthesis both in the control and the porphyric animal was enhanced with a corresponding fall-off of free porphyrin levels in the liver. Thus, Lottsfeldt et al (279) came to the conclusion that inosine exerted its effect by making more iron available for haem synthesis, thereby lowering the porphyrin content of the livers of AIA-treated rats. In addition, inosine was also shown to stimulate Fe^{59} incorporation into haem in bone marrow and red cells of both control and porphyric rats (279).

The link-up between purine and iron metabolism was studied by Green, Mazur et al both in-vitro (157) and in-vivo (290). It was shown that anaerobic incubation of ferritin with liver slices resulted in an increase in its ferrous content which was now capable of dissociation and combination with iron binding agents, e.g. plasma iron-binding protein (157). They also noted that there was an increased production of uric acid by these anoxic cells which by diffusing out could directly reduce the ferric iron of ferritin. This excessive formation of uric acid under oxygen tensions of less than 20% was thought to reflect both the increased catabolism of high energy nucleotides involved in synthetic reactions and the decreased activity of uricase, and enzyme sensitive to hypoxia.

Ferritin has been shown to act as an electron acceptor during the oxidation of purines by xanthine oxidase, especially in the absence of oxygen (157). Thus, xanthine oxidase whilst oxidising hypoxanthine and xanthine reduced the ferric iron of ferritin to its more utilisable ferrous form (157).

In summary, therefore, in-vitro studies of Green and Mazur suggested that under hypoxic conditions, there was increased catabolism of high energy nucleotides with an associated increase in xanthine oxidase activity. This, by reducing ferritin, made more iron

....P.T.O.

available in its ferrous form. In addition, with increased production of uric acid, more ferric ferritin was reduced by a direct action of this oxypurine, thus potentiating the xanthine oxidase effect. On these lines, the authors explained their earlier observations that in dogs made hypotensive by haemorrhage, not only were blood uric acid levels raised, but there was also a significant increase in plasma iron.

To date, evidence for the participation of xanthine oxidase in the release of ferrous iron had been based on in-vitro studies using rat liver slices subjected to lowered oxygen tension. To test their hypothesis in-vivo, Masur, Green et al (290) administered xanthine oxidase substrates to a variety of animals in-vivo and hoped thereby, that by increasing the activity of the enzyme in the tissues, an increase in plasma iron would be recorded.

The effect of post-traumatic and post-haemorrhagic shock in raising blood uric acid levels was confirmed (290). As the elevation in blood urate was far greater in hypotensive dogs than in arenal dogs, the authors concluded that the hyperuricaemia represented increased purine catabolism rather than diminished renal excretion of uric acid in the shocked animals. They went on to show that following the I.V. infusion of hypoxanthine,

not only was there a transient rise in serum uric acid but the plasma iron in guinea pigs, dogs and rabbits tested increased significantly, usually for more than two hours after administration. This elevation in plasma iron was noted in response to I.V. hypoxanthine, AMP and inosine. However, iron levels in the blood fell following the infusion of uric acid, glycine, butyric acid and saline. This provided good evidence that the administered purine compounds were causally related to the release of ferrous iron from stores to the plasma and that this was mediated via enhanced xanthine oxidase activity rather than production of its end product, uric acid (290). Thus again, in-vivo studies led to the same conclusions that in shocked animals, increased catabolism and oxidation of purines was the possible underlying mechanism behind the observed increases in plasma iron levels.

As the liver is the main site of xanthine oxidase activity in the body, one would expect the main release of ferrous iron following injection of purine compounds, to take place in the liver. Mazur et al (290) showed that blood from the liver of dogs was richer in iron and uric acid following shock, than blood from the femoral artery. After I.V. infusion of purine, hepatic venous blood again had more iron than femoral arterial blood. As splenectomised rabbits also responded in the same way to

purine injections, the authors confirmed the role of the liver in the release of ferrous iron.

How do all these findings link up with experimental animal porphyria? Lottsfeldt et al (278,279) felt that inosine, by making more ferrous iron available to protoporphyrin in the liver enhanced the formation of haem, thereby lowering hepatic porphyrin levels. However, as haem synthesis is already increased in experimental porphyria (239,279) inosine hardly corrected the basic defect of increased porphyrin synthesis unless other mechanisms were operative.

Experimental porphyria is probably the result of an induced increase in ALA synthetase activity (446). The influence of iron on ALA synthesis has already been discussed at length (Chapter II, pages 42-46). Lascelles et al (244,247) showed that in induced iron deficiency, suspensions of Rhodopseudomonas spheroides produced increased amounts of porphyrin. On addition of small amounts of ferrous iron, the excessive porphyrin formation was almost entirely suppressed. Although ferrous iron enhanced the synthesis of bacteriochlorophyll and haem, these could only account for 10% and 1% respectively of the porphyrins which failed to appear. Burnham and Lascelles (51) noted that haem markedly inhibited ALA synthesis and felt that the main effect of iron above was mediated via a negative feedback inhibition

of ALA synthesis by the extra haem and bacteriochlorophyll formed.

The analogies to the observations in experimental animal porphyria are obvious. While Lottsfeldt et al (279) noted a small increase in liver haem following inosine, the grossly excessive production of porphyrins in the porphyric animal was reduced to virtually normal values. Thus, inosine and other purine compounds may, by making more ferrous iron available in the liver, not only enhance more haem synthesis, but they may by a negative feedback mechanism inhibit ALA synthetase to a degree virtually reversing the disease process.

Thus there has merged very definite evidence for the potential value of inosine and other purine compounds as a therapeutic agent in porphyria even though purine metabolism in this disease may be entirely normal.

Saunders (377) showed that in experimental porphyria induced in rats by HCB, serum iron levels were consistently elevated despite no apparent increase in iron absorption. If one is going to relate this to altered purine metabolism, one would expect this to reflect increased purine catabolism by the porphyric liver, i.e. a situation directly opposite to the one observed by Talman, Labbe et al in chick embryos (234,437). However, as de Matteis et al have shown no disorder of purine metabolism in animal porphyria induced by HCB, some other

mechanism must be operative. It is of interest that in porphyria induced in rats by 3,5-diethoxycarbonyl-1, 4,-dihydrocollidine (DDC), there was no abnormality in the serum iron levels or absorption of Fe^{59}.

Gajdos et al (115) and Palma-Carlos et al (335) have been impressed by the fact that purine compounds such as ATP and inosine decrease the excessive porphyrin synthesis in chick embryo and experimental animal porphyria. This has stimulated them to study the reverse problem, i.e. the effect decreasing levels of hepatic ATP have on porphyrin formation.

1% orotic acid in the diet causes a 50% decrease in the hepatic level of acidosoluble nucleosides (453). Gajdos et al (115) administered orotic acid to rats and showed significant decrease in hepatic ATP but an increase in red cell ATP. These authors demonstrated increased hepatic, stool, bone marrow and RBC porphyrin content with normal levels in the urine. After two weeks, enhanced ALA synthetase activity was noted in liver mitochondria. Intra muscular AMP reduced significantly the excess porphyrin formation and the increased ALA synthetase activity. Gajdos et al (115) also quote earlier work where 6 mercaptopurine caused experimental porphyria, reducing biosynthesis of ATP at the same time. Imuine alleviated the metabolic disorder in this experiment. There is also a structural similarity

between griseofulvin and purine ribosides, suggesting that this drug may act as a competitive purine analogue when inducing porphyria. Inosine and AMP help griseofulvin induced experimental porphyria.

Palma-Carlos et al (335) induced porphyria in rats with ethionine, a powerful inhibitor of hepatic ATP synthesis even after a small dose. It also inhibits protein synthesis. These authors demonstrated a statistically significant accumulation of porphyrins and their precursors in urine, faeces, hepatic tissue and RBC. Whilst hepatic ATP levels were reduced, red cell levels of ATP were unchaged. In contrast to the orotic acid experiments above, after ethionine, no increase in liver mitochondrial ALA synthetase was reported and the administration of inosine, AMP and ATP had no effect on the disturbance of porphyrin metabolism.

The reasons for these differences are not known. In Rhodopseudomonas spheroides (128) ethionine causes increased porphyrin synthesis not only from glycine and succinic acid but from ALA as well. Thus, ethionine may not work primarily by inducing ALA synthetase activity thus explaining why exogenous purines, which presumably act by inhibiting ALA synthetase, do not influence the experimental porphyria. The other possibility is that ethionine by impairing protein

synthesis also prevents enzyme induction and inhibition both of which may be mediated by a protein activator or inhibitor (114) respectively.

de Matteis (74) however, noted no relation between porphyria and decreased levels of hepatic ATP. In his experiments of rats, PIA decreased hepatic levels of ATP without being porphyrinogenic whilst HCB produced porphyria without interfering with the formation of ATP. As mentioned earlier, barbiturates also reduce intracellular ATP levels (5) without affecting porphyrin synthesis. As haem-proteins are so intimately linked with biological oxidation, it is possible that in porphyia, decreased tissue ATP levels are compatible with a co-existent respiratory defect (446).

D. HUMAN PORPHYRIA.

While purine metabolism has been studied extensively in the chick embryo and experimental animal with porphyria, hitherto, there has been no attempt to measure purine synthesis from common haem/purine precursors in human porphyria. One of the main aims of this thesis is to measure purine synthesis from glycine-2-C^{14} in South African genetic and symptomatic forms of porphyria.

(1) Purine synthesis in human porphyria.

In 1961, Taxay (440) reported 2 of 6 patients with acute porphyria having serum uric acid levels between 1 and 2 mg/100 ml without increase in the urinary uric acid excretion. Of routine uric acid estimations done 5 of 16 hypouricaemic patients were thought to have suffered from acute porphyria although evidence for the suppositions were not given in the actute.

The first impression given by the article was that, as in the chick embryo experiments of Talman et al (234, 437), this reflected decreased purine synthesis in acute porphyria. Taxay (440) considered other possibilities. Aminoaciduria has been described in acute porphyria (292). Hypouricaemia has been reported in many generalised proximal tubular disorders, some with amino acid uria, e.g. Fanconi syndrome or Wilson's disease (300).

However, if there were a temporary disorder of proximal tubular function in porphyria, one would expect larger amounts of uric acid to be excreted in the urine. Another possibility was that urinary uric acid may form a complex with PBG therefore escaping detection by enzymatic spectrophotometric techniques. In uric acid isolated from the urine of acute porphyrics studied in this thesis, considerable difficulty was experienced in extracting the uric acid by copper precipitation or charcoal absorption methods (see later). Whether this was due to uric acid being complexed with one of the metabolic products excreted by the acute porphyric subject, is not known. However, one can envisage hypouricaemia from proximal tubular disease where the increased urinary urate excretion is not detected for the above reasons.

Howell and Wyngaarden (195) studied uricolysis in man and showed that verdoperoxidase and cytochrome C-cytochrome oxidase destroyed uric acid in-vitro. These enzymes are present in red cells and leucocytes. The active uricolytic substance was identified as the oxidised haem prosthetic group, haematin. As in animal porphyria (239,279) and in human porphyria (78)(this thesis) haem synthesis probably increased, there may be more endogenous ferrihaem in the body available to break down

uric acid in the liver. Thus, significant uricolysis in man is a possible cause of both hypouricaemia and lowered urate excretion in human porphyria.

Ludwig (281) studied four patients with acute porphyria and showed that when serial uric acid concentrations were determined, levels dropped from normal to as low as 1.0 mg/100 ml. The following important associations were noted. The decreased blood uric acid levels co-incided with increased excretion as shown by an increase in the urate/excretion. Excretory pattern of aminoacids and uric acid correlated better with ALA excretion than with that of PBG or porphyrins suggesting that this organic acid may alter the renal handling of both aminoacids and urate. However, other organic acids, e.g. lactic and β hydroxybutyric acids (300) interfere with urate secretion and cause hyperuricaemia. It is more likely that as with Fanconi syndrome and Wilson's disease, the aminoaciduria, urate excretion and hypouricaemia are all manifestations of a temporary disorder of proximal tubular function in acute porphyria.

Ludwig (281) made another interesting observation. Maximal urate and α-NH_2-nitrogen excretion coincided with maximal severity of the hyponatraemic state, a syndrome possibly due to inappropriate secretion of antidiuretic hormone.

Therefore the dilutional effects of the net water retention will, with the renal loss of urate, adequately account for the decreased serum urate observed in acute porphyria without invoking diminished purine synthesis in this disease.

Kramer (237) in discussing iron metabolism in the porphyrias noted that in acute phases of S.A. genetic porphyria, 9 of 12 cases had raised serum iron whilst in all, plasma iron turnover fell within the normal range. He postulated two possible causes, viz. reduced utilisation of iron by the bone marrow or release of iron from ferritin stores in the liver. In support of the former, 6 of 7 subjects showed diminished utilisation of the isotope iron by the bone marrow. If the latter mechanism were operative along the lines discussed earlier (157,290), then one would anticipate increased purine synthesis and breakdown in acute porphyria.

The snag of discussion so far is that all evidence is inferential and the answer can only be obtained by direct measurement of purine synthesis in this disease.

(2) <u>The Therapeutic Effect of Administered Purines</u>.

Gajdos and Gajdos-Török have reported the effects of AMP administration in human porphyria (89,111). In one of these papers (111), the authors give full clinical

and biochemical data for each of the 10 cases reported. In one, a case of erythropoietic porphyria, there was no effect. In another, possibly symptomatic, possibly S.A. genetic porphyric on the data, there was marked clinical and moderate biochemical therapeutic response to AMP. 8 cases of acute porphyria were studied. Taking into account the natural history of acute porphyria, bearing all facets of the cases in mind, it appeared from analysis of data in this paper, that 3 of the 8 patients (cases 1,3 and 8) had a very convincing clinical and biochemical response to daily I.M. AMP. In 3 cases, results were most unconvincing and in the remaining two, there was a moderate and a possibly disputable effect of the administered nucleotide.

It is difficult to draw conclusions from the above series other than to say that whilst administered AMP may be of therapeutic value, a larger double blind control study is necessary to test the efficiency of the drug.

E. RECAPITULATION.

Under special experimental conditions, Gajdos et al (112) demonstrated that the addition of purine nucleotides and nucleosides, especially ATP markedly impaired porphyrin synthesis in Rhodopseudomonas spheroides. However, the work of Kikuchi et al (220,221,422) under more natural conditions, showed that in fact, ATP was a necessary cofactor in the formation of succinyl coenzyme A from succinic acid and coenzyme A, thus enhancing porphyrin synthesis in both Rhodopseudomonas spheroides and Rhodospirillum rubrum. The observations of Gajdos et al are therefore of little significance.

In chick embryos, Talman, Labbe et al (234,437) convincingly demonstrated decreased purine synthesis and thereby uric acid formation following Sedormid or ALA induced porphyria. The administration of adenine but not guanine or xanthine improved the clinical and biochemical aspects of the disease. Whilst there may be certain limitations to the experimental techniques employed, the results are convincing and have led to the postulate that a block in the utilisation of the δ carbon of ALA in purine synthesis is the cause of both the symptoms and the biochemical changes of the porphyric chick.

In animals, there is no impairment of purine synthesis

in porphyria induced by Sedormid, AIA or HCB (74). However, inosine most certainly diminishes the excessive production of porphyrins to near normal levels in rats made porphyric with AIA (278,279). Work by Green and Mazur in-vitro (157) and in-vivo (290), suggest that inosine, in being oxidised to uric acid by xanthine oxidase, provides electrons for hepatic ferritin, thus reducing the iron to its utilisable form. It has also been shown (239,279) that inosine thereby stimulates haem synthesis over and above that already noted in experimental porphyria. This, by a feedback inhibition of ALA synthetase, as described by Lascelles and her group (51,244,247), may account for the dramatic and almost complete reversal of excessive porphyrin excretion brought about by AIA. Thus, in experimental porphyria, exogenous inosine and other purine compounds may reverse the fundamental defect, viz. increased ALA synthetase activity.

The inhibitory effect on porphyrin synthesis by exogenous purines has led workers to study the converse situation, the effect of decreasing ATP on porphyrin formation. Orotic acid (115), ethionine (335) and AIA (74) all decrease hepatic ATP levels and cause experimental animal porphyria. However, PIA and barbiturates impair ATP formation without producing porphyria (74)

whilst other porphyrinogenic drugs, e.g. HCB do not lower hepatic ATP levels (74). As haem-containing enzymes and ATP are intimately linked with biological respiration, and energy trapping, certain respiratory defects could induce ALA synthetase activity (446) and interfere with hepatic ATP levels.

In human porphyria, circumstantial evidence points to a low blood uric acid level as being due to renal loss of urate via the proximal tubule and a dilution effect as part and parcel of the hyponatraemic syndrome (281). There is no available data on purine synthesis in this disease. There may be a beneficial therapeutic effect from AMP in acute porphyria (109,111), but the study reported is not ideal for drawing definite conclusions.

Thus, purine metabolism and porphyria are linked in two probably unrelated ways. Firstly there is the possibility of reduced purine synthesis, the only striking evidence being the observations in chick embryo porphyria. As uricotelic avian purine metabolism probably differs markedly from that in the ureotelic mammals, the observations in experimental porphyria in animals such as the rat and rabbit are more applicable to man. The observations by de Matteis et al (74) of unimpaired purine synthesis in porphyric rats probably

represents what is happening in human porphyria.

The second link is a definite one. There is experimental evidence that purine compounds can be of potential value in the treatment of porphyria.

In this thesis, the postulates of Talman, Labbe et al (234,437) have been tested in human porphyria, i.e. evidence for a block in the utilization of the δ carbon of ALA has been explored in cases of S.A. genetic porphyria and symptomatic porphyria using glycine-2-C^{14} as the common haem/purine precursor. In addition to the evidence just reviewed, as the activity of the succinate-glycine cycle is low in normal liver (446), it is unlikely that the basic defect of acute human porphyria will lie in this pathway.

CHAPTER VI.

EXPERIMENTAL DESIGN AND TECHNIQUES.

The metabolism of purines and haem in the liver has been assessed by studying the incorporation of glycine-2-C^{14} into urinary uric acid and serum bilirubin in normal and porphyric human subjects. In this chapter, relevant clinical details and the materials and methods employed will be discussed under the following headings: Subjects studied, experimental design and technical procedures.

A. SUBJECTS STUDIED.

25 human subjects were given glycine-2-C^{14} I.V. and thereafter incorporation and disposition of the C^{14} label into uric acid and/or the pattern of C^{14} activity in serum bilirubin was determined. The list of patients involved in this study is as follows:-

Urinary uric acid-C^{14} experiments.

(a) Normal Subjects.

(i) H.J.

(ii) A.J.

(iii) A.M.

(iv) R.B.

(b) Symptomatic porphyrics.

(i) A.A.

(ii) L.M.

(iii) B.P.

(iv) W.B.

(c) South African genetic porphyries.

(i) A. v R. - remission.

(ii) C. v H. - acute attack

(iii) M.N. - acute attack

(iv) M. de J. - acute attack

(v) Z.M. - acute attack

(vi) A. Jov. - acute attack

Serum bilirubin-C^{14} studies.

(a) Normals.

(i) D.D.

(ii) E.J.

(iii) O.M.

(iv) C.R.

(v) F.R.

(b) Symptomatic porphyries.

(i) R.N.

(ii) P.T.

(iii) T.M.

(c) S.A. Genetic porphyries.

(i) A. Jov. - acute attack

(ii) Z.M. - acute attack

(iii) M.S. - acute attack

1. Normal subjects.

With the exception of E.J. who had haemolytic anaemia, none of the normal controls suffered from liver, renal, metabolic or any other diseases likely to affect porphyrin, purine or glycine metabolism. H.J., A.J., D.D., E.J., C.R., and P.R. were all of Cape Coloured extraction whilst A.M., R.B., and O.M. were of the Bantu racial group.

2. Symptomatic porphyrics.

All the symptomatic porphyria subjects studied had histories of excessive alcoholic intake, typical skin lesions, biochemical and histological evidence of liver disease and the laboratory evidence necessary to substantiate the diagnosis. None of the patients had renal or other disease likely to influence the experimental data. With the exception of Bantu males R.N. and P.T., the subjects were all adult Cape Coloured males.

3. South African genetic porphyrics. (S.A.G.P.)

A diagnosis of South African genetic porphyria was made in all the patients of this group by the usual clinical and laboratory criteria (89,90). As the patients in the throes of an acute attack were extremely ill, it was not possible to maintain constant experimental conditions. Brief summaries of each of the cases studied will be presented in the following chapter.

B. EXPERIMENTAL DESIGN.

South African genetic porphyrics were treated in general medical wards, their fluid and food intake varying according to their clinical state. As soon as they were fit enough to eat, they were put on to ward diets except for CvH who, being uraemic, was placed on a 20 gm protein diet.

The normal and symptomatic porphyric subjects were all studied under Metabolism Ward conditions. Each was placed on a Step 8 diabetic diet which comprised 100 gm protein, 66 gm fat and 240 gm carbohydrate. The hepatic, haematological and renal states of each patient were investigated and urine and stool was examined for its porphyrin and, in the urine, porphyrin precursor content.

At 8 a.m. on day 1 of the experiment, each subject received an intravenous injection of an accurately weighed amount (approx. 5 gm) of a solution of glycine-2-C^{14} in normal saline containing a total of about 70 μc.

In the uric acid degradation experiments, twenty-four hour urine samples were collected daily for 2 weeks following glycine-2-C^{14} administration for measurement and isolation of uric acid. The uric acid recovered from the urine was recrystallised until pure crystals were obtained. A portion was degraded into fractions containing C4 + 5 and C2 + 8. Blood was also taken daily for uric

acid assay.

All specimens of urine were stored in a cold room at 4° C. Blood was centrifuged and serum stored in the dark deep freeze at 15° C, the packed cells being kept at 4° C.

In the serum bilirubin-C^{14} studies, about 20 to 50 ml of heparinized blood was drawn at frequent intervals on the first day and thereafter daily, for the following 5-10 days for estimation of serum bilirubin and isolation of haem-C^{14} and bilirubin-C^{14}.

C. TECHNICAL PROCEDURES.

The following technical procedures have been adopted during the course of the experimental work.

These will now be presented and discussed in full detail.

(1) Enzymatic spectrophotometric method for determination of uric acid.

This method has largely been based on the one described by Liddle et al (266).

(a) Principle.

One of the physical properties of the uric acid molecule is its capacity, in aqueous solution, to absorb a definite quantity of light from the ultra violet region of the spectrum. At pH 9.4 the maximum absorption occurs at 292 mμ. The enzyme uricase catalyzes the oxidation of uric acid to allantoin which does not absorb light at 292 mμ. By measuring the optical density of a biological solution at this wavelength before and after the action of uricase, one can determine the amount of uric acid that it contains. To ensure activity of uricase, the enzyme is also added to a standard solution of uric acid. Before discussing the reagents, procedure, etc., it is necessary to mention how purified uric acid is obtained from the commercial product. A Beckman Du spectrophotometer was used to measure O.D. of samples at 292 mμ.

(b) Purification of Commercial uric acid.

This is done as follows:-

(i) Dissolve 1.1 g of lithium carbonate in 75 ml cold distilled water and heat to 90°C. Dissolve 2.5 g of uric acid in this solution, add 0.1 g activated charcoal and mix thoroughly.

(ii) Filter the mixture by gravity into a flask con-

taining 700 ml of distilled H_2O, preheated to 55-60° C and mix.

(iii) Precipitate uric acid crystals by the successive addition at 15 min intervals of 2.5 ml, 2.0 ml and 4.0 ml of glacial acetic acid.

(iv) Thirty minutes after the last addition, decant supernatant liquid and filter off the crystals.

(v) Wash the uric acid with distilled water until the filtrate is neutral to litmus paper and dry in a dessicator.

(c) Reagents: for determining uric acid.

(i) Uric acid: A standard solution of uric acid is necessary to assay the activity of uricase preparations. Uric acid is first purified as described. Then dissolve 30.0 mg of purified uric acid in 10 ml hot water by the addition of approximately 1 ml of a hot saturated solution of lithium carbonate, and bring the solution to a volume of 100 ml with distilled water. This solution is stable when frozen but will deteriorate gradually at room temperature. A 25 fold dilution (12 ug uric acid/ml) is used in calibrating the method.

(ii) Glycine buffer: (Stock solution 2/3M pH 9.4). Dissolve 25 G of glycine and 4.4 G of sodium hydroxide in CO_2-free distilled water and make the final volume 500 ml.

Add 3 ml of chloroform as a preservative. Store in a refrigerator. For use in the assay, dilute the stock solution to 0.1 M for urine determinations and 0.067 M for serum determinations. The diluted buffer can be stored at room temperature for a week before microbial growth makes it unsuitable for use.

(iii) Uricase. (SIGMAC Laboratories).

100 Prateorius units per ampoule.

(d) Cuvettes.

Silica cuvettes with a 10 mm light path are used.

The following cuvettes are prepared for assay.

(i) Assay cuvette: This contains 2.0 ml of 0.1 M glycine buffer pH 9.4 and 1.0 ml of test solution containing uric acid to be determined.

(ii) Enzyme blank cuvette: Contains 3 ml of 0.067 M glycine buffer.

(iii) Biological fluid blank cuvette: Some biological fluids such as serum and spinal fluid contain substances which undergo spontaneous changes in O.D. under conditions of assay. This curvette is a duplicate of the assay curvette but no uricase is added, i.e. it contains 2.0 ml of 0.1 M glycine buffer pH 9.4 and 1.0 ml of test solution containing the uric acid.

(iv) Reference cuvette: This cuvette is used for setting the spectrophotometer to zero. The large

amounts of nonurate material with absorption at 292 mμ which are present in fluids such as serum can be cancelled out by substituting a reference cell of corresponding degree of O.D. instead of water, bringing the initial O.D. reading in the assay cuvette to the range of 0.200-0.400: This is only used for serum or certain specimens of urine with high optical density at 292 mμ due to non-urate material. Otherwise spectrophotometer set to zero against a water blank.

The solutions are mixed in the cuvettes and O.D. values are determined at 292 mμ prior to the addition of uricase; this is termed the Initial O.D. Sufficient uricase is added to produce an initial rate of change in O.D. in the assay cuvette of 0.020 to 0.030 per min. For commercial preparations of uricase about 0.005 to 0.10 ml suffices. The reaction is allowed to proceed until there is no further change in O.D. in the assay cuvette, usually a period of 25 min. O.D. readings are taken at 3, 25, and 30 minutes. The O.D. at the end is the Final O.D.

Calculations: The following values must first be available:

1. Change in O.D. assay cuvette = Initial O.D. - Final O.D.
2. " " " enzyme blank cuvette = Final O.D. - Initial O.D.

3. Change in O.D. biological fluid blank = Final O.D. - Initial O.D.

4. Change in O.D. Uric Acid = (1) + (2) + (3)

(e) Optical Density equivalent to 1 mg/ml uric acid.

On referring to the standard solution of uric acid used, it can be seen that every one ml of the diluted standard added to the "assay cuvette" has twelve ug of uric acid. The drop in optical density after uricase has acted is usually about 0.300 units on the spectrophotometer. Thus it can be calculated that as 12 ug uric acid are present in 3.01 ml of fluid in the cuvette, the concentration of uric acid is ± 4 ug/ ml. Thus 1 ug per ml would produce a change in optical density of 0.300/4 = 0.075. Liddle et al (266) found a figure usually 0.0745. This figure is determined for every group of specimens assayed by including a standard solution of uric acid with each batch.

(f) Assay of Uric Acid in Urine.

Cuvettes as for blood. The assay cuvette should contain 2.0 ml of 0.1 M glycine buffer and 1 ml of diluted urine. The urine is collected over 24 hours at room temperature with 4 ml toluene added as a preservative. A dilution in the range 1:100 to 3:100 generally produces a satisfactory change in O.D. uric acid. A biological fluid blank is not necessary. A silica cuvette containing water acts as a reference cuvette.

To calculate the number of mg uric acid/ml urine, it is best to consider a hypothetical sum. Suppose the change in O.D. urine sample after uricase = 0.298 units. Then 1 ml of solution in the cuvette has 0.298/0.0745 mg uric acid = 4 ug uric acid. Therefore 3.01 ml will have about 12 ug uric acid. All the uric acid in the cuvette is present in the 1 ml of diluted urine added. Thus 1 ml of diluted urine has 12 ug of uric acid. If a 1 in 100 dilution of urine was used, concentration of uric acid in urine = 1,200 ug or 1.2 mg/ml.

Expressed as a formula:-

$$\text{mg uric acid/ml urine} = \frac{\text{Change in O.D. uric acid}}{0.0745} \times \frac{3.01}{1} \times \frac{1}{1000} \times \text{Dilution factor.}$$

(g) Assay of uric acid in serum.

Assay cuvette contains 3 ml 0.067 M glycine buffer pH 9.4, 0.1 ml serum and 0.01 ml uricase. Thus total volume in cuvette = 3.11 ml. As serum has a large quantity of non-urate material, reference blank made up usually of urine, it is used to zero the spectrophotometer in such a way as to make the initial O.D. assay cuvette about 0.2 to 0.4. Uricase blank is now 3.1 ml of 0.067 M glycine buffer + 0.01 ml uricase and the standard uricase cuvette is set up as before, namely, 2 ml of 0.1 M glycine buffer + 1 ml of diluted standard

uric acid solution. The latter is read against a water blank, as with urine.

It can be calculated, on lines similar to that described for urine, that the concentration of uric acid in serum follows the formula below:-

$$\text{mg uric acid/100 ml serum} = \frac{\text{Change O.D. uric acid}}{0.0745} \times 3.11$$

(h) Accuracy of method.

As duplicate assays agreed closely (urine to within 1 to 2%; serum to within about 5%), all uric acid estimations performed were done singly. A Beckman DU spectrophotometer was used to obtain all O.D. readings.

(2) Isolation and purification of uric acid from urine.

Urinary uric acid was purified by the method of Hawke, Oser and Summerson (183). Uric acid after isolation was purified by a method, virtually a scaled down version of the one described by Folin (104) with certain modifications.

(a) Reagents:

1. Sodium acetate crystals (Lab. reagent).
2. Sodium metabisulphite (Lab. reagent).
3. 10% $CuSO_4$ solution in water.
4. Concentrated HCl.
5. Saturated lithium carbonate solution (1.1 g lithium carbonate in 75 ml water)

(b) Procedure:

Measure urine volume and add sodium acetate crystals (6 g/100 urine) and sodium metabisulphite powder (5 g/100 ml urine) and bring to the boil in a "Corningware" vessel.

To the boiling urine add approximately 1/10th volume of 10% $CuSO_4$ solution. Maintain temperature at the boiling point for at least 3 minutes. Remove vessel from hotplate and allow to stand for a few mins. Filter through Whatman's No. 541 filter paper and wash precipitate with hot water until filtrate is colourless-
Return washed precipitate to a conical flask by puncturing tip of filter paper and washing through the precipitate. To the precipitate suspended in 200-600 ml water, add 1 ml conc. HCl for every 200 ml water. Boil solution vigorously whereupon all, or the greater part of the precipitate, should go into solution. Remove copper and expel excess of H_2S by renewed boiling. Determine completeness of H_2S with H_2S expulsion by holding strip of filter paper moistened with lead acetate solution over the neck of the conical flask. Boil until no further black discolouration of filter paper can be detected.

Filter through Whatman's No. 541 filter paper to remove CuS and concentrate filtrate to approx 1/20th volume by boiling (N.B. Boiling chips in beaker).

Allow to stand for a few hours whereupon uric acid crystallises out. Harvest crystals on Hirsch funnel and recrystallise as follows:

Place crystals in small (50 ml) beaker and add sufficient boiling lithium carbonate solution to dissolve crystals noting carefully the volume of lithium carbonate solution used.

Heat solution to just below boiling point, add a spatula of activated charcoal and mix thoroughly. Keep temperature of the solution just below boiling point with stirring for 10 - 15 minutes.

Filter off activated charcoal using Whatman's No. 42 filter paper and dilute clear filtrate to 60-120 ml depending on amount of lithium carbonate solution used, and the amount of crude uric acid isolated. The more dilute the uric acid solution, the better the crystals obtained.

Heat to 60°C and mix.

Precipitate uric acid crystals by successive addition at 15 min. intervals of 0.25 ml, 0.2 ml and 0.4 ml of glacial acetic acid for every 7.5 ml lithium carbonate solution used originally to dissolve the crystals.

Thirty mins after the last addition, filter crystals on Hirsch funnel using Whatman's no. 541 filter paper. Wash crystals with distilled water.

The second recrystallisation is carried out as above omitting the addition of charcoal.

The pure crystals are dried in a vacuum dessicator over silica gel.

Where the specific activity of uric acid must be determined, pure crystals are weighed directly into a tared counting vial and suspended in scintillator solution containing thixotrophic gelling agent.

(c) Comment.

In general, this method worked extremely well. The crude uric acid crystals yellow, pink, straw-coloured, etc., came down as glistening white crystals after the first recrystallisation. They were then washed thoroughly and recrystallised one again before counting or degradation. In a few of the acute porphyric subjects studied, e.g. C.v.H., M. de J., when the acid copper-urate solution had H_2S bubbled through it, instead of forming a dense copper sulphide precipitate settling to the bottom and easily filtered off (leaving urate in solution), the black precipitate remained in suspension and had to be "salted out" of this by adding large quantities of sodium chloride. When the sodium chloride/urate solution was boiled down and allowed to cool, the urate crystals obtained were mixed with large quantities of the added salt. The latter was removed by washing the crude crystals with hot water until bulk remained constant. This impure uric acid was then recrystallised three times to ensure purity of sample.

The reason for this difficulty, only experienced with acute porphyrics, could not be ascertained.

(3) Degradation of Uric Acid (to separate C4,5 from C2, 8.

(a) Principle: i. Uric Acid.

Shaken in air with MnO_2
(pH 13; 40°)

ii. Allantoin

Alkaline hydrolysis (100°
pH 10; 45 mins)

iii. Allantoic Acid

Acid hydrolysis (100°;
pH2; 10 mins)

iv. Urea + Glyoxylic acid.

(b) Reagent:

Manganese Dioxide (MnO_2) solution: A fresh solution should be prepared every 1 ~~-2~~ weeks.

To 500 ml of 1.82% K MnO_4 slowly add 500 ml of 20 vol % H_2O_2, using magnetic stirrer. When effervescence has subsided, the precipitate is separated by centrifugation. Wash precipitate with H_2O, then with 0.1 N NaOH until clear. Suspend it in a convenient volume of 0.1 N NaOH and allow it to settle. When the precipitate has settled, decant the supernatant into a separate bottle and use the precipitate and the supernatant as described in the method.

(c) Method.

(1) Weigh out exact amount of uric acid into a 50 ml. thick-walled centrifuge tube. The weight should be in the neighbourhood of 220-270 mg. If the uric acid yield is small, make up to ~ 210 mg with a non-radioactive carrier

uric acid, noting the weight added and the consequent dilution of the sample.

(2) To the tube, add 7.5 - 10 ml of MnO_2 precipitate and 20 ml of the MnO_2 supernatant (or 0.1 N NaOH). Subject the contents of the tube to 7-10 mins ultrasound. Spin down the MnO_2 and test the supernatant for unchanged uric acid. This is done by reading the optical density at 292 nm. If the reaction is not complete, a fresh aliquot of MnO_2 must be added and the sample again treated with ultrasound.

(3) When complete, remove MnO_2 by centrifugation. pH is now ± 11-12.

(4) Neutralise the solution to pH 7 with 10 N HNO_3. Make it 0.1 M with respect to Na_2CO_3 by adding 10.6 mg/ml solution. This brings the pH to between 10 and 10.5.

(5) Heat it on a boiling waterbath for 45 mins to convert the allantoin to allantoic acid.

(6) Add 10 N HNO_3 slowly until a pH of 2 using pH paper, avoiding Avoid excess effervescence.

(7) Place it on a boiling waterbath for additional 10 mins.

(8) Cool the solution, neutralise it to pH 7 and add 7½ ml of 25% basic lead acetate to precipitate the glyoxylic acid as the lead salt. Centrifuge.

(9) Supernatant contains urea. Pour off and store in "fridge" until later (Step 11).

(10) Preparation of Glyoxylic Acid Carbazone Crystals.

(a) Add 9 ml H_2O to the precipitate in the centrifuge tube.

(b) Add 4½ ml of 4N H_2SO_4 i.e. total volume 13½ ml.

(c) Stir well on shaker for 3-5 mins and remove the $PbSO_4$ by centrifuging.

(d) Filter the supernatant (Whatmans no 541) into a 30 ml beaker in which 1200 mg of semicarbazide HCl had previously been weighed.

(e) Stir with glass rod. White crystals of the glyoxylic acid-semicarbazone form. Leave in fridge at 4^0C for crystals to form fully overnight.

(f) Filter crystals on Hirsch funnel, wash well with water, dry weigh into counting vial. Glyoxylic acid semicarbazone represents C4 and 5 of uric acid nucleus.

(11) Extraction and Digestion of Urea (C 2 and 8 atoms).

(a) Evaporate to dryness the supernatant fluid obtained from the basic lead precipitation (Step 9).

(b) Extract urea from residue with $\pm$ 20 ml absolute ethanol.

(c) Evaporate off the ethanol with Rotary evaporator and dissolve urea in 15 ml water.

(d) Acidify solution by adding ½ ml glacial acetic acid, warm, and aerate it with excess of H_2S to remove any lead

ions which might be present. Filter off the lead sulphide (Whatmans No 541).

(e) Neutralise the solution to pH 7 with CO_2-free 10 N NaOH.

(f) Add 1 ml of 1 M Na acetate buffer (pH 5) for every 10 ml of urea solution. pH of solution drops to $\pm$ 5.7-6.

(g) Transfer to special urea digestion apparatus (fig.6-1) Add 3 crushed urease tablets. Add ½ ml conc H_2SO_4 to side arm. Add 5 ml 1N CO_2-free NaOH to small flask. Assemble apparatus and create partial vacuum by suction.

~~Degradation of Uric Acid (To separate C4,5 from C2, 8) (contd~~

(h) Incubate at 37-55° for 1 - 1½ hours. Pour acid into solution to lower pH and liberate CO_2 from the NH_4CO_3 formed.

(i) Allow 1 hr for CO_2 to diffuse across to the NaOH.

(j) Collect Na_2CO_3 formed as $BaCO_3$ by first adding 2½ ml 2M NH_4Cl and 5 ml 25% solution of $BaCL_2$.

(k) Collect $BaCO_3$ by millipore filter and dry. Weigh into counting vials. $BaCO_3$ represents C2 and 8 of uric acid nucleus.

(d) Comments.

The complicated and involved degradation procedure is based in principle on the method described by Buchanan et al (46) but has been considerably modified to degrade the larger quantities of uric acid used in the experiments.

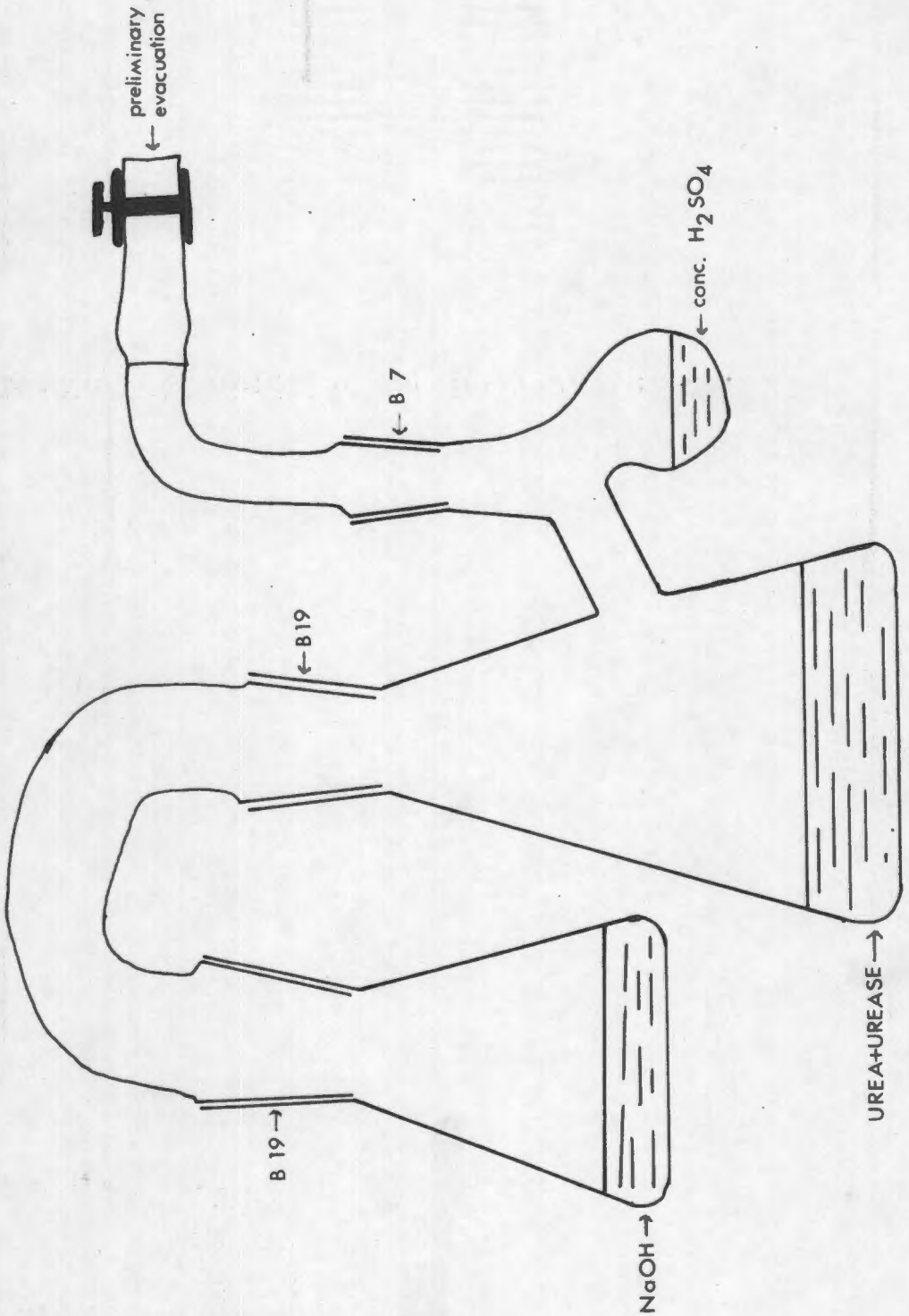


Fig. 6-1: Urea digestion apparatus.

The activity of the C-6 carbon of the uric acid molecule was not studied and this step was omitted completely.

The manganese dioxide solution was very much more concentrated than the one described by Buchanan (46). Initially, the uric acid/manganese dioxide solution was agitated at 38° C in 50 ml conical flasks in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago) for 8 to 12 hours. This step was cut down to 7 to 10 minutes with the use of ultrasound (Sonifier, Branson Sonic Power, Branson Instruments Incorporated, Connect. U.S.A.).

To test the extent of degradation, urea estimations were performed on the supernatent obtained following the lead precipitation step. The amount of urea present in the sample at this stage was often close to 50% of the maximum amount theoretically obtainable.

1 m mole of uric acid if completely degraded, should give rise to 2 m moles of urea and 1 m mole of glyoxylic acid semicarbazone. The usual yield (corrected to 1 m mole uric acid) was about 2/3 m moles urea (as barium carbonate) and 1/3 m moles glyoxylic acid as the semicarbazone. In later experiments where 2 m moles of uric acid were degraded, amounts of barium carbonate and glyoxylic acid semicarbazone were often over 250 mg and 60 mg respectively, i.e. amounts that could be accurately weighed with enough radioactivity to give significant counts above

background.

(4) Combustion of organic samples.

(a) General principles.

The following procedures deal with the determination of the specific activity of bilirubin and haemin. These organic substances, and others e.g. protoporphyrin, stercobilin etc., can be combusted to carbon dioxide by heating with a chemical mixture of high oxidising potential. Carbon dioxide evolved diffuses across a vacuum and is trapped in CO_2 - free sodium hydroxide and counted as barium carbonate. The total weight of organic sample combusted should give rise to about 1½ m moles of CO_2 i.e. equivalent to about 300 mg barium carbonate.

With haemin, the weight combusted is about 30 mg, which gives rise to adequate amounts of CO_2. However, with bilirubin 0.1 mg or less is all that can be isolated and as a consequence unlabelled or "cold" glycine is usually mixed with the sample to produce sufficient carbon dioxide.

In calculating the specific activities of the original sample, the following points must be borne in mind. Where the entire sample combusted is radioactive (e.g. haemin), the activity of the isotopic carbon in barium carbonate is equivalent to that of each of the thirty-four carbon atoms of the haemin moiety. However, when carrier is

added, e.g. glycine to bilirubin, not only must the exact weight of the bilirubin be known, but the <u>total</u> barium carbonate obtained must be ascertained, the latter, as one must determine the total specific activity coming from that accurately known weight of bilirubin combusted.

The method to be described is based on the one used by van Slyke et al (449).

(b) <u>Reagents:</u>

(i) Make a mixture of KIO_3 and $K_2Cr_2O_7$ in the proportion 2:1. Grind with a mortar and pestle to mix thoroughly.

(ii) Liquid reagent: 67 ml fuming H_2SO_4
33 ml H_3PO_4
1 g KIO_3

Heat to 160-180° C till KIO_3 has dissolved. Keep dust free.

(iii) CO_2-free NaOH.

First make a quantity of CO_2-free distilled water by boiling water for approx. 3 hrs. Store in an aspirator bottle, closed with a rubber bung complete with a drying tube containing indicator soda lime. Make a saturated solution of NaOH using the above CO_2 free water ($\pm$ 20 N). Store in a Winchester bottle with a siphon protected by a drying tube with soda lime.

To make the 1 N NaOH used in the method, sufficient

*SCWP millipore filter, 8 u pore size, white plain. Diam. 47 m.m. B.D.H. (Millipore filter corp. Mass. U.S.A.)

NaOH is removed from the Winchester by means of a disposable syringe and this is diluted with CO_2 free distilled water to the correct normality.

(c) Procedure:

Add 1 g of powder reagent (a) and 5 ml of liquid reagent (b) to a B24 tube containing the sample to be combusted. The exact weight of the sample must be known and it should be sufficient to produce 1½ m moles of carbon on combustion.

To a B19 tube, add 5 ml of 1 N NaOH(CO_2- free) (c). Join the B19 and B24 tubes with an adaptor which has a side-piece for evacuation (See Fig. 6-2).

Using water pump evacuate the system.

Warm the B24 tube gently over a Bunson flame and gradually heat contents to boiling (removing from flame now and again to allow effervescence to subside).

When white fumes appear the reaction is finished and the tubes are left for 6 hours or overnight to allow diffusion of CO_2 to the NaOH to take place.

After 6 hours, the vacuum is released and 2 ml of 2 M NH_4 CL and 3 ml 25% $BaCl_2$ are added to the NaOH. A white precipitate of $BaCO_3$ forms. This is collected on a previously weighed *millipore filter and placed in a weighed counting vial. The $BaCO_3$ is then dried, removed from the filter paper, crushed and weighed into the counting vial. Add 15 ml scintillator and count.

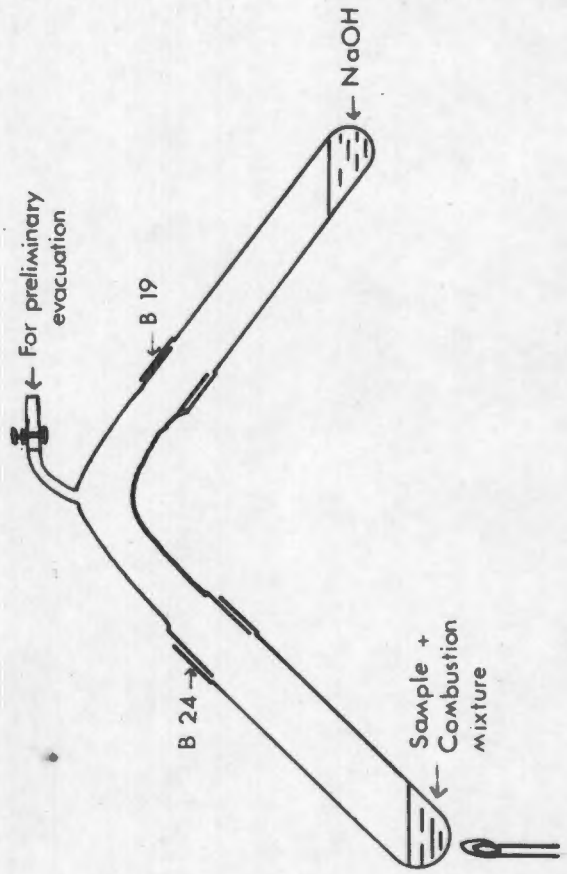


Fig. 6-2: Apparatus for combustion of organic samples.

(d) Yield of barium carbonate-efficiency of combustion and the collection of CO_2 evolved.

One millimole of haemin (ferrihaem chloride) has 34 milliatoms of carbon. As the molecular weight of haemin is 652 (651.969) and that of carbon 12, it can be calculated that 408/652 i.e. 62% of the weight of haemin can be accounted for by its carbon content.

As the molecular weight of $BaCO_3$ is 197, the total weight of carbon evolved from the combustion of haemin is:-

$$\frac{BaCO_3 \times 12}{197}$$

This, expressed as a percentage of the weight of haemin combusted should be 62.7% if both the combustion and the technique for trapping and collecting the evolved CO_2 are 100% efficient.

50 random successive values were calculated and most values were between 59.5 and 63.5% with a mean of 61.9%. Thus the average loss of carbon during the whole procedure was only 1.28%, i.e. an efficiency of a high order.

This efficiency is not important where the whole compound combusted is isotopic e.g. haemin. However, when minute quantities of an isotopic organic compound, e.g. bilirubin plus carrier e.g. glycine, are combusted, the total activity evolved must be measured (see section (a) above). The accuracy of measuring C^{14} activity in bilirubin is dependent therefore upon a virtually 100%

yield of $BaCO_3$. I feel that the combustion method fulfils these needs adequately.

(5) Determination of specific activity of serum bilirubin.

Two methods of isolating bilirubin from serum have been employed in this study. The first is modified from the extraction and crystallization procedure reported by Ostrow et al (331) and the second is identical to that used by Yamamoto et al (498) and Israels (208).

In the former method, the concentration of bilirubin in a known volume of serum is estimated, a known amount (approximately 1 to 1½ mg) of carrier bilirubin is added and the concentration of the carrier-serum mixture is determined. In this way, the exact dilution of serum bilirubin by the added carrier can be calculated. Bilirubin is then extracted from the acidified plasma into ether, recrystallized from chloroform and then from dry methanol and a known weight is combusted to carbon dioxide which is counted as barium carbonate. In the procedure described by Yamamoto, Israels et al (498,208), bilirubin is extracted from serum into chloroform and adsorbed on to an aluminium oxide column. It is washed, eluted with chloroform, its concentration is determined spectrophotometrically and a known volume is evaporated to dryness in a combustion tube where, after combustion to CO_2, C^{14} activity is counted as barium carbonate. The yield from this method is only 15% compared to the 25 to 30% yield by the procedure of Ostrow et al, but it has the advantage of being simpler

and, as there is no dilution of the "hot" serum bilirubin by "cold" carrier, inaccuracies in the azobilirubin assay of serum bilirubin will not influence the final result.

(a) Collection of specimen.

Approximately 40 ml of heparinized blood is taken into a "Lusteroid" tube sealed with a plastic cap and is centrifuged in a refrigerated centrifuge at 10,000 rpm for 10 minutes. Serum is separated and stored in the dark at -15° C if delay of more than one hour is anticipated as serum bilirubin is unstable in light at room temperature.

(b) Bilirubin carrier.

About 100 ml of pooled human serum is cleared by centrifugation at 10,000 rpm for 20 minutes and passage through an OSWP (10mu) millipore filter (B.D.H; Millipore filter corp. Mass. U.S.A.). 50 mg of bilirubin (B.D.H.) are weighed into a 50 ml beaker wrapped in aluminium foil to exclude light. The bilirubin is dissolved rapidly in 7.5 ml of 0.05 N NaOH with gentle swirling. Immediately it has gone into solution, 20 ml serum and 3.5 ml of 0.1 N HCl are added in this order. The contents of the beaker are transferred to a 100 ml volumetric flask which is made up to the mark with serum. Solution is then stored in a dark bottle in the deep freeze.

(c) Reagents.

(1) Reagents for azobilirubin assay of serum bilirubin.

Caffeine mixture: Caffeine 100 gm, sodium benzoate 150 gm,

sodium acetate 250 gm, made up with water to 2L in a volumetric flask.

Sulphanilic acid solution: Sulphanilic acid 5.0 gm, 15 ml conc. HCI made up to 1L with distilled water.

Sodium nitrite solution: Prepare stock solution of 25% Analar sodium nitrite in water. Dilute 50 times (i.e. 0.2 ml of stock solution + 9.8 ml water) immediately before use.

Diazo reagent: 0.25 ml of 0.5% sodium nitrite + 10 ml sulphanilic acid. Make fresh reagent each day and use within 30 minutes of preparation.

Ascorbic acid solution: 200 mg ascorbic acid are dissolved in 5 ml water. Make fresh solution each day.

Fehlings solution: 100 gm. NaOH, 300 gm sodium potassium tartrate, made up to 1 litre with distilled water.

(ii) Reagents used in extraction bilirubin from serum.

Dry methanol: prepare as in Vogel pg. 164 (452).

Peroxide free ether: first prepare following solution.

Ferrous sulphate 60 gm, cone H_2SO_4 6 ml, water 110 ml.

Shake 1L of ether with 20 ml of this solution and thereafter wash once with water. Ether must be cleared of peroxide at least every two days.

(d) Procedure for assay of bilirubin in serum.

Allow plasma sample and bilirubin carrier solution to thaw in the dark in warm water.

To 2 ml of the plasma sample add 3 ml water and mix (i.e. prepare 5 ml of a 4:10 dilution of plasma).

If the plasma is haemolysed make a 1:10 dilution.

Measure volume of remainder of plasma and mix with 3.0 ml bilirubin carrier solution to give mixture for extraction. Set this on one side with exclusion of light.

To 0.5 ml of carrier-plasma mixture, add 4.5 ml water (i.e. prepare 1:10 dilution of mixture).

Prepare 5 ml of 1.25 dilution of the carrier solution by adding 4.8 ml of 1:25 dilution of the carrier solution by adding 4.8 ml of water to 200 ul of the standard.

Set out 2 rows of 6 test tubes in each row and make additions as indicated in the table below. The front row represents the test solutions and the back row the blanks. The assay is carried out in duplicate.

The contents of each tube mixed and read on a spectrophotometer at 600 mu slit width 0.05.

TUBE NO.

	1	2	3	4	5	6
Plasma	1 ml 4/10 test	-	1 ml 1/10 mixture	-	1 ml 1/25 carrier	-
Caffeine (ml)	2.0	2.0	2.0	2.0	2.0	2.0
Diazo Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5
	Wait exactly 10 mins for colour to develop.					
Ascorbic Acid (ml)	0.1	0.1	0.1	0.1	0.1	0.1
Fehlings Solution (ml)	1.5	1.5	1.5	1.5	1.5	1.5
Plasma	-	1 ml 4/10 test	-	1 ml 1/10 mixture	-	1 ml 1/25 carrier

Calculations:

Total mg bilirubin per 100 ml plasma =

$$\frac{\text{Mean reading of test - mean reading of blank x 43}}{\text{Dilution of plasma}}$$

Total mg bilirubin per 100 ml plasma-carrier mixture =

$$\frac{\text{Mean reading of test - mean reading of blank x 43}}{\text{Dilution of plasma-carrier mixture}}$$

Total mg bilirubin per 100 ml carrier solution =

$$\frac{\text{Mean reading of test - mean reading of blank x43}}{\text{Dilution of carrier solution}}$$

Accuracy of assay is checked by comparing carrier concentration of bilirubin as determined directly and as determined indirectly from difference between plasma alone and plasma + carrier mixture.

This method is modified from the work of Michaëlson (296).

(e) Extraction of Bilirubin. (331)

Serum-carrier mixture is brought to pH 5.0 with 0.3 M Acetate buffer (pH 5) using pH meter.

Bilirubin extracted in 250 ml separating funnel with repeated small volumes of peroxide-free ether until all the yellow colour extracted (usually 1 L peroxide-free ether is required for complete extraction.

Extracts pooled in a 1 L separating funnel covered with brown paper to exclude light and washed five times with approx. 100 ml portions of distilled water.

Ether extract shaken vigorously with 5.0 ml of 0.02 M sodium pyrophosphate solution. The yellow pigment should be extracted completely into this layer. If it is not, add 0.1 M NaOH drop by drop until all yellow pigment is extracted into aqueous phase. Repeat the extraction with sodium pyrophosphate solution until clear.

Pooled pyrophosphate extracts are added to 100 ml separating funnel containing 1 ml glacial acetic acid and bilirubin extracted repeatedly into small volumes of chloroform.

Combined chloroform extracts washed 6 times with water, once with an equal volume of 10% NaCl solution and 4 times with water.

Filter chloroform solution through Whatman's No. 40 filter paper moistened with chloroform and evaporate filtrate to a small volume in an evaporating dish over boiling water in a beaker. Transfer the concentrate quantitatively to a graduated centrifuge tube and evaporate off the remainder of the chloroform under suction until crystals just begin to form.

Add 2 volumes of dry methanol and continue evaporation slowly until bilirubin crystallises out as orange needles. Separate by centrifugation and remove supernatant fluid with a Pasteur pipette. Wash crystals with 0.5 ml cold dry methanol. Centrifuge, decant methanol and dry crystals in vacuo over silica gel and paraffin wax shavings.

Recrystallise bilirubin by dissolving in a few ml of chloroform, evaporating down and adding ½ volume of methanol as above.

Dissolve crystals in a few ml of chloroform and run onto 1 x 2 cm columns of aluminium oxide. The bilirubin is absorbed as a thin layer on the top of the column. Wash repeatedly with chloroform to extract all fatty material and finally elute bilirubin using a solution of 1% acetic acid in chloroform.

Transfer eluate to 250 ml separating funnel and wash repeatedly with distilled water until neutral. Recrystallise bilirubin from chloroform solution after evaporation as above.

Dissolve final crystals in about 3 ml chloroform and transfer to combustion tube.

Measure volume of solution and transfer to combustion tube. Measure bilirubin concentration in solution by reading 0.1 ml of solution + 2.9 ml chloroform in stoppered cuvette against chloroform blank at 453 mu in spectrophotometer.

Calculations:

Total bilirubin in combustion tube = volume of solution x Beckman reading x 0.28896

Evaporate chloroform in combustion tube. Add approxi-

mately 40 mg glycine to combustion tube to provide carrier CO_2, combust and harvest barium carbonate as usual.

(6) Estimation of Specific Activity of Plasma Bilirubin (Alternate Method).

This method is based on personal communication with Israels (208) and from a paper by Yamamoto et al (498).

(a) Procedure.

(1) To 10-25 ml plasma add 0.18 vols of $(NH_4)_2SO_4$ solution (saturated at room temperature) and 2.5 vols of 95% ethanol.

(2) Stand overnight in refrigerator (4°C) and centrifuge at 10,000 r.p.m. for 10 min.

(3) Decant supernatant and to it add further 2.5 x original volume of 95% ethanol to secure complete precipitation of protein.

(4) Stand for 1 hour at 4°C and centrifuge at 10,000 r.p.m. for 10 min.

(5) Add approx. 100 mg ascorbic acid to the ethanol supernate and take to dryness in rotary evaporator (N.B. Temperature not >40°C).

(6) Dissolve residue in chloroform and filter on to 1 x 2 cm column of aluminium oxide. Bilirubin is absorbed as a yellow band at top of column.

(7) Wash column with 50 ml chloroform and elute bilirubin band with 1% glacial acetic acid in chloroform.

(8) Wash eluate 3 times with distilled water and filter through chloroform moistened filter paper into 10 ml graduated measuring cylinder.

(9) Read optical density in Beckman (DB Spectrophotometer) at 453 mu against chloroform blank (= R).

(10) Return contents of cuvette to measuring cylinder and record volume (= V).

(11) Transfer contents of measuring cylinder quantitatively to combustion tube and evaporate to dryness.

(12) Add approx 40 mg glycine (to provide carrier CO_2) and combust.

(13) Harvest $BaCO_3$ quantitatively and count.

Calculation:

$$\text{S.A. of bilirubin (dpm/mg} = \text{dpm} \frac{\times 10^3}{R \times V \times 9.73}$$

Assuming E mol for bilirubin in chloroform of 6×10^4 (296).

(b) Comments on methods (5) and (6) for measuring plasma bilirubin-C^{14} activity.

A number of difficulties beset any method of measuring plasma bilirubin-C^{14} activity following glycine-2-C^{14}. Firstly, even at peak values, the isotopic activity of bilirubin is only a few hundred counts per minute/mg. As normal plasma only has about 0.5 mg bilirubin/100 ml and as the 20 ml of plasma obtained has, therefore, approximately 0.1 mg per 100 ml, one is working with minute quantities.

With a yield of 20% the 0.02 mg of bilirubin counted would only have 10 to 20 counts above background at peak values. Thus, methods of isolation must be carried out meticulously as tiny errors are grossly magnified. Strict precautions must be taken against radioactive contamination.

Method No. 5, after adding carrier bilirubin, has a yield of about 25 to 30%. Therefore, if one starts with 0.1 mg of isotopic bilirubin, 0.025 to 0.03 mg of "hot" bilirubin will be available for counting. Method No. 6, adapted from Isreals (208) only has an average yield of about 15%, providing usually 0.015 mg of "hot" bilirubin or less for counting, while at first glance, method No. 5 therefore appears superior, method No. 6 has other important advantages. As no carrier bilirubin is added, the accuracy of the serum bilirubin assay is not important and, even though in method 5 these were done in duplicate and agreed well with each other, there must be a fair measure of inaccuracy when measuring quantities of bilirubin less than 1 mg/100 ml. Method No. 6 has far fewer steps than No. 5 and besides being easier, it provides less opportunity for contamination.

By both methods, the final solution of bilirubin in chloroform had an absorption spectrum, which when expanded with the use of the Beckman DB spectrophotometer, showed a specific sharp peak at 453 mu with no other peaks between 320 and 700 mu (See fig. 6-3).

Serum Bilirubin extracted in chloroform

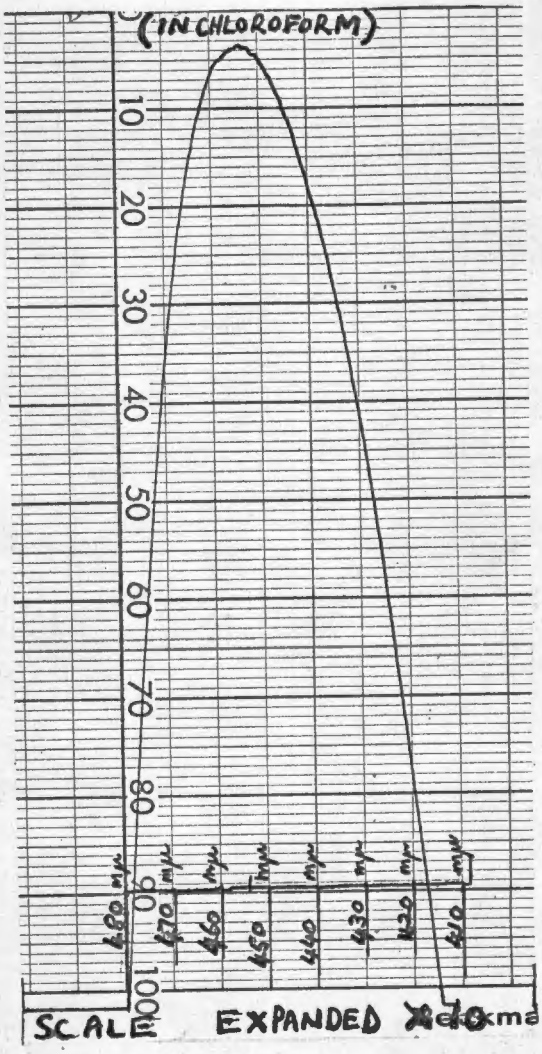


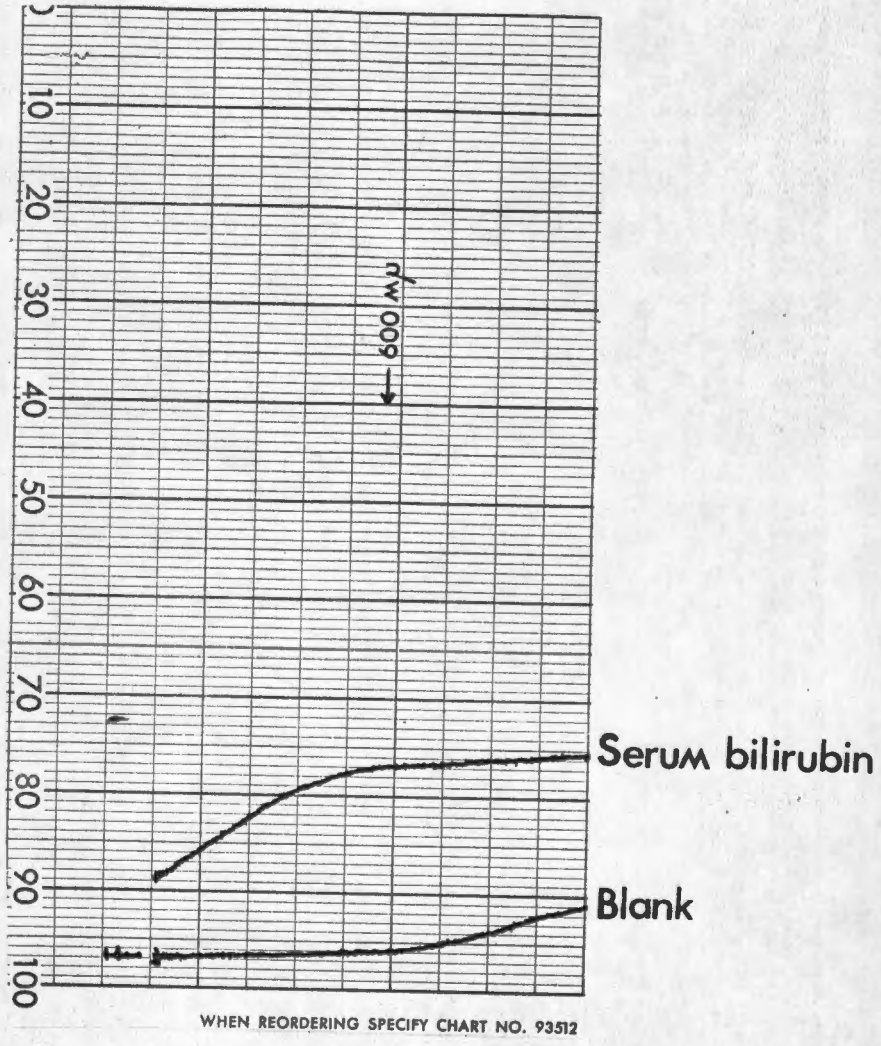
Fig. 6-3: Absorption spectrum of bilirubin in chloroform after extraction from serum (expanded 10 times).

When extracting bilirubin from plasma by method 5, on crystallizing bilirubin from the serum, fat globules often came down with the bilirubin crystals. However, following adsorption onto an aluminium oxide column, washing with chloroform and elution with acidified chloroform, re-crystallization resulted in pure clean looking orange crystals of bilirubin with the single absorption peak in chloroform at 453 mu.

(c) Comments on assay procedures.

The azobilirubin assay procedures was adapted from the work of Michaëlson (296). He showed that the azobilirubin was best read at 600 mu. I confirmed that when the absorption spectra of an azobilirubin sample and blank were compared, the maximum difference in optical density was at about 600 mu. (See fig. 6-4). The absorption peak of the azobilirubin expanded 10 times was at between 590 mu and 600 mu (See fig 6-5). I also showed that the colour obtained was proportional to bilirubin present in a standard sample and that the conversion factor to convert optical density into mg bilirubin/100 ml was 43.2 (See fig. 6-6). This agreed closely with figures of 43 accepted by Michaelson (296). Assays were always done in duplicate and agreed to within 2 to 4% of each other.

The assay of bilirubin in chloroform was adapted from the recommendations for uniform bilirubin standard as discussed by the Standards Committee of the College of American



Azobilirubin

Fig. 6-4: Absorption spectrum azobilirubin (test).

Azobilirubin

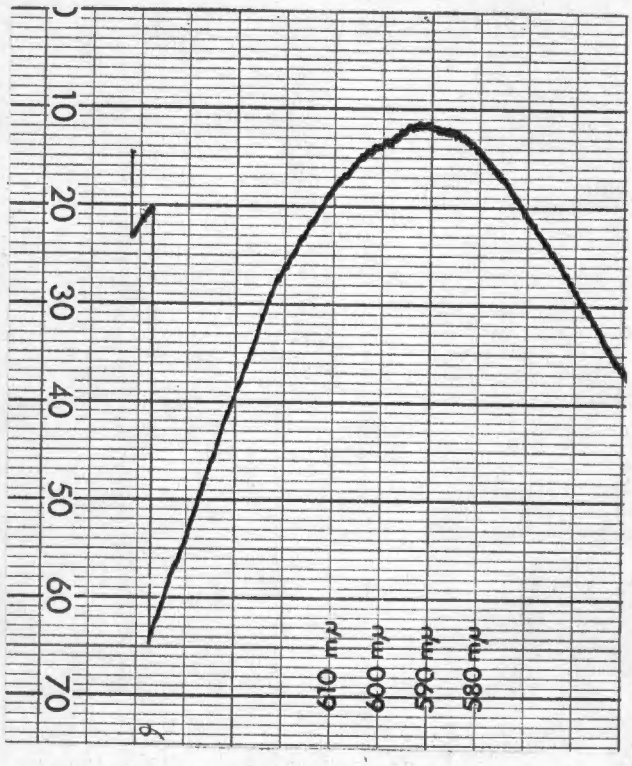


Fig. 6-5: Absorption spectrum azobilirubin expanded 10 times.

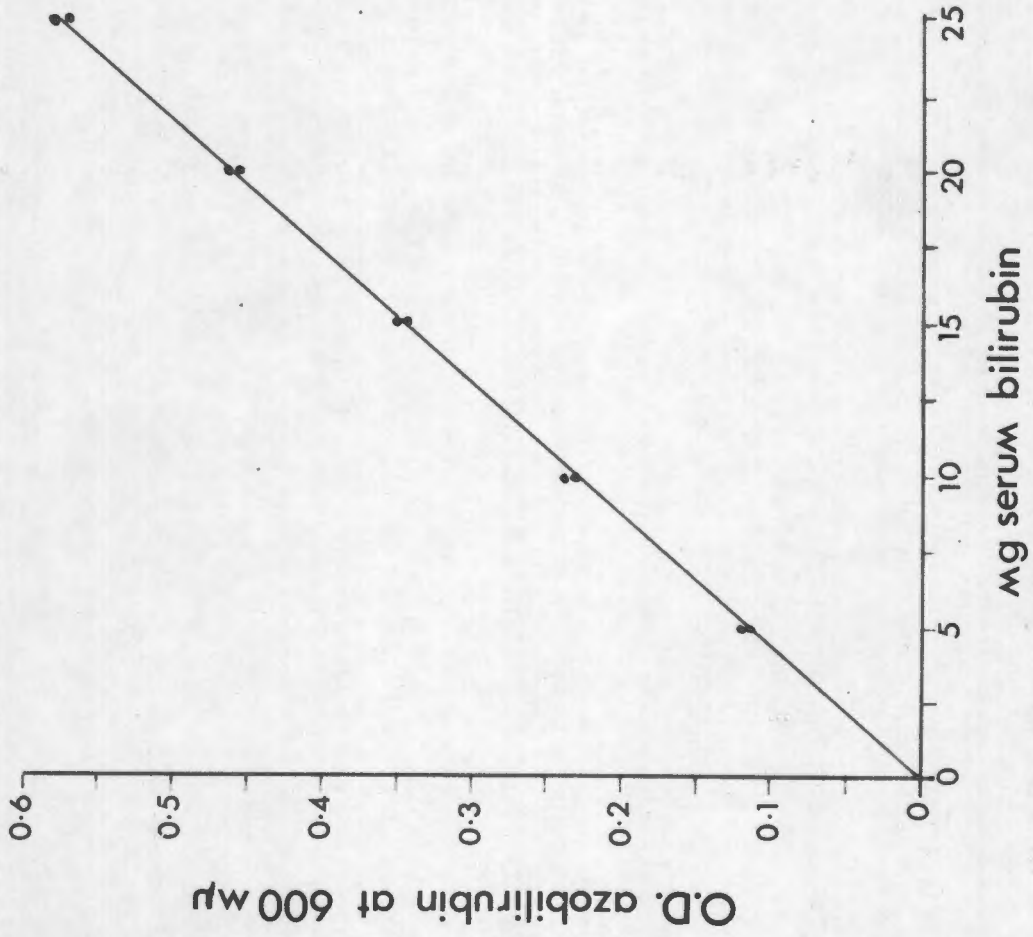


Fig. 6-6: Standard calibration curve for azobilirubin method of serum bilirubin assay.

Pathologists (408). Six highly purified commercial and 2 privately purified crystalline preparations were measured in triplicate by 3 different laboratories and gave a 1 cm molar absorptivity of 60,700 $\pm$ 800 (mean $\pm$ 1 standard deviation) when dissolved in chloroform and read at 453 mu at 25° C. The committee recommended as acceptable a bilirubin sample giving a molar absorptivity of between 59,100 and 62,300.

To calculate the total amount of bilirubin in the combustion tube, both an accurate assay and the volume of the solution at the time of assay must be known. Usually, the bilirubin solution is present in a finely graduated test-tube (from which volume can be read) before transferring to a combustion tube. 0.1 ml of this solution is added to 2.9 ml chloroform and the cuvette stoppered, thus making a 1 in 30 dilution of the bilirubin solution. As 60,000 mg/litre (approximately molar solution) would read about 60,000 on the spectrophotometer, 0.1 mg bilirubin/100 ml chloroform would have an optical density of about 0.1. More accurately, a reading of 0.1 would be equivalent to 584/607 x 0.1 mg/100 ml i.e. 0.09632 mg/100 ml chloroform.

From the above, the calculation formula is as follows:-

(a) Reading x 0.9632 = no mg bilirubin/100 ml chloroform in assay cuvette.

(b) Reading (R) x 0.9632 x 30 (i.e. R x 0.28896)= mg bilirubin/100 ml chloroform in combustion tube.

(c) Volume (V) x reading (R) x 0.28896 i.e. VR x 0.28896 = Total weight of bilirubin to be combusted.

The standard calibration curve measuring known amounts of bilirubin (BDH) is chloroform was a straight line in the range of concentrations encountered during experimental procedures (See fig. 6-7).

(7) Isolation and purification of haemin.

This rather straight forward isolation procedure is derived from a method described by Labbe et al (236).

(a) Reagents:

(1) 2% $SrCl_2.6H_2O$ in glacial acetic acid.

(2) Acetone (Analar).

(3) Working solvent: One part of (1) + three parts (2) prepared immediately before use.

(4) Pyridine (Analar).

(5) Chloroform (Analar).

(6) Acetic acid: water (1:1).

(7) Ethanol 96%.

(8) Ether (Analar).

(b) Procedure:

To 1 volume of blood or lysed red cells add, with stirring, 12 volumes of working solvent.

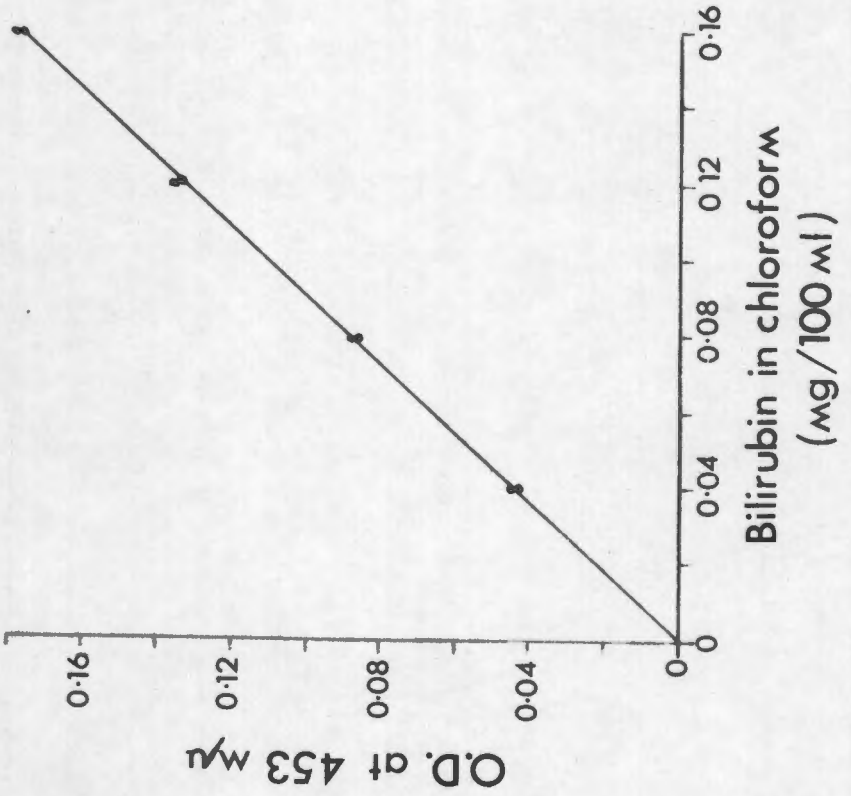


Fig. 6-7: Standard calibration curve of bilirubin in chloroform.

Stand for 20 mins with occasional stirring. Heat to boiling point briefly during this period of standing to increase protein precipitation.

Filter and wash the residue on filter paper twice with 1 volume of solvent.

Pooled filtrates are heated to 100°C with a boiling chip in a beaker. Stir with thermometer and do not allow temperature to exceed 102°C.

Crystallisation begins when the solvent concentrates and is complete on cooling to room temperature.

Harvest crystals by filtration (Hirsch funnel) and wash twice with 50% acetic acid in water, once with ethanol and once with ether.

Recrystallise by taking up in minimum amount of pyridine and chloroform.

Filter and add 2% $SrCl_2$ in acetic acid. Heat to 100°C and allow to cool.

Harvest crystals and wash as before. Dry in vacuum dessicator over silica gel. Weigh accurately into combustion tubes for counting as barium carbonate.

(c) Comment.

Yield after recrystallisation - approx. 60%.

Thus, starting with about 8 ml of packed cells about 30 mg of purified haemin were obtained for combustion.

(8) Measurement of radioactivity.

The radioactivity of samples from all the uric acid degradation experiments and two of the bilirubin-C^{14} studies was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer. For the remaining bilirubin-C^{14} experiments, all measurements of radioactivity were made with the Beckman Liquid Scintillation System.

Uric acid, glyoxylic acid semicarbazone and barium carbonate were weighed accurately into counting vials and suspended in a scintillator of the following composition: 2,5 - diphenyloxazole (PPO), 0.3 gm; 1,4 - bis - 2- (5 phenyloxazole) - benzene (POPOP), 0.03 gm; Cab - O - Sil (Packard), 4.0 gm; toluene 100 ml.

Preliminary experiments indicated that there was no self-absorption of the soft β-emissions over the range of weights used in the counting vials. The efficiency with which the Packard-Tri-Carb counter and the Beckman Liquid Scintillation counter detected C^{14} in the various samples was about 53 to 57% and 65 to 68% respectively. The efficiency of counting every batch was determined by adding a known number of dpm as n-Hexadecane-1-C^{14} (The radiochemical centre, Amersham, Buckinghamshire, England) to several samples. The radioactivity of the samples counted (c.p.m.) was thereby accurately converted to disintegrations per minute (d.p.m.) Radioactivity of the individual samples was expressed as d.p.m. or as

percentage dose glycine given.

CHAPTER VII.

PRESENTATION OF EXPERIMENTAL RESULTS.

1. URINARY UCIC ACID DEGRADATION STUDIES.

The experimental methods have been discussed in detail in Chapter VI. In brief, 70 to 80 uc of glycine-2-C^{14} were administered I.V. to 14 human subjects of whom 4 were normal controls, 4 had proven symptomatic porphyria and 6 suffered from S.A. genetic porphyria. Of the latter group, 5 were studied during an acute attack and 1 during remission. Uric acid was isolated from daily specimens of urine for the ensuing two weeks and degraded in such a manner as to allow for the separate counting of carbon atoms 4 and 5 as glyoxylic acid semicarbazone and carbon atoms 2 and 8 as urea. The urea was digested with urease and the CO_2 was trapped and counted as barium carbonate.

In this chapter, the validity of the experimental findings has been evaluated with regard to the patients clinical state, the conditions under which the study was performed and the accuracy of isolation and assay procedures.

The results have been presented along the following lines:-

(a) Day to day excretion of C^{14} activity of the whole urinary uric acid molecule and its C4 + 5 and C2 + 8 components,

(b) cumulative excretion of urinary uric acid-C^{14} activity and the C^{14} activity of its component carbon atoms,

(c) the daily ratios of C4 + 5: C2 + 8, showing how they scatter around the mean and its standard error and

(d) the daily percentage of the molecule's activity residing in C4+5 and C2+8 respectively, and how this varies according to mean values for the experiment.

The above results are expressed graphically and in tabulated form from which salient features have been extracted and summarized. These will be correlated and discussed in Chapter VIII.

A. NORMAL SUBJECTS.

(1) H.J.

(a) Evaluation of Experimental findings.

H.J. was a coloured male aged 48 years, who was admitted for a right below knee amputation for peripheral vascular disease. He was not hypertensive. His renal function was good and he was not anaemic (haemoglobin 14.0G/100 ml). Thus he was a good subject as a normal control.

4.7832 G of glycine-2-C^{14}, equivalent to 1.8176 x 10^{9} dpm were injected I.V. on day 1 of the experiment.

Blood uric acid levels ranged between 6 and 7 mg/100 ml and 24 hour excretion of urinary uric acid ranged between 458 and 625 mg on a step 8 diabetic diet containing 100 G protein. The daily recoveries of uric acid from the urine were good, usually more than 200 mg, often more than 400 mg being isolated. Thus adequate amounts were available for both counting and degradation.

On day 5, the degradation procedure was marred by breakage of apparatus and had to be repeated on 38.2 mg of "hot" labelled uric acid diluted to 193.0 mg by the addition of "cold" unlabelled carrier uric acid, the dilution factor being 5.052. 24.7 mg of uric acid were available for counting. On day 6, owing to a difficulty in isolation, only 33.2 mg uric acid were obtained of which 13.6 mg were counted and 19.6 mg were diluted by a factor of 10.413 with carrier uric acid prior to

TABLE 7-1.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	cumul.	Daily	Cumul.	Daily	Cumul.
1	14.800	14.800	9.468	9.468	3.489	3.489
2	14.737	29.537	11.267	20.735	3.649	7.138
3	16.849	46.386	9.003	29.738	2.072	9.210
4	15.191	61.577	6.362	36.100	2.846	12.056
5	15.859	77.436	10.340	46.440	2.831	14.887
6	15.424	92.860	10.094	56.534	2.845	17.732
7	13.663	106.523	7.866	64.400	2.339	20.071
8	18.156	124.676	10.191	74.591	2.836	22.907
9	16.959	141.634	11.615	86.206	2.383	25.290
10	13.235	154.869	8.539	94.745	2.073	27.363
11	14.390	169.259	12.812	107.557	1.483	28.846
12	15.332	184.591	8.821	116.378	2.210	31.056
13	11.961	196.552	11.395	127.773	1.862	32.918
14	13.461	210.013	10.036	137.809	2.102	35.020

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul). as uric acid, C4+5 and C2+8 by a normal subject, H.J. Values in the table as percentage dose x 10^{-3}.

DAILY EXCRETION URIC ACID

H.J.

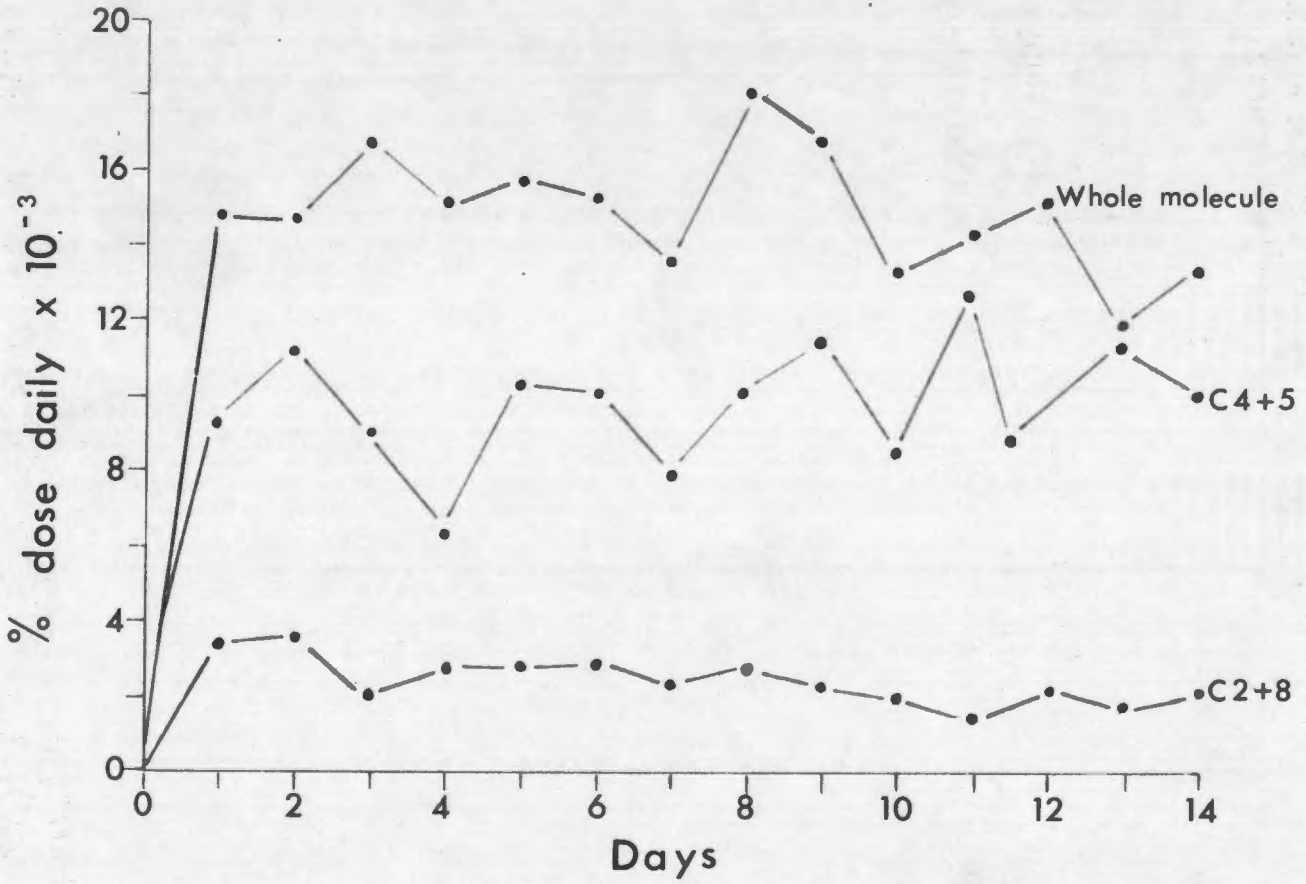


Fig. 7-21: The daily incorporation of ^{14}C into urinary uric acid, C4+5, C2+8 in normal, H.J.

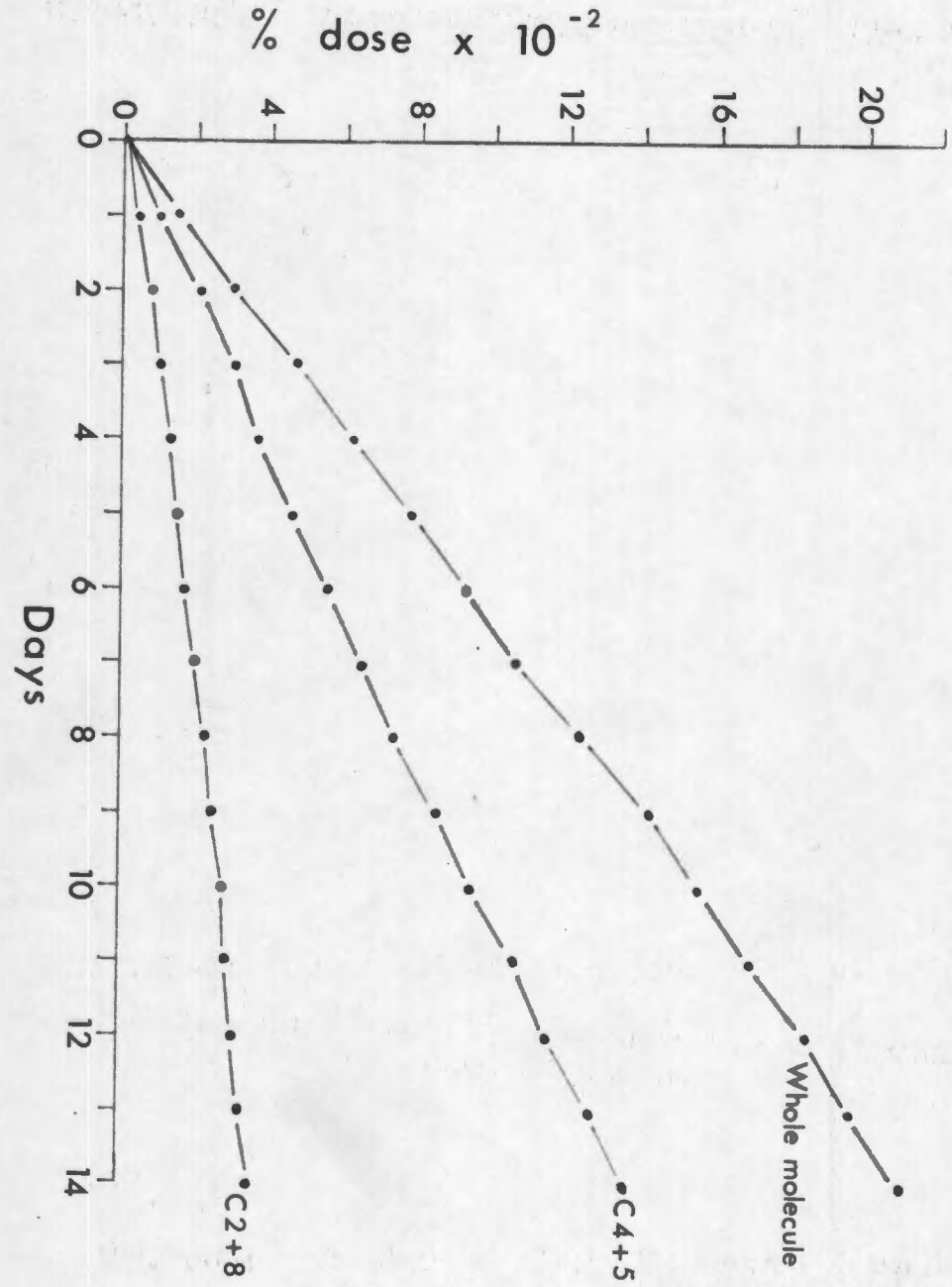


Fig. 7-2: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, H.J.

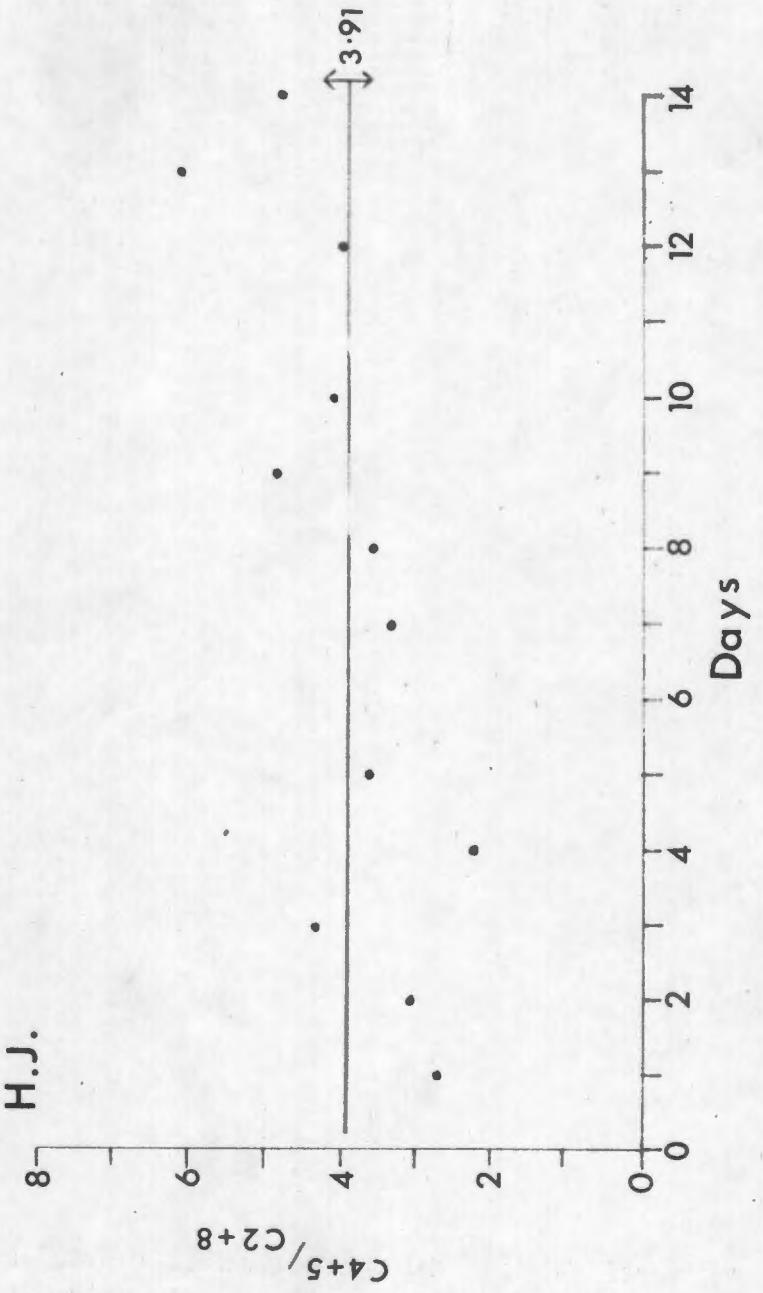


Fig. 7-3: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in normal, H.J.

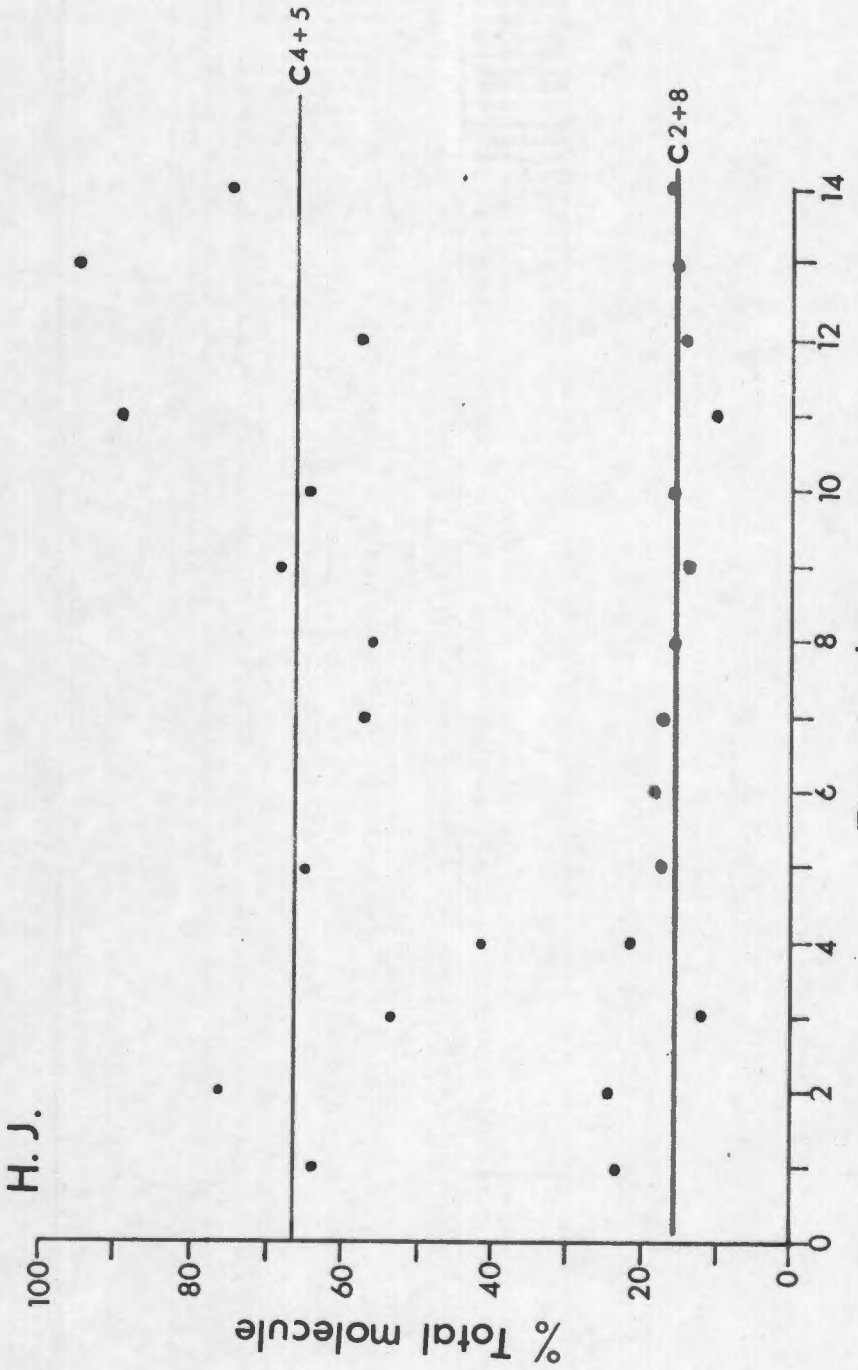


Fig. 7-4: Disposition of radioactivity within uric acid molecule, daily values around mean, in normal, H.J.

degradation.

Usually more than 100 mg of uric acid and barium carbonate were obtained for counting whilst the weights of glyoxylic acid semicarbazone ranged between 20 and 70 mg. On days 5 and 6, counts significantly above background were obtained. Thus from all angles, this was a valid and satisfactory experiment.

(b) Results.

The percentage administered dose excreted daily and cumulatively as uric acid, C4 + 5 and C2 + 8 is set out in Table 7-1 and expressed graphically in fig. 7-1 and 7-2.

Note how in fig. 7-1 the peak values of excreted isotopic activity are reached on the first day of the experiment, the slowly declining plateau occurring from this point. There is a diurnal fluctuation in activity which clearly reflects both the physiological variations of activity excreted from day to day and the limitations of the experimental techniques employed. Whilst the plateau of C4+5 activity remains fairly steady over the 14 days of the study, there is an early hump in C2+8 activity over the first few days after glycine-2-C^{14} administration.

This early hump in C2+8 excretion is reflected in the C4+5: C2+8 ratios (fig. 7-3) and the percentage activity residing in the ureido carbons of the uric acid moiety (fig.7-4). In fig. 7-3, note how ratios scatter around the mean of 3.91 ± S.E. of 0.30. In the first few days

values are significantly lower, the average for the first 4 days being 3.1.

Whilst for the whole experiment C2+8 comprises a mean of 16.88% of the molecule's activity, over the first 2 days the average figure is 24.16%, considerably higher than the mean (fig. 7-4).

The significance of these findings will be discussed in Chapter VIII.

Fig. 7-2 depicts the cumulative excretion of uric acid-C^{14}, C4+ 5 and C2+8 activity. The excreted activity mounts steadily showing no tendency to plateau off. Note how after 7 days (Table 7-1, Fig. 7-2), 0.106% of the administered dose was excreted as urinary uric acid-C^{14} comparing with the average value of 0.18% obtained by Wyngaarden(406) on his analysis of the results of other workers with glycine-1-C^{14}.

(c) Summary.

(i) This was a valid and satisfactory experiment.

(ii) Peak excretion of C^{14} activity as uric acid and its constituent carbons was noted by day 1. (fig.7-1)

(iii) This peak was followed by a plateau of activity extending over the 14 days of the study.

With regard to C2+8, a small hump of excreted activity was noted over the first few days following glycine-2-C^{14} administration.

(iv) 0.21%, 0.137% and 0.036% of the total dose was excreted as urinary uric acid, C4+5 and C2+8 respectively over 14 days (fig.7-2).

(v) The C4+5:C2+8 ratios averaged 3.91 ± S.E. of the mean of 0.30. Ratio values were lower (owing to the early hump of excreted C2+8 activity) over the first 4 days the average being 3.1. (fig. 7-3).

(vi) An average of 66.40% and 16.8% of the uric acid molecule's activity was found to reside in C4+5 and C2+8 respectively over the 14 days of study. Values above the mean were noted in the percentage activity present in C2+8 over the first week (fig.74) with values of 23.57% and 24.76% on days 1 and 2 respectively.

(2) A.J.

(a) Evaluation of experimental findings.

A.J. a 23 year old coloured male had been treated in the medical wards for a partial post-traumatic Brown-Sequard syndrome. With good hepatic and renal function and a haemoglobin of 13 G/100 ml, he was an ideal subject for a normal control. He could void urine without difficulty.

4.9004 G of glycine-2-C^{14} equivalent to 1.8622×10^8 dpm were injected I.V. on day 1 of the experiment.

As this was one of the first studies performed, many

TABLE 7-2.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	4.177	4.177	2.583	2.583	1.359	1.359
2	13.023	17.200	6.094	8.677	2.345	3.704
3	15.956	33.156	7.788	16.465	2.355	6.059
4	*9.186	42.342	* 5.864	22.329	*1.413	7.472
5	20.144	62.486	13.623	35.952	3.471	10.943
6	18.751	81.237	14.519	50.471	4.123	15.066
7	12.953	94.190	8.137	58.608	2.097	17.163
8	9.636	103.826	7.311	65.939	1.957	19.120
9	* 9.956	113.782	* 7.649	73.588	*2.024	21.144
10	10.204	123.986	7.804	81.392	2.153	23.297
11	*13.741	137.727	* 9.944	91.336	*2.729	26.026
12	20.655	158.382	14.286	105.622	3.404	29.430
13	15.437	173.819	9.915	115.537	1.045	30.475
14		187.186		124.422		32.819

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4+5 and C2+8 by a normal subject, i.v. Values in table expressed as percentage dose x 10^{-3}. Those values marked by an asterisk were estimated from the corresponding graph of the above data. The 14-day cumulative incorporation figure is derived by extrapolation from other values.

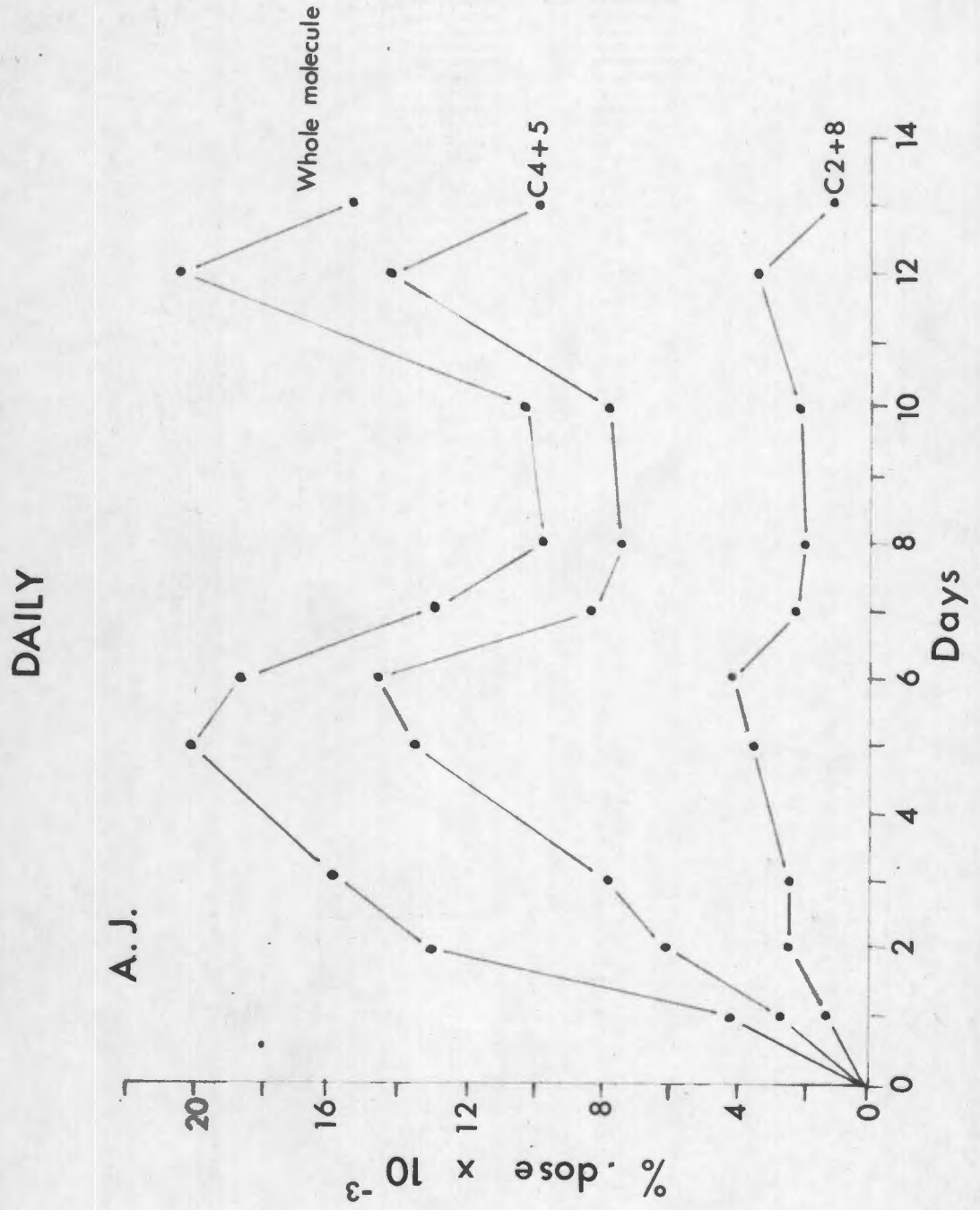


Fig. 7-5: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, A.J.

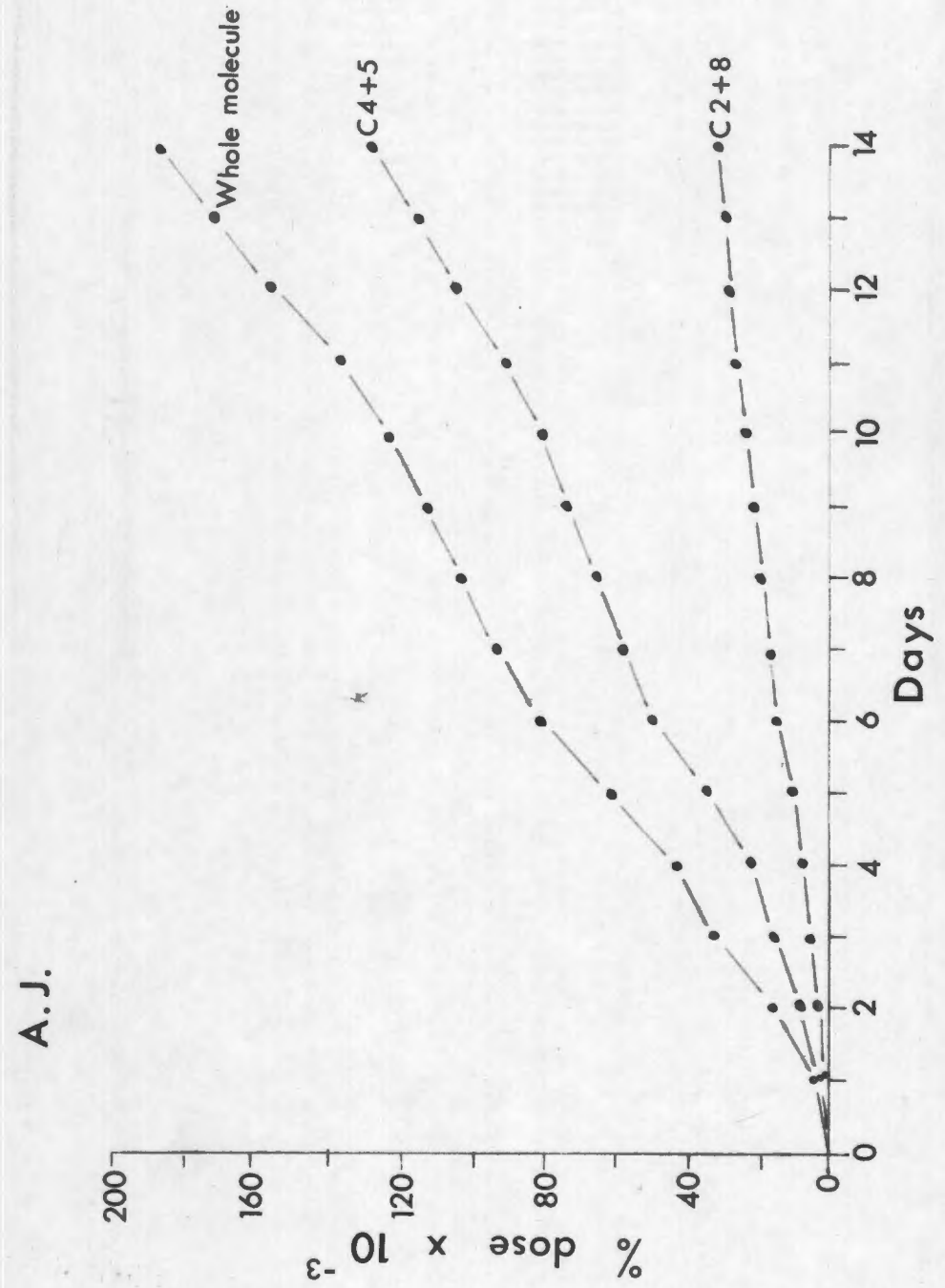


Fig. 7-6: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, A.J.

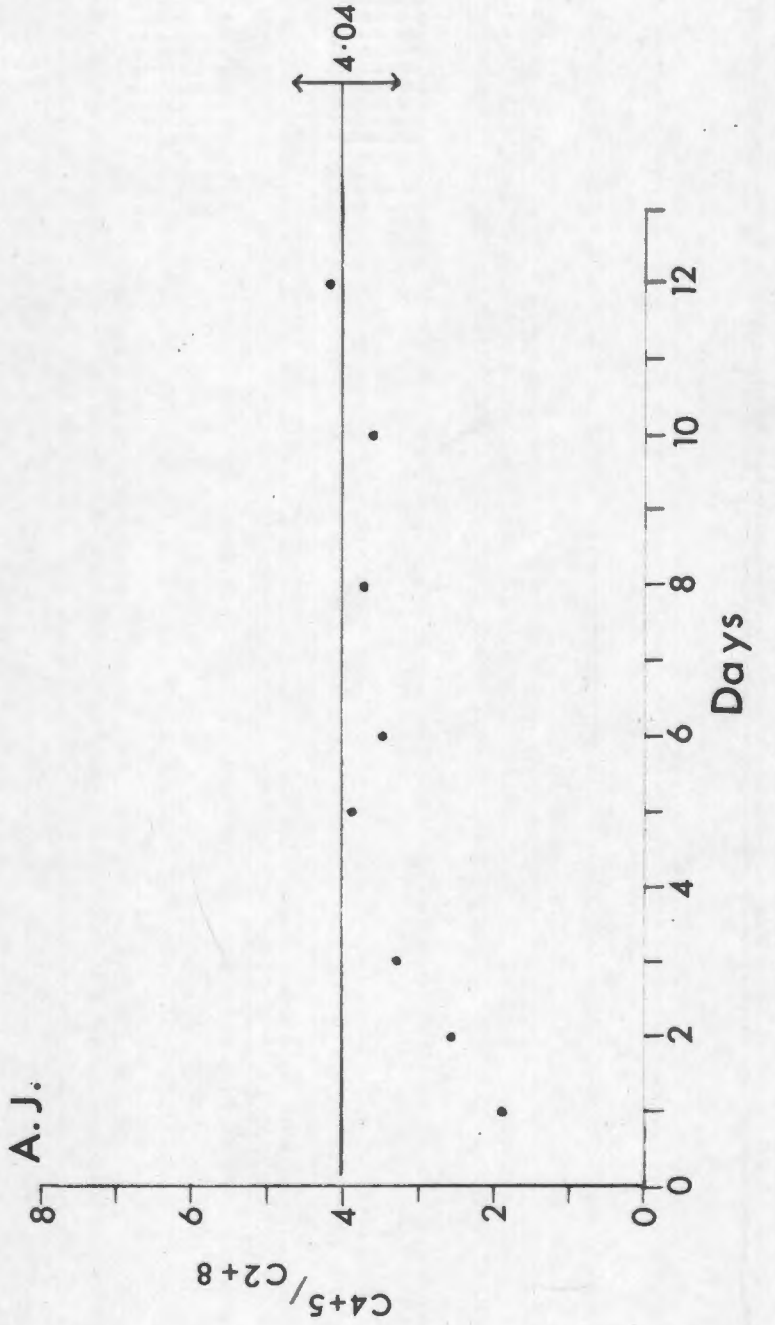


Fig. 7-7: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in normal, A.J.

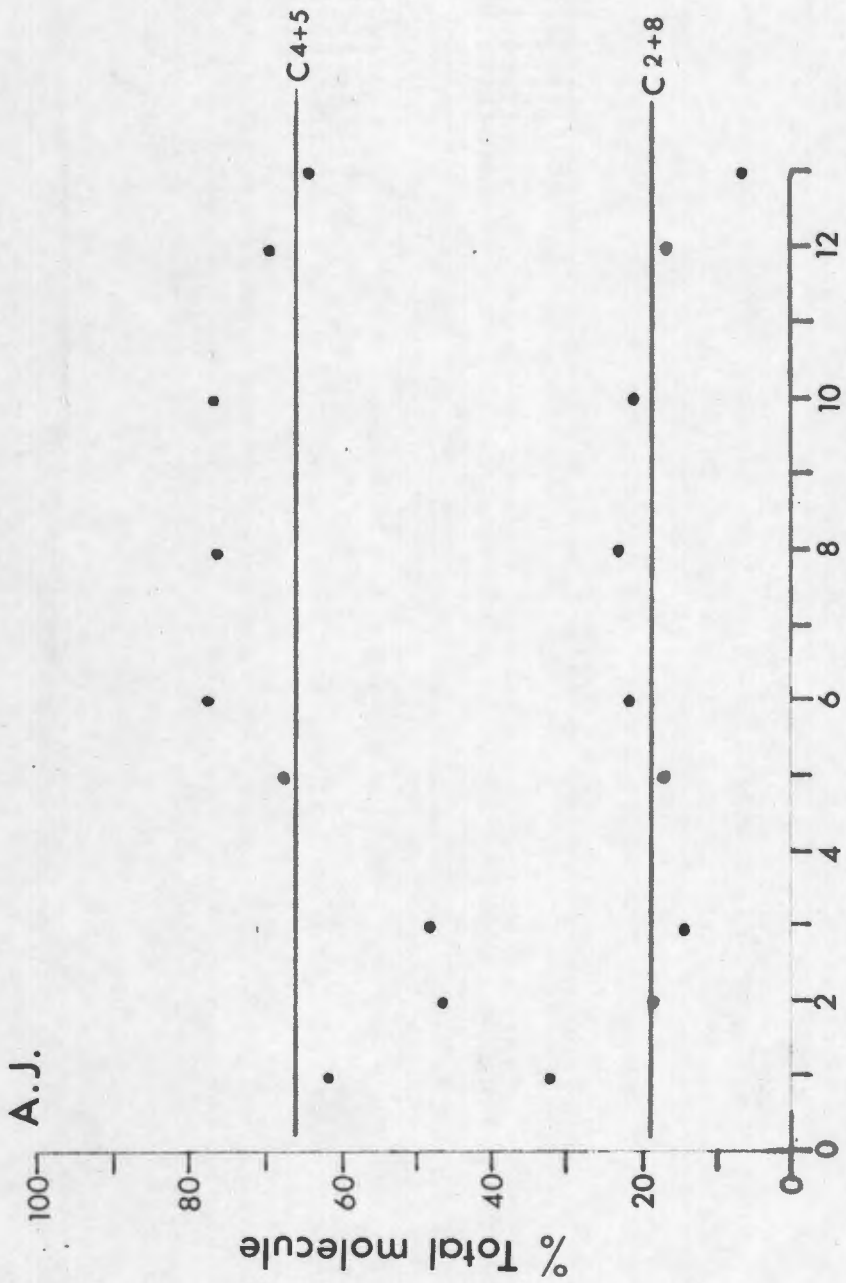


Fig. 7-8: Disposition of radioactivity within uric acid molecule, daily values around mean, in normal, A.J.

technical difficulties were encountered which made some of the results inaccurate.

Daily uric acid excretion varied between 200 to 500 mg per day of which only 50 to 150 mg pure uric acid were isolated. On days 4,7,9 and 11 too little uric acid was obtained for accurate analysis. As most of recrystallized uric acid obtained was kept for degradation, amounts weighed out for counting ranged between 5 and 22 mg with only 1.8 mg on day 1. Dilution with cold carrier uric acid varied between 8 and 12 and amounts of barium carbonate and glyoxylic acid semicarbazone available for counting were usually more than 50 mg.

Thus, results are far from ideal but are probably reasonably accurate and valid. Only c.p.m. significantly above background have been accepted.

(b) Results.

The daily and cumulative excretion of urinary uric acid-C^{14} and its component carbons is shown in Table 7-2, figs. 7-5 and 7-6. On those days where samples were not obtained, approximate values estimated from the graph in fig.7-5 were used. These values have been marked by an asterisk.

Note the big trough in excreted C^{14} activity during days 7 to 10 with a late secondary peak thereafter (fig.7-5). The early peak is also delayed to the 5th day. As in H.J., activity in C2+8 is more prominent in the first few days

following glycine-2-C^{14} administration accounting for lower C4+5: C2+8 ratios during this period (fig.7-7).

In fig. 7-7 the ratios of acceptable degradation experiments are shown graphically. Whilst the mean value is 4.04, the average ratio for the first 3 days is 2.6.

Fig. 7-8 shows the intramolecular distribution of activity within the uric acid-C^{14} moiety. Note once again how on day 1, significantly more activity (32.56%) resides in C2+8 than the mean value for the study (18.8%).

(c) Summary.

(i) Although not an ideal experiment, results are probably acceptable and valid.

(ii) Corrected for 14 days, 0.187%, 0.12½% and 0.033% of the administered dose was excreted as urinary uric acid, C4+5 and C2+8 respectively (fig 7-6).

(iii) The average C4+5:C2+8 ratio was 4.04 ± S.E. of 0.72. The mean value for the first 3 days was 2.6 (fig. 7-7).

(iv) An average of 65.38% and 18.8% of the total uric acid molecule's activity resided in C4+5 and C2+8 respectively. On day 1, 32.56% lay in C2+8, significantly higher than during the rest of the experiment (fig.7-8).

(3) A.M.

(a) Evaluation of experimental findings.

Table 7-1.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	cumul.	Daily	cumul.
1	3.741	3.741	2.761	2.761	0.826	0.826
2	6.927	10.668	4.998	7.759	1.377	2.203
3	7.785	18.453	5.988	13.747	1.603	3.806
4	7.975	26.428	5.054	18.801	0.993	4.799
5	9.334	35.762	6.467	25.268	1.041	5.840
6	9.269	45.031	6.679	31.947	1.211	7.051
7	7.358	52.389	5.757	37.704	1.060	8.111
8	9.232	61.621	8.295	45.999	1.590	9.701
9	8.580	70.201	6.047	52.046	1.492	11.193
10	9.892	80.093	6.849	58.895	1.776	12.969
11	11.136	91.229	6.249	65.144	1.953	14.922
12	10.691	101.920	5.965	71.109	1.746	16.668
13	8.704	110.624	5.896	77.005	1.691	18.359
14	8.616	119.240	6.785	83.790	1.578	19.937

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 +8 by a normal subject, A.M. Values in table expressed as percentage dose x 10-3.

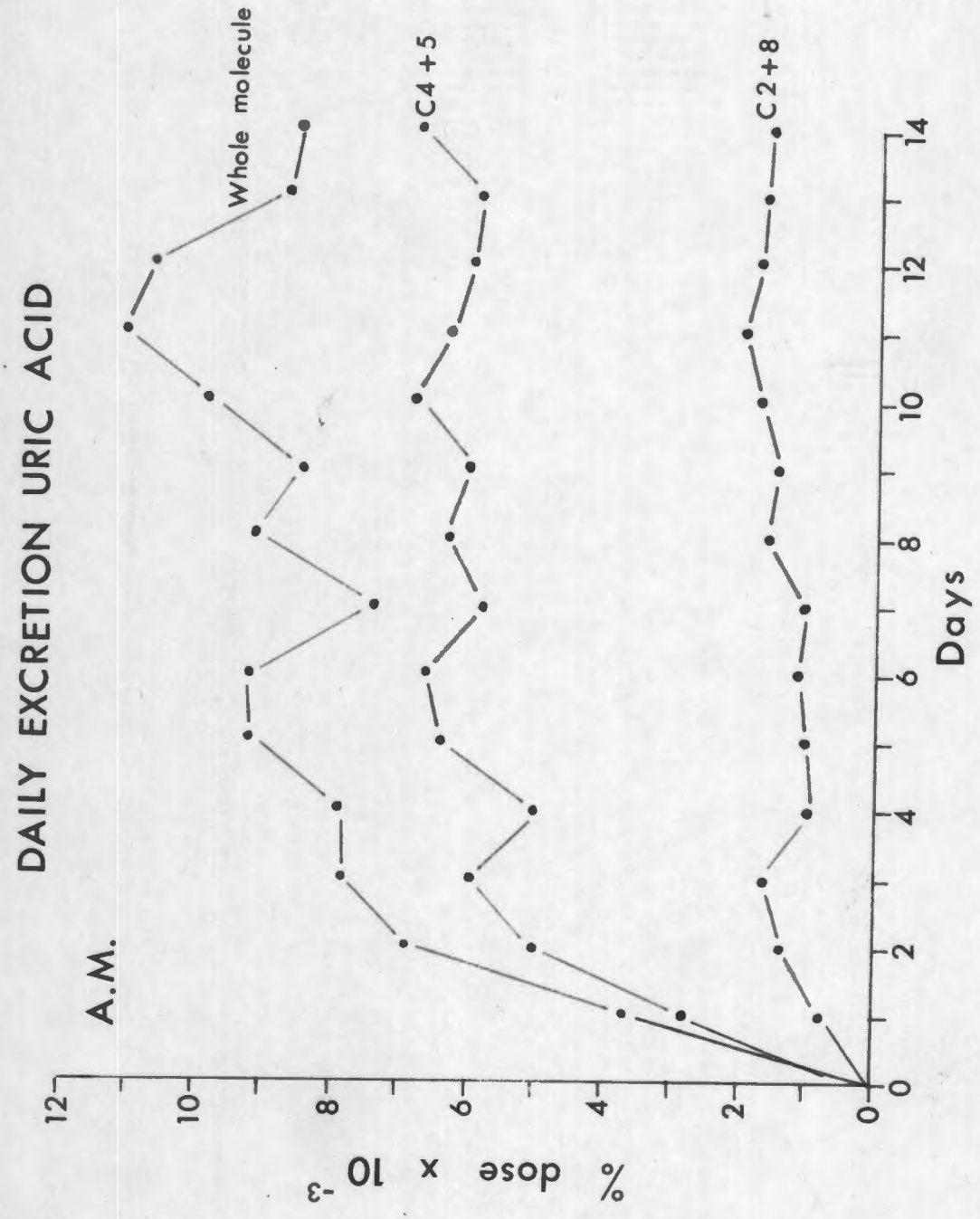
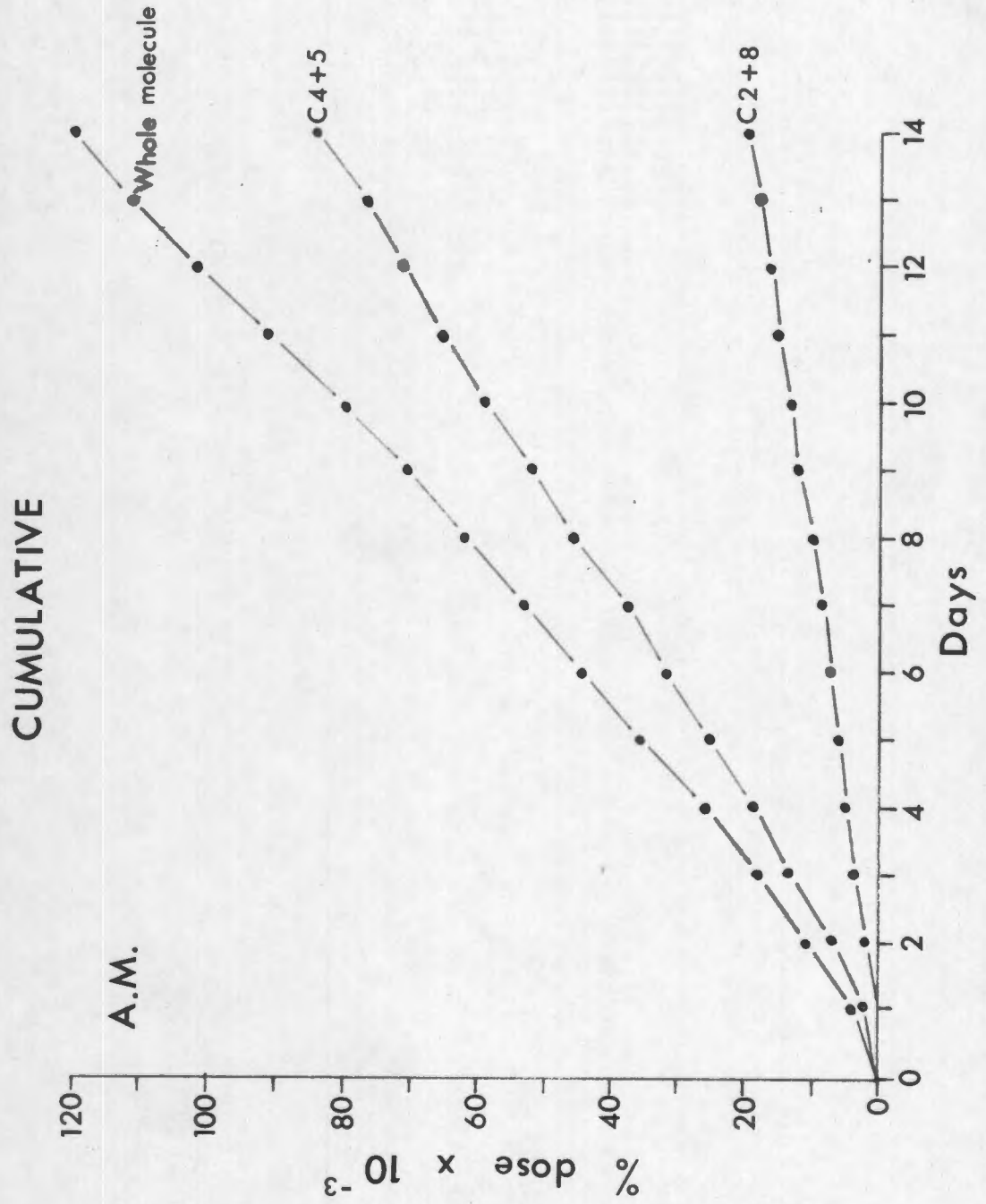
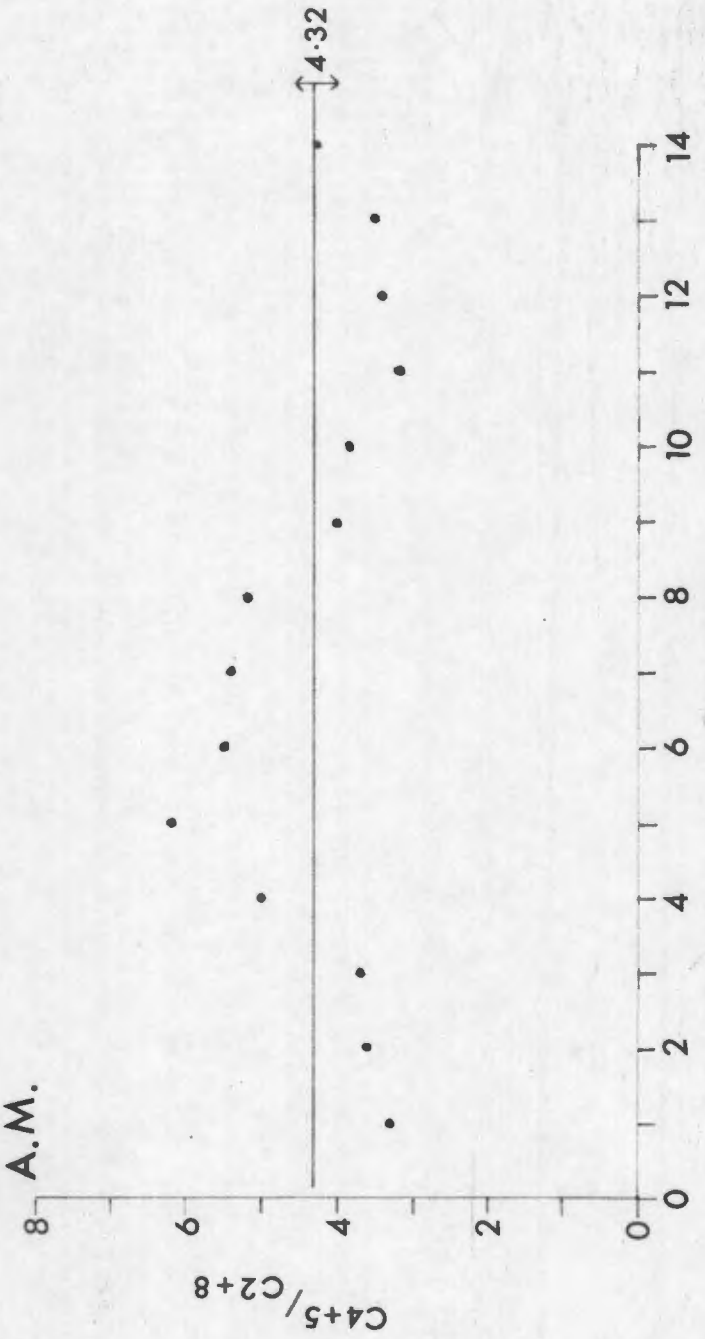


Fig. 7-9: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, A.M.



7-10: The cumulative incorporation glycine2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, A.M.



7-11: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in normal, A.M.

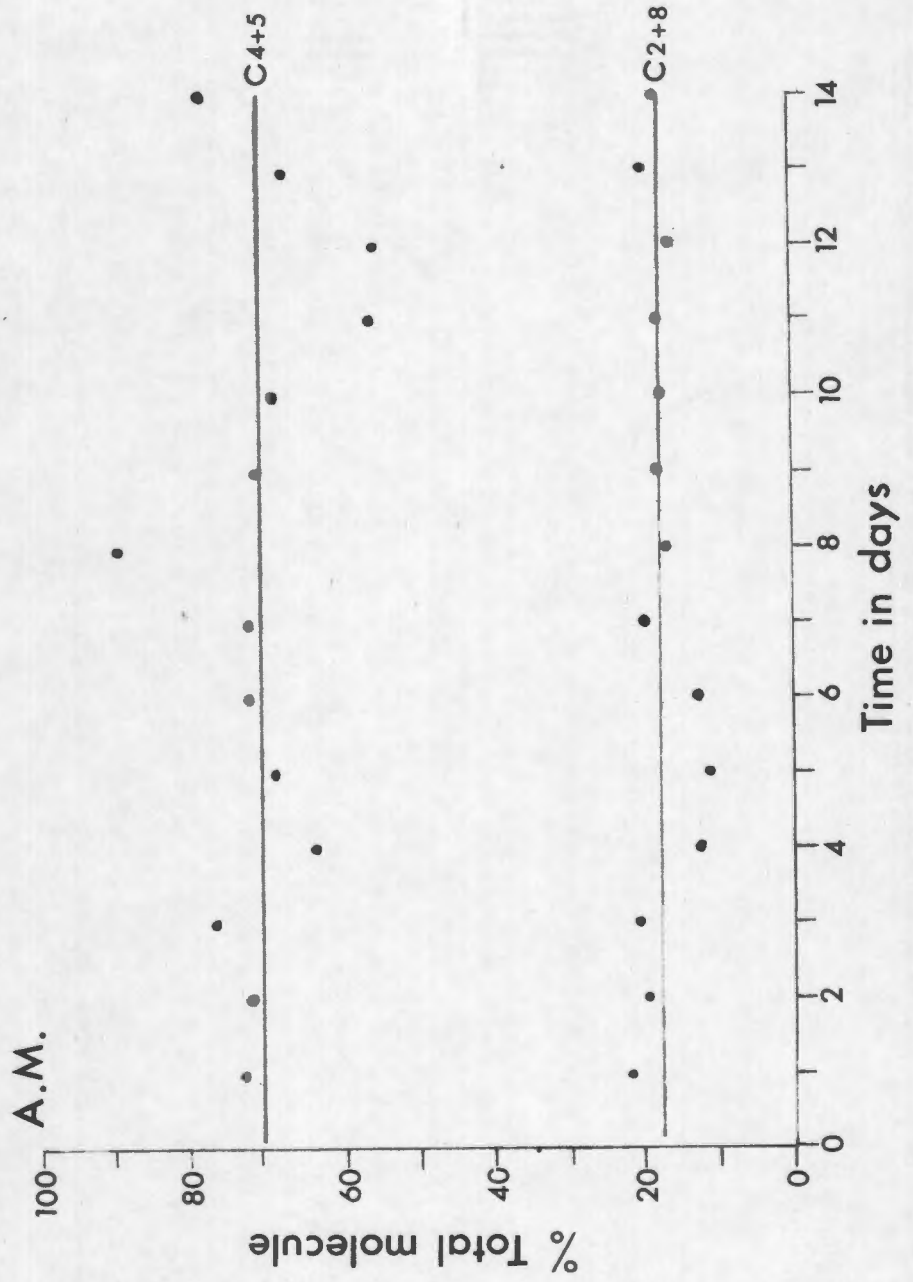


Fig. 7-12: Disposition of radioactivity within uric acid molecule, daily values around mean, in normal, A.M.

This 32 year old Bantu male had osteitis of the neck of his femur. When convalescent, he received 1.9964 x 10^8 dpm glycine-2-C^{14} I.V. At this stage he was well from a renal, hepatic and haemopoietic standpoint and his haemoglobin was 14 gm/100 ml.

This was a most satisfactory experiment from every point of view. Blood uric acid levels varied from 2.75 to 4.44 mg/100 ml. Daily 24 hour urinary uric acid values were usually between 500 and 700 mg of which, on most occasions, more than 400 mg were isolated. There was no dilution factor in any of the degradation procedures and over 100 mg uric acid and barium carbonate and more than 50 mg glyoxylic acid semicarbazone were obtained for counting.

(b) Results.

The results have been expressed as in the last two experiments (see table 7-3, figs. 7-9, -10, -11, and -12.)

Whilst C2+8 on average comprises 17.37% of the molecule's activity, on days 1 to 3, values are 22.06,, 19.89 and 20.59% respectively (fig. 7-12). Thus the early hump in C2+ 8 activity noted in H.J. and A.J. is again noted in A.M.

(c) Summary.

(i) This was a valid and acceptable experiment.

(ii) The plateau of urinary uric acid-C^{14} was

reached by day 3 with a small secondary peak on days 10 to 13 (fig. 7-9).

(iii) As in H.J. and A.J., the relative activity in C2+8 was greater during the first three days after giving glycine-2-C^{14} (fig.7-9).

(iv) 0.119%, 0.0199% and 0.083% of the activity was excreted in 14 days as urinary uric acid, C2+8 and C4+5 respectively (fig.7-10)

(v) The mean ratio ± S.E. of C4+5: C2+8 was 4.32 ± 0.26 whilst the average value for the first 4 days was 3.9 (fig.7-11)

(vi) An average of 70.60% and 17.37% of the total molecule's activity came from C4+5 and C2+8 respectively (fig.7-12).

(4) R.B.

(a) Evaluation of experimental findings.

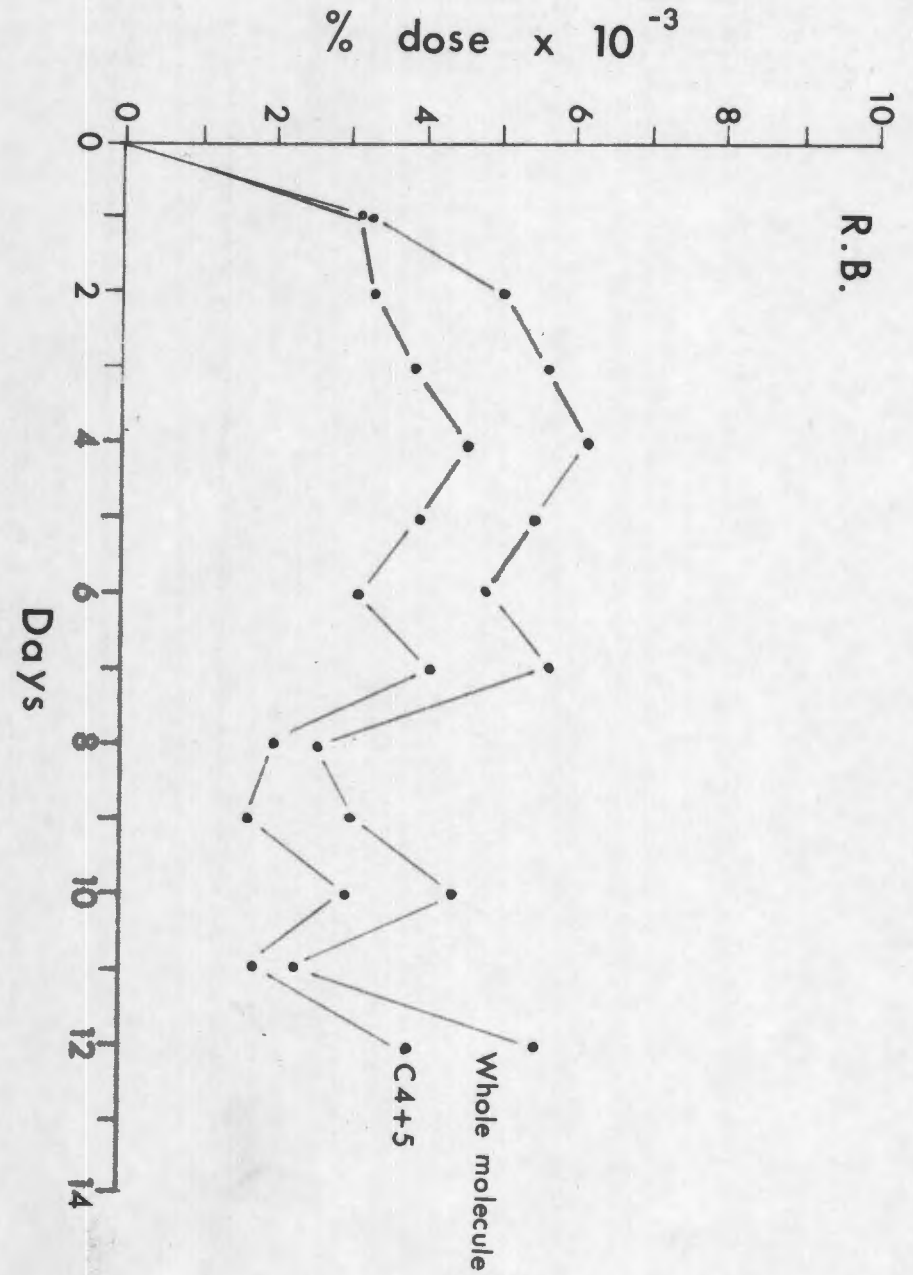
This 35 year old Bantu male had a small granuloma near his left shoulder. He was otherwise fit with good renal and hepatic function. Haemoglobin was 12.8 G/100 ml.

1.8722 x 10^8 dpm glycine-2-C^{14} were injected I.V. and uric acid was isolated from the urine for the ensuing 12 days. 24 hour urinary uric acid excretion varied between 100 and 300 mg/day of which less than 50 mg were isolated, of which most was used for degradation. The

TABLE 7-4.

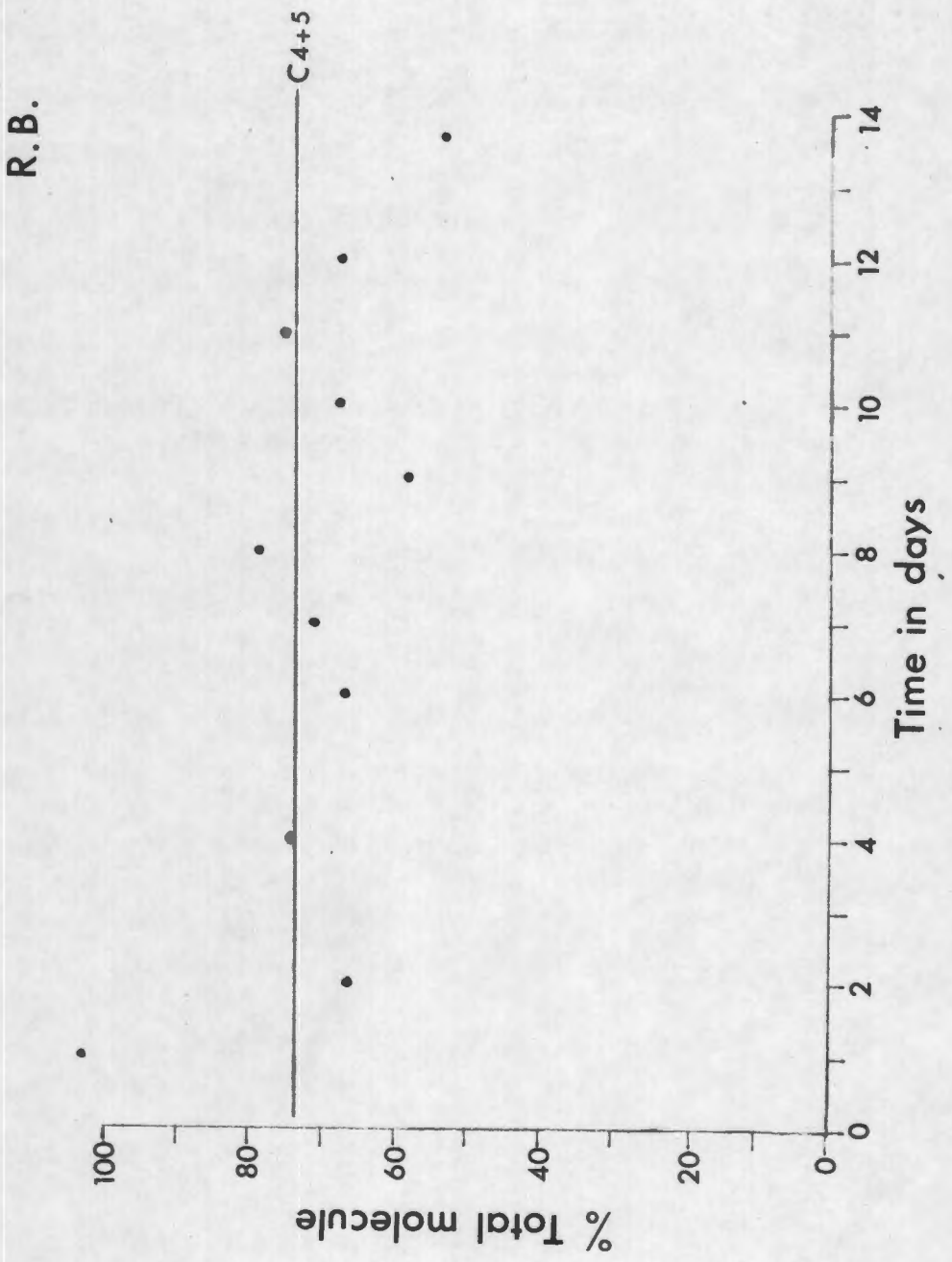
Days	URIC ACID	C 4 + 5
	Daily	Daily
1	3.180	3.288
2	5.068	3.888
3	5.638	3.981
4	6.216	4.622
5	5.490	3.942
6	4.812	3.249
7	5.707	4.100
8	2.627	2.077
9	3.028	1.778
10	4.409	3.012
11	2.377	1.804
12	5.589	3.806

The percentage dose glycine-2-C^{14} excreted in the urine daily as uric acid and C4 + 5 by a normal subject, R.B. Values in table expressed as percentage dose x 10^{-3}.



7-13: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in normal, R.B.

Fig. 7-14: Disposition of radioactivity within uric acid molecule, daily values around mean, in normal, R.B.



uric acid assays were done on specimens which were weeks old and the extremely low 24 hour urinary urate excretion values were thought to be due to technical error. Their cumulative excretion of activity has therefore not been calculated.

This was one of the first experiments performed and many of the results have been discarded as inaccurate. Barium carbonate samples were contaminated from a radioactive point of view and were not acceptable.

In summary, this was a poor experiment giving little information.

(b) Results.

Results are expressed in Table 7-4 and figs. 7-13 and 7-14. The only important information arising from the experiment is that on average, 73.4% of the total molecule's activity resided in C4+5 (fig. 7-14).

B. SYMPTOMATIC PORPHYRIC SUBJECTS.

(1) A.A.

(a) Evaluation of experimental findings.

A.A., a 46 year-old Cape Coloured male, had skin lesions of porphyria for 7 years. He was a chronic alcoholic subject with clinical and biochemical evidence of liver failure. Liver biopsy showed increased iron pigment, hepatocellular damage with regeneration but no fibrosis. He had a normal stool porphyrin content but excreted excessive amounts of uroporphyrin (6392 ug) and coproporphyrin (528 ug) in the urine daily. He was therefore a classical case of symptomatic porphyria.

Renal function was good (creatinine clearance 142 ml/min.) and his haemoglobin 15.4 gm/100 ml. 7 serum uric acid levels ranged between 3.65 and 5.92 mg/100 ml during the course of the experiment. He was placed on a step VIII diabetic diet.

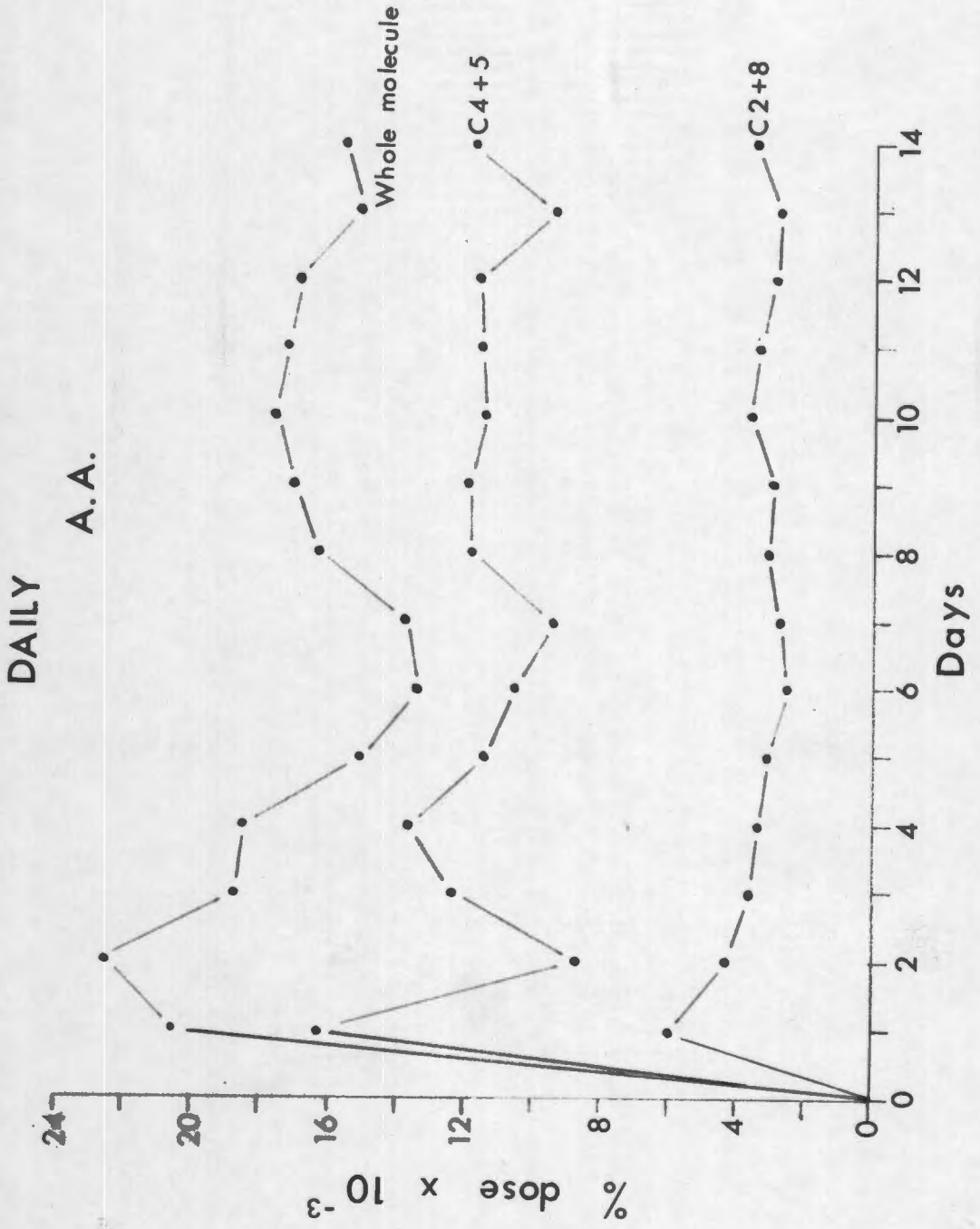
24-hour urinary urate excretion ranged between 471 and 639 mg of which more than 400 mg purified uric acid were usually obtained. Thus adequate amounts of isotopic uric acid were available for degradation and counting and generally more than 50 mg glyoxylic acid semicarbazone and 100 mg barium carbonate were obtained for counting.

The dose of glycine-2-C^{14} was 1.9964×10^8 dpm. From

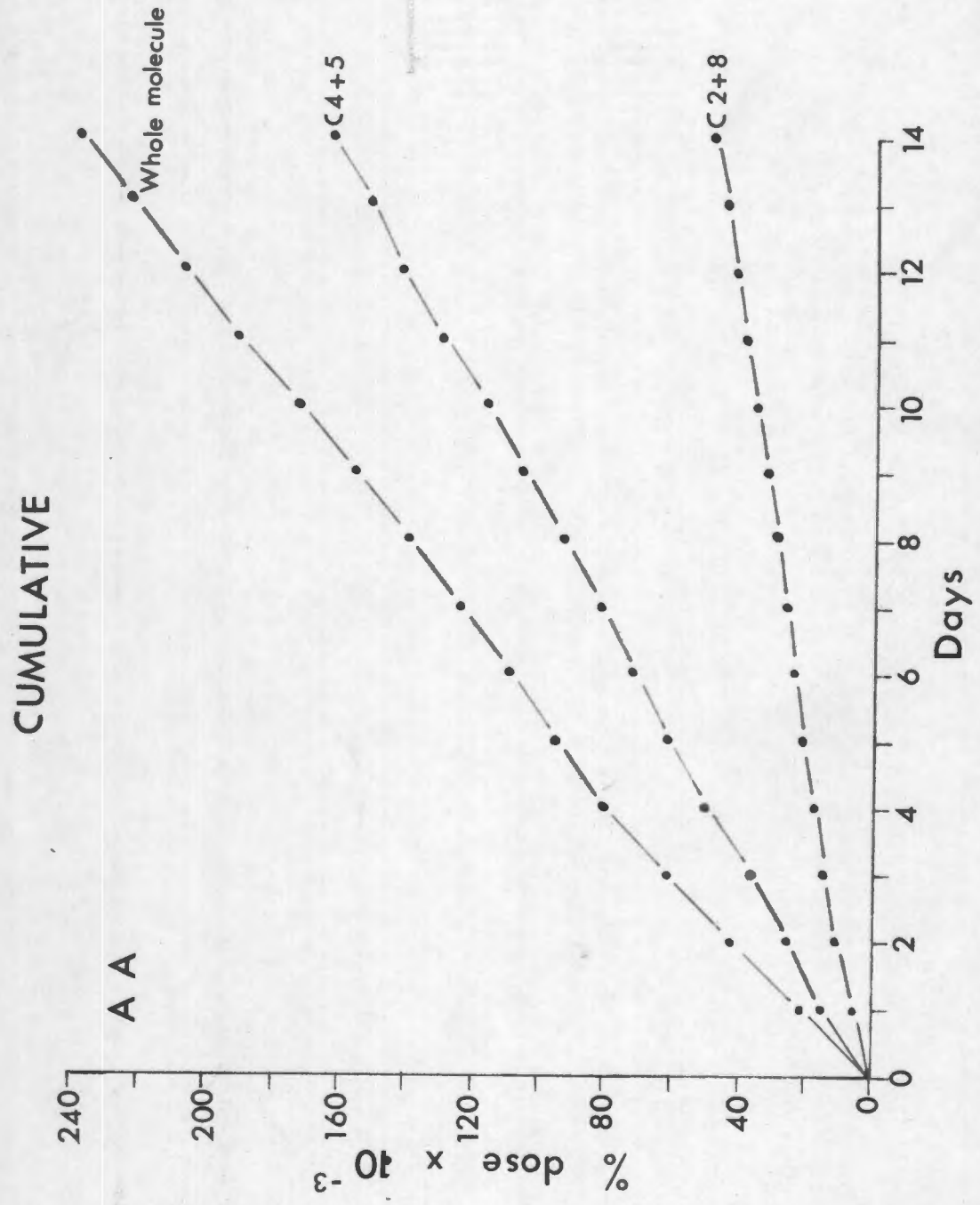
TABLE 7-5.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	cumul.	Daily	Cumul.	Daily	Cumul.
1	20.544	20.544	16.213	16.213	5.966	5.966
2	22.553	43.097	8.552	24.765	4.328	10.294
3	18.732	61.829	12.382	37.147	3.785	14.079
4	18.633	80.462	13.756	50.903	3.515	17.594
5	15.135	95.597	11.466	62.369	3.080	20.674
6	13.447	109.044	10.488	72.857	2.515	23.189
7	13.858	122.902	9.418	82.275	2.708	25.897
8	16.470	139.372	11.965	94.240	3.093	28.990
9	17.090	156.462	12.059	106.299	2.985	31.975
10	17.685	174.147	11.587	117.886	3.846	35.821
11	17.280	191.427	11.819	129.705	3.497	39.318
12	16.907	208.334	11.855	141.560	3.002	42.320
13	15.136	223.470	9.392	150.952	2.912	45.232
14	15.739	239.209	12.928	163.880	3.547	48.779

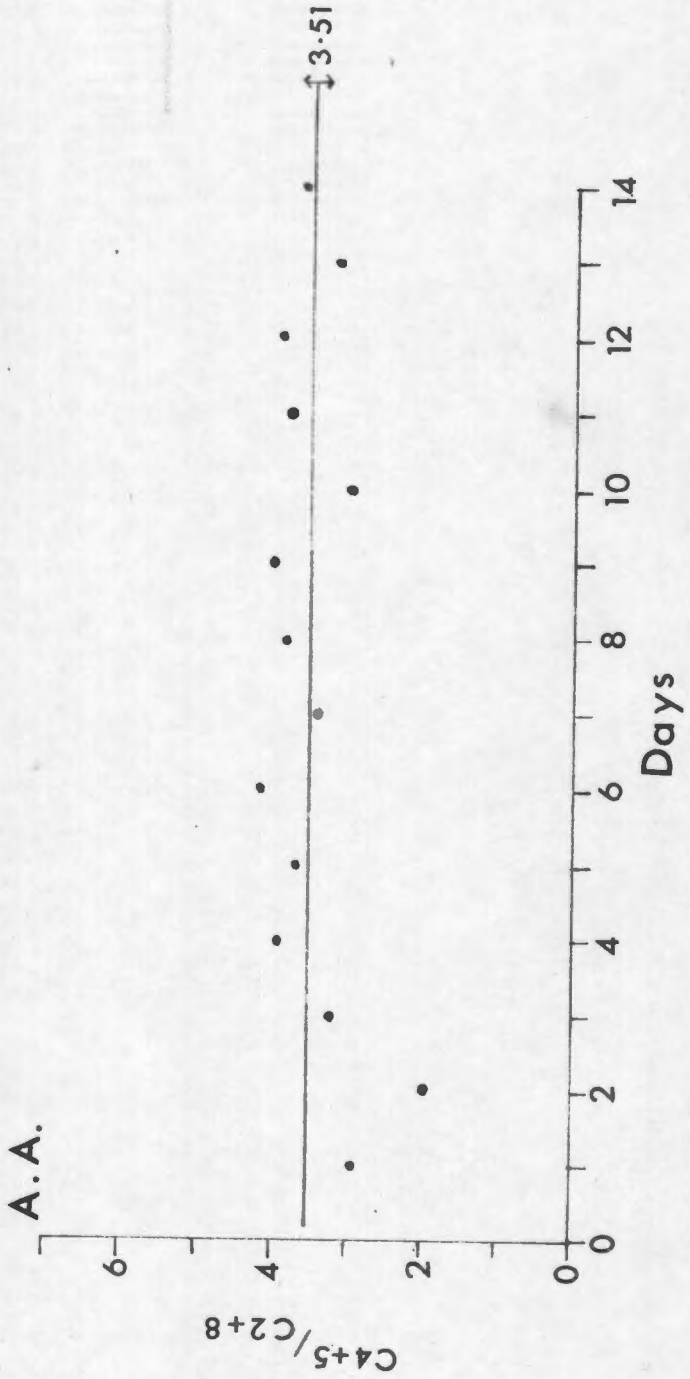
The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4+5 and C2 + 8 by a symptomatic porphyric subject, A.A. Values in the table expressed as percentage dose x 10^{-3}.



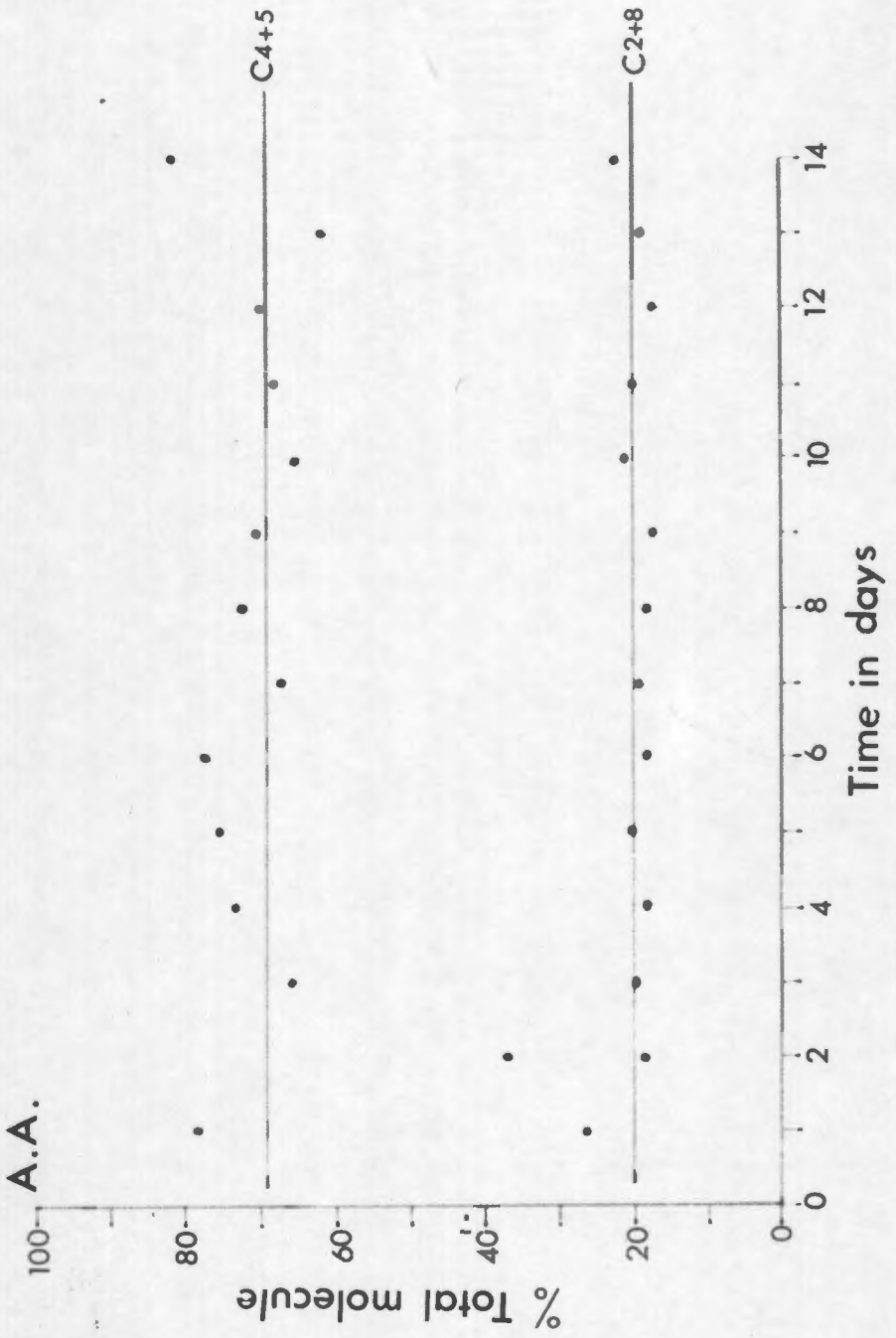
7-15: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in symptomatic porphyrie, A.A.



7-16: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in symptomatic porphyric, A.A.



7-17: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (⇕) in symptomatic porphyric, A.A.



7-18: Disposition of radioactivity within uric acid molecule, daily values around mean, in symptomatic porphyric, A.A.

all points of view, this was an excellent experiment.

(b) Results.

These are summarized in Table 7-5, figs. 7-15, -16, -17, -18. Note the distinct early peak of activity on day 2 (fig. 7-15) dropping to a plateau after 4 days. Note how the C4+5: C2+8 ratios hover closely around the mean of 3.51± S.E. 0.15.

(c) Summary.

(i) This was an accurate and valid experiment.

(ii) Early peak of urinary uric acid-C^{14} activity noted on day 2 falling to a plateau of activity thereafter (fig.7-15).

(iii) 0.239%, 0.164% and 0.049% of the dose were excreted over 14 days as uric acid-C^{14}, C4+5 and C2+8 respectively. (fig. 7-16).

(iv) The C4+5: C2+8 ratios varied closely around the mean ± S.E. value of 3.51± 0.15. Theaverage for the first four days was 3.02, a value lower than the mean. (fig.7-17).

(v) 69.28% and 20.11% of the molecule's activity resided in C4+5 and C2+8 respectively (fig.7-18).

(2) L.M.

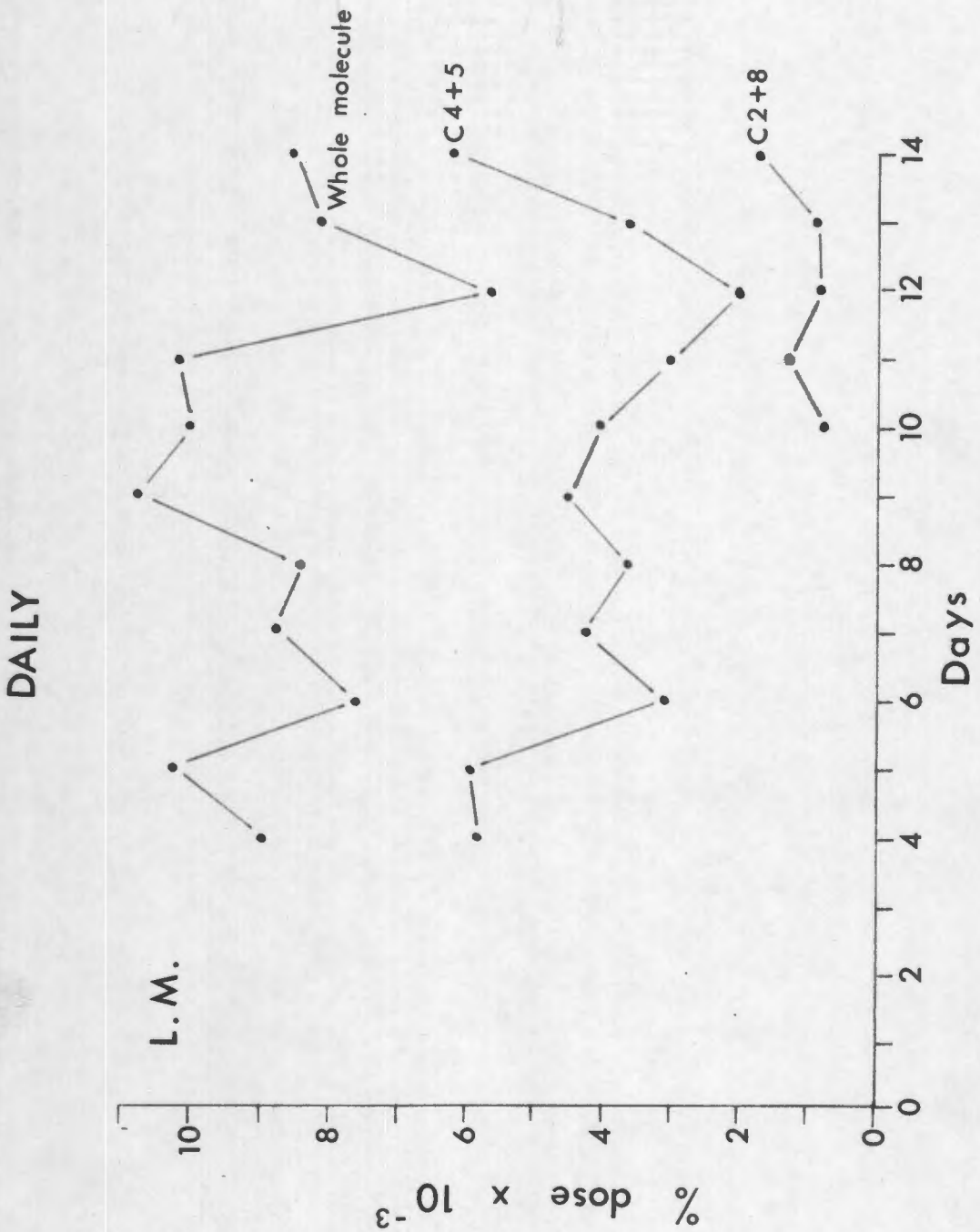
(a) Evaluation of experimental findings.

L.M. was a classical symptomatic porphyric Coloured male aged 57. As expected, his liver function was deranged

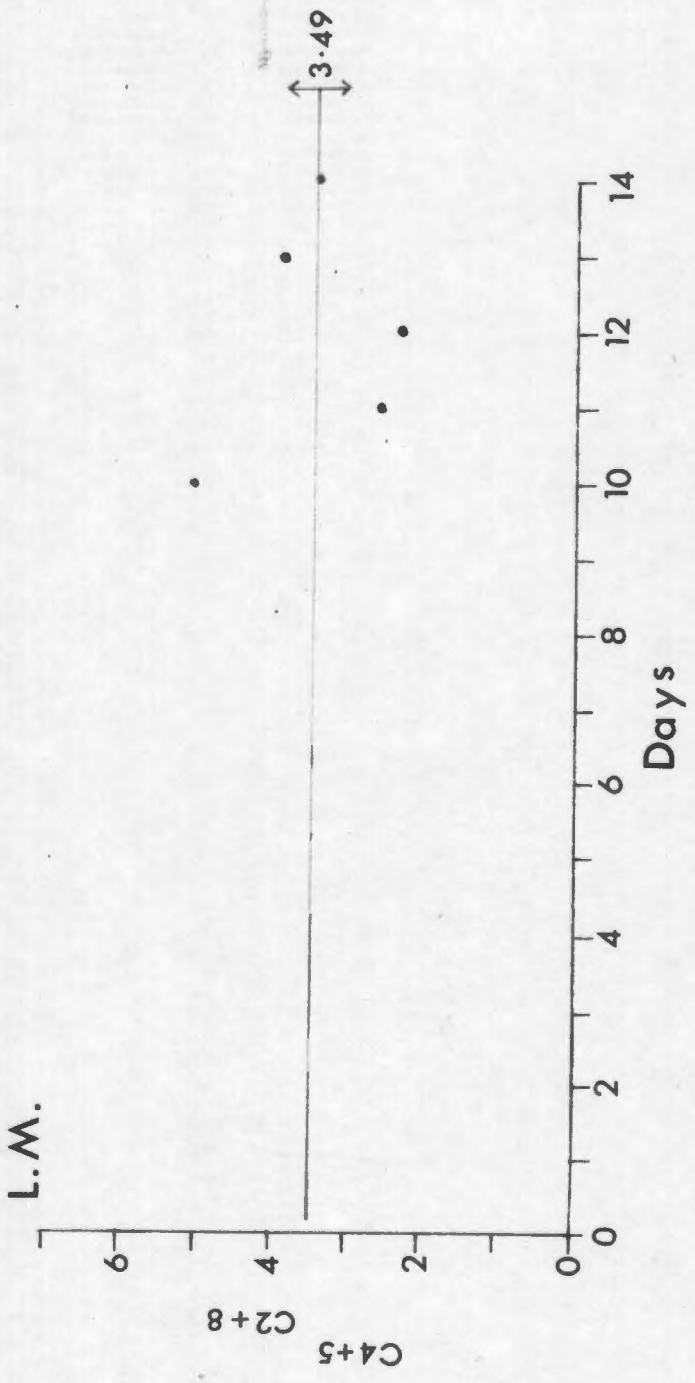
TABLE 7-6.

Days	URIC ACID	C4 + 5	C2 + 8
	Daily	Daily	Daily
1	—	—	—
2	—	—	—
3	—	—	—
4	6.834	5.990	—
5	10.338	5.906	—
6	7.532	3.128	—
7	8.857	4.255	—
8	8.477	3.604	—
9	10.839	4.524	—
10	10.171	4.161	0.804
11	10.259	3.054	1.337
12	5.601	2.094	0.883
13	8.276	3.743	0.955
14	8.684	6.387	1.860

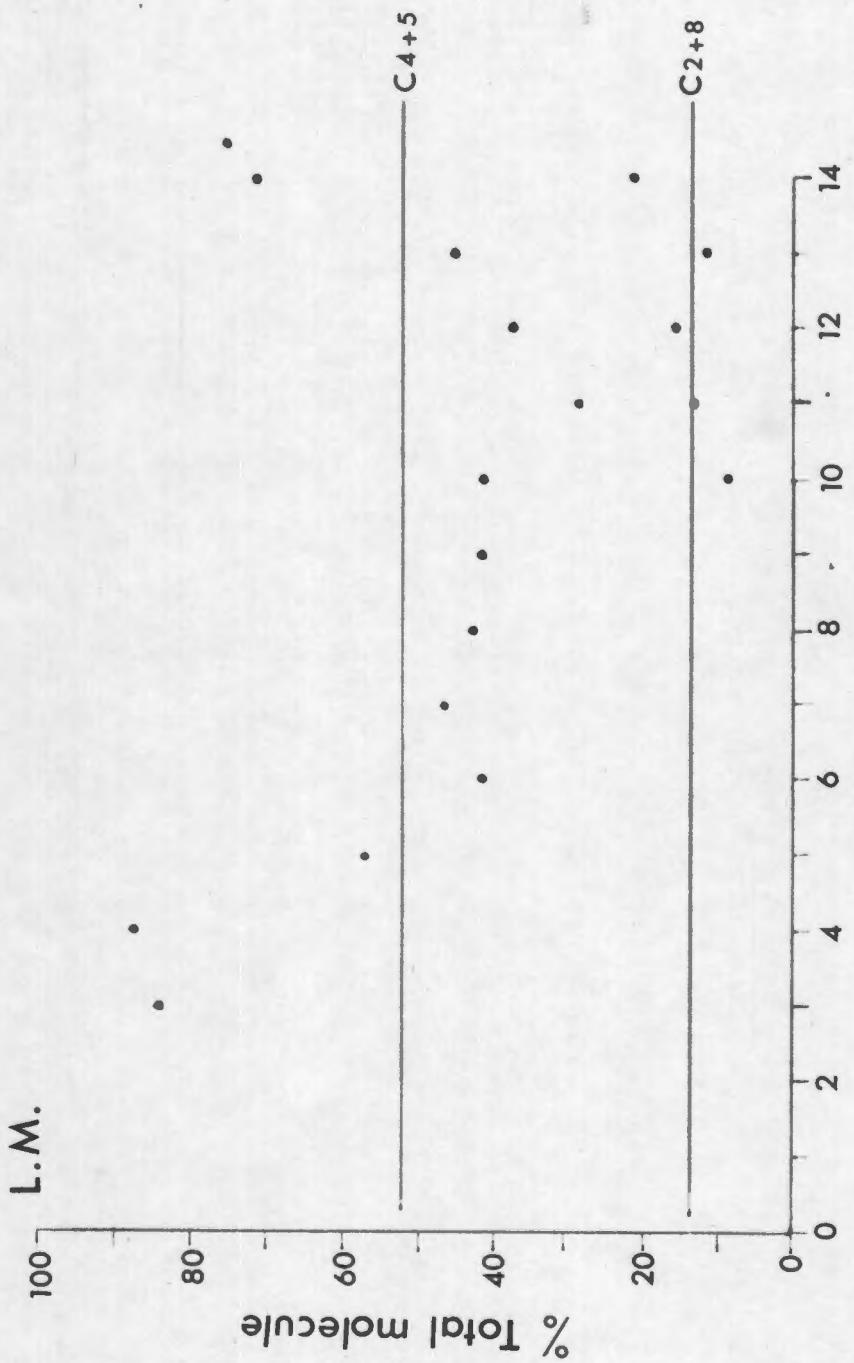
The percentage dose glycine-2-C^{14} excreted in the urine daily as uric acid, C4 + 5 and C2 + 8 by a symptomatic porphyric subject, L.M. Values in the table are expressed as percentage dose x 10^{-3}.



7-19: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in symptomatic porphyric, L.M.



7-20: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in symptomatic porphyric, L.M.



7-21: Disposition of radioactivity within uric acid molecule, daily values around mean, in symptomatic porphyric, L.M.

but his renal function (creatinine clearance 112) was normal. Haemoglobin was 14.5 gm/100 ml.

1.8441×10^8 dpm glycine-2-C^{14} were injected I.V. at the beginning of the experiment. Blood uric acid levels ranged between 3.38 and 5.10 mg/100 ml and the 24-hour urinary urate levels were between 250 mg and 350 mg/day. About 100 to 200 mg of uric acid were isolated daily except for days 1 and 2 where, owing to breakage of apparatus, specimens were lost.

Amount of uric acid isolated from the urine ranged between 94.3 and 251 mg allowing adequate amounts for counting and degradation with a small dilution factor of less than 2. Usually, more than 100 mg barium carbonate were obtained for counting whilst most of the glyoxylic acid semicarbazone samples were between 20 and 35 mg in weight.

Unfortunately, a good experiment was spoilt by contamination of a whole batch of barium carbonate samples from days 3 to 9. Thus C2+8 values were only obtained from days 10 to 14.

(b) Results.

Experimental data are depicted in Table 7-6, figs. 7-19, 7-20 and 7-21.

(c) Summary.

(i) A moderately good experiment marred by radioactive contamination of a batch of 7 barium carbonate

samples.

(ii) 52.12% of the molecule's activity (average of 12 values) and 13.9% (average of 5 values) resided in C4+5 and C2+8 respectively (fig. 7-21).

(iii) Of the 5 fully acceptable degradation experiments, C4+5: C2+8 ratios were 3.49 ± S.E. 0.45. (fig.7-20).

(3) B.P.

(a) Evaluation of experimental findings.

B.P.,a symptomatic porphyric coloured male, received 2.0344×10^8 dpm glycine-2-C^{14} I.V. on day 1 of the experiment. Renal function was good and blood uric acid levels ranged between 4.07 and 4.45 mg/100 ml (6 values). 24-hour urate excretion lay between 449 and 795 mg, usually about 600 mg/day. Of this, more than 400 mg uric acid were usually isolated, adequate for counting and degradation. With 250 to 350 mg uric acid available for degradation, Large amounts of barium carbonate (> 200 mg) and of glyoxylic acid semicarbazone (> 100 mg in 3 samples) were obtained for counting.

From all points of view, this was a highly satisfactory experiment.

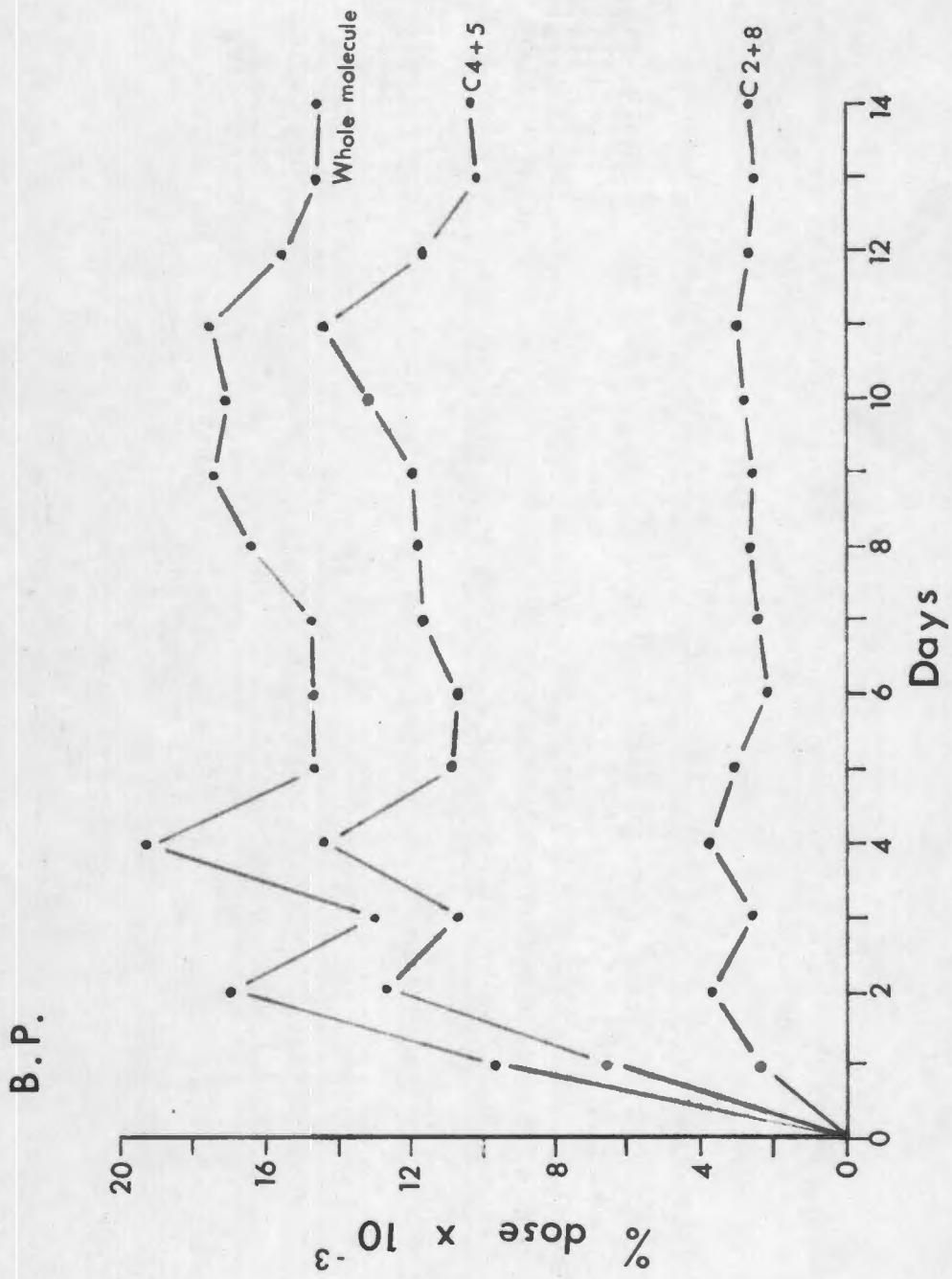
(b) Results.

These are summarized in Table 7-7, figs. 7-22,23,24 and -25. Note the early peak of activity on day 2 (fig. 7-22) followed by a plateau. The C4+5: C2+8 ratios are close to the mean ± S.E. of 4.17 ± 0.17 (fig. 7-24). The average

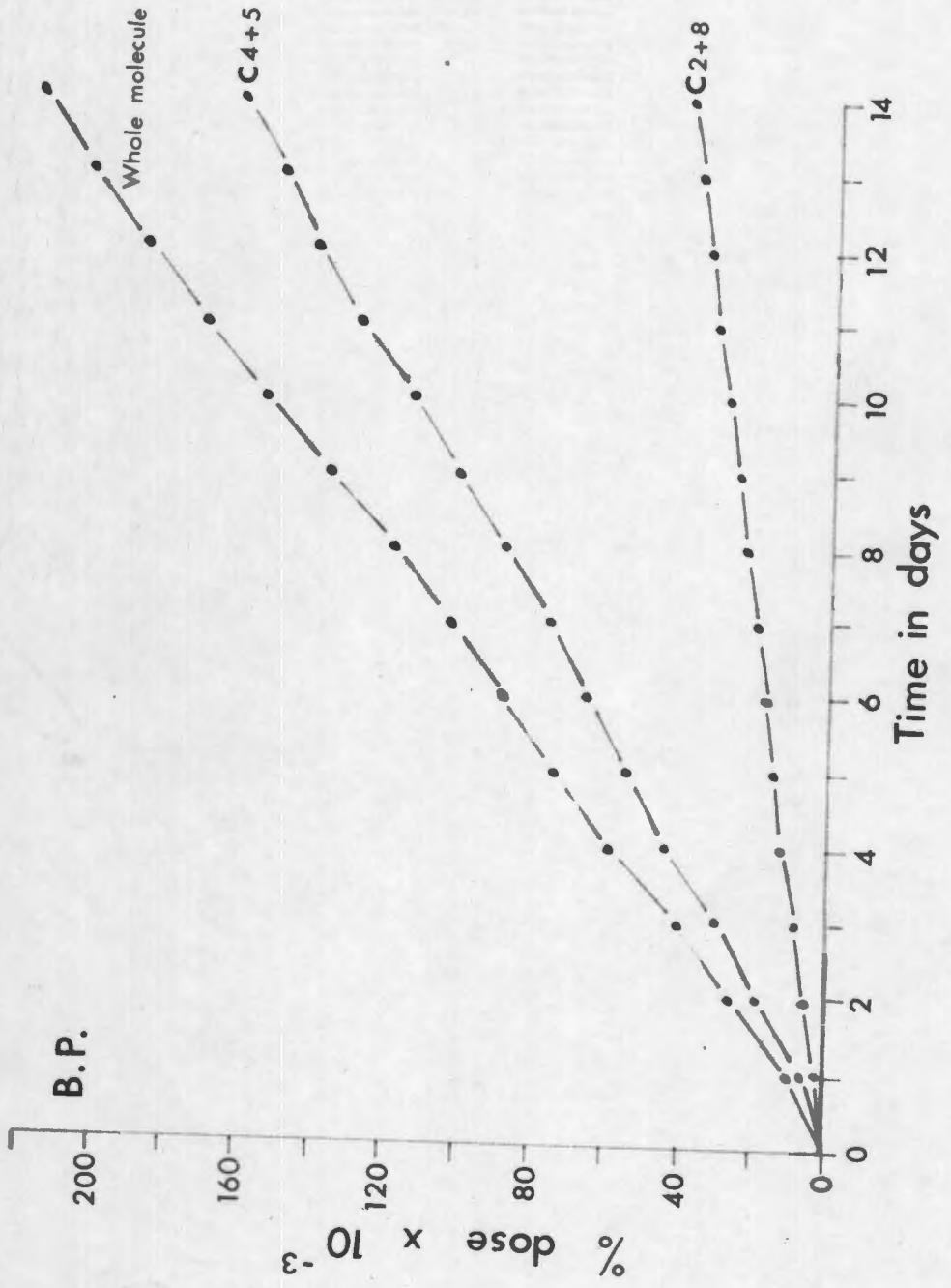
TABLE 7-7.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	9.717	9.717	6.620	6.620	2.397	2.397
2	16.938	26.655	12.716	19.336	3.661	6.058
3	13.010	39.665	10.555	29.890	2.538	8.596
4	19.382	59.047	14.361	44.251	3.804	12.400
5	14.606	73.654	10.782	55.033	3.003	15.402
6	14.624	88.278	10.561	65.594	2.100	17.502
7	14.717	102.995	11.798	77.393	2.438	19.940
8	16.325	119.320	11.891	89.284	2.561	22.502
9	17.429	136.749	12.034	101.318	2.632	25.134
10	17.044	153.794	13.169	114.487	2.886	28.020
11	17.468	171.262	14.355	128.843	3.017	31.037
12	15.571	186.832	11.666	140.509	2.701	33.738
13	14.505	201.337	10.090	150.600	2.496	36.235
14	14.629	215.967	10.381	160.980	2.699	38.934

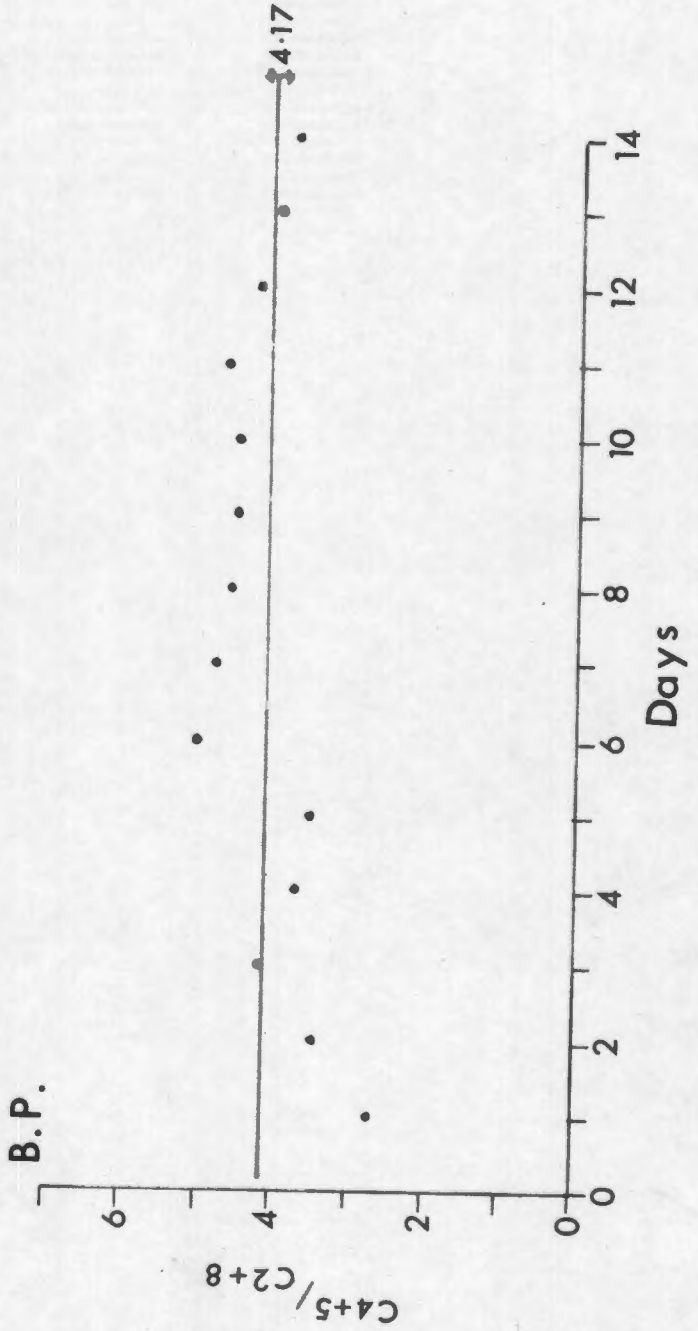
The percentage dose glycine-2-C^{14} excreted in the urine daily as uric acid, C4 +5 and C2 + 8 by a symptomatic porphyric subject, B.P. Values in the table expressed as percentage dose x 10^{-3}.



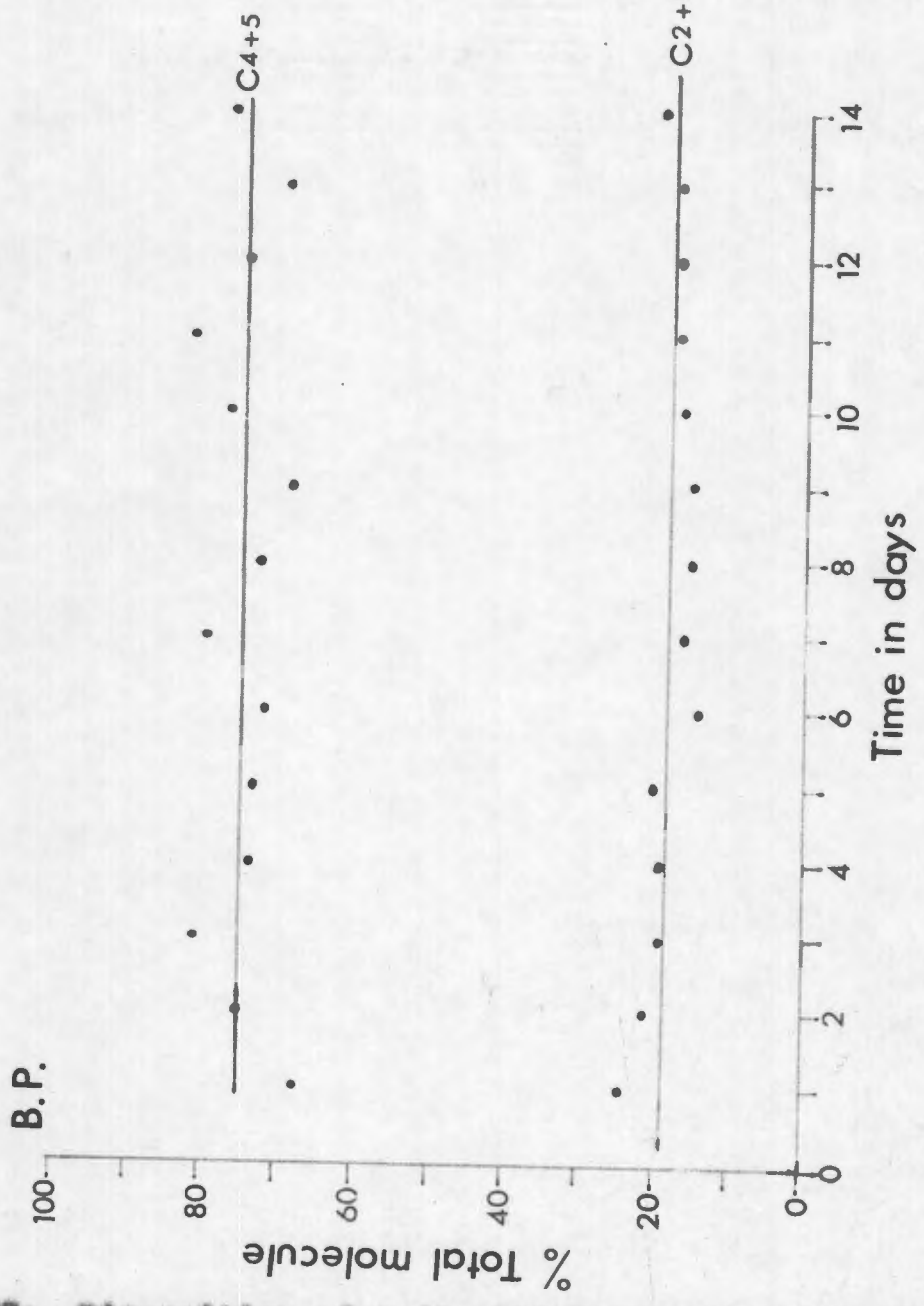
7-22: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+ 8 in symptomatic porphyric, B.P.



7-23: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in symptomatic porphyric, B.P.



7-24: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in symptomatic porphyric, B.P.



7-25: Disposition of radioactivity within uric acid molecule, daily values around mean, in symptomatic porphyric, B.P.

ratio for the first 4 days is lower than the mean, viz. 3.5.

(c) Summary.

(i) An accurate experiment.

(ii) Early peak noted on day 2 followed by a plateau of urinary uric acid-C^{14} activity (fig.7-22).

(iii) 0.216%, 0.161% and 0.0389% of the dose was excreted as urinary uric acid-C^{14}, C4+5 and C2+8 respectively over the 14 days of the experiment (fig. 7-23).

(iv) C4+5: C2+8 ratios averaged 4.17 ± S.E. 0.17 over 14 days whilst the mean of the first 4 days was 3.5 (fig. 7-24).

(v) An average of 74.8% of the molecule's activity resided in C4+5 and 18.31% in C2+8 over the duration of study. (fig.7-25).

(4) W.B.

(a) Evaluation of experimental findings.

W.B., a Coloured male aged 36 was a known alcoholic with increased pigmentation and porphyric skin lesions. He was shown to have the typical biochemical findings of symptomatic porphyria with evidence of chronic liver disease. Renal function was normal (creatinine clearance 124.1 ml/min.) and his haemoglobin 17.1 gm/100 ml.

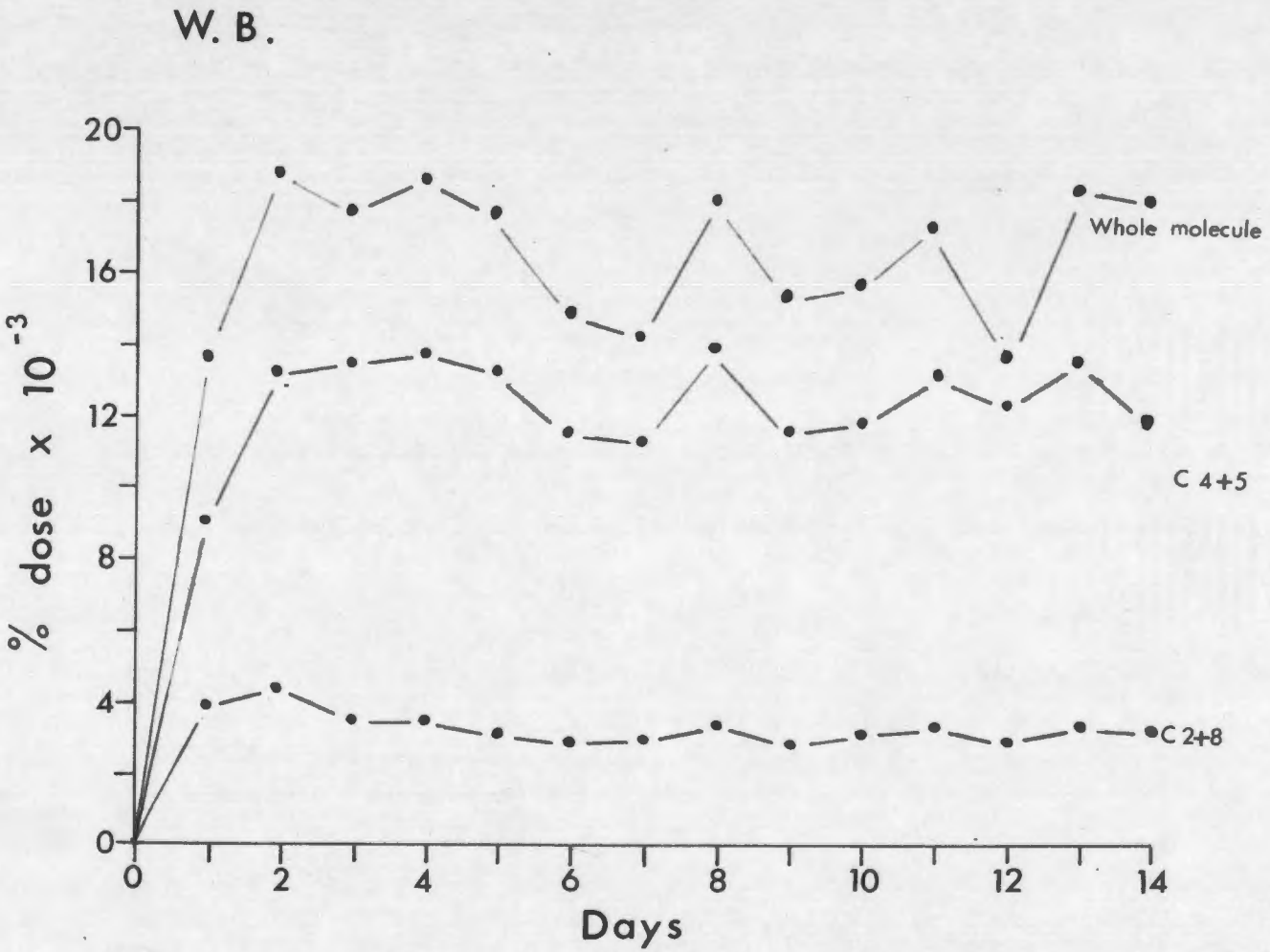
1.9128×10^8 dpm glycine-2-C^{14} were injected I.V. on the first day of the experiment. Blood uric acid levels lay between 2.70 and 5.03 mg/100 ml (5 values) with 24-hour

TABLE 7-8.

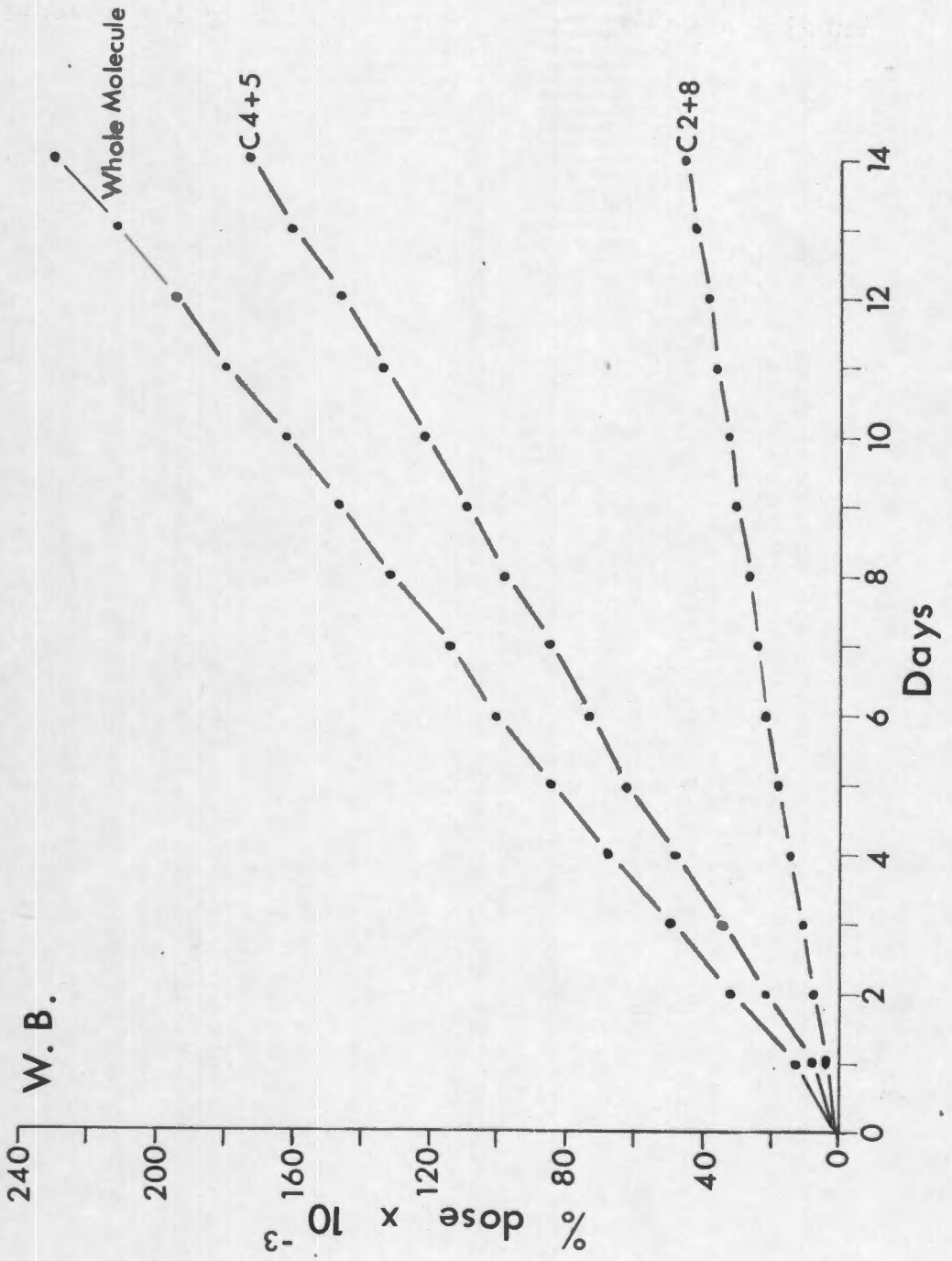
Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	cumul.	Daily	Cumul.
1	13.749	13.749	9.010	9.010	3.869	3.869
2	18.437	32.186	13.226	22.236	4.321	8.190
3	17.869	50.055	13.450	35.686	3.475	11.665
4	18.320	68.375	13.624	49.310	3.535	15.200
5	16.840	85.215	13.218	62.528	3.162	18.362
6	14.855	100.070	11.569	74.097	2.862	21.224
7	14.112	114.182	11.318	85.415	2.984	24.208
8	18.098	132.280	13.926	99.341	3.368	27.576
9	15.331	147.611	11.509	110.850	2.756	30.332
10	15.619	163.230	11.767	122.617	3.138	33.470
11	17.390	180.620	13.034	135.651	3.239	36.709
12	13.678	194.298	12.222	147.873	2.881	39.590
13	18.362	212.660	13.481	161.354	3.251	42.841
14	17.991	230.651	11.904	173.258	3.192	46.033

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 + 8 by a symptomatic porphyric-subject, W.B. Values in the table are expressed as percentage dose x 10-3.

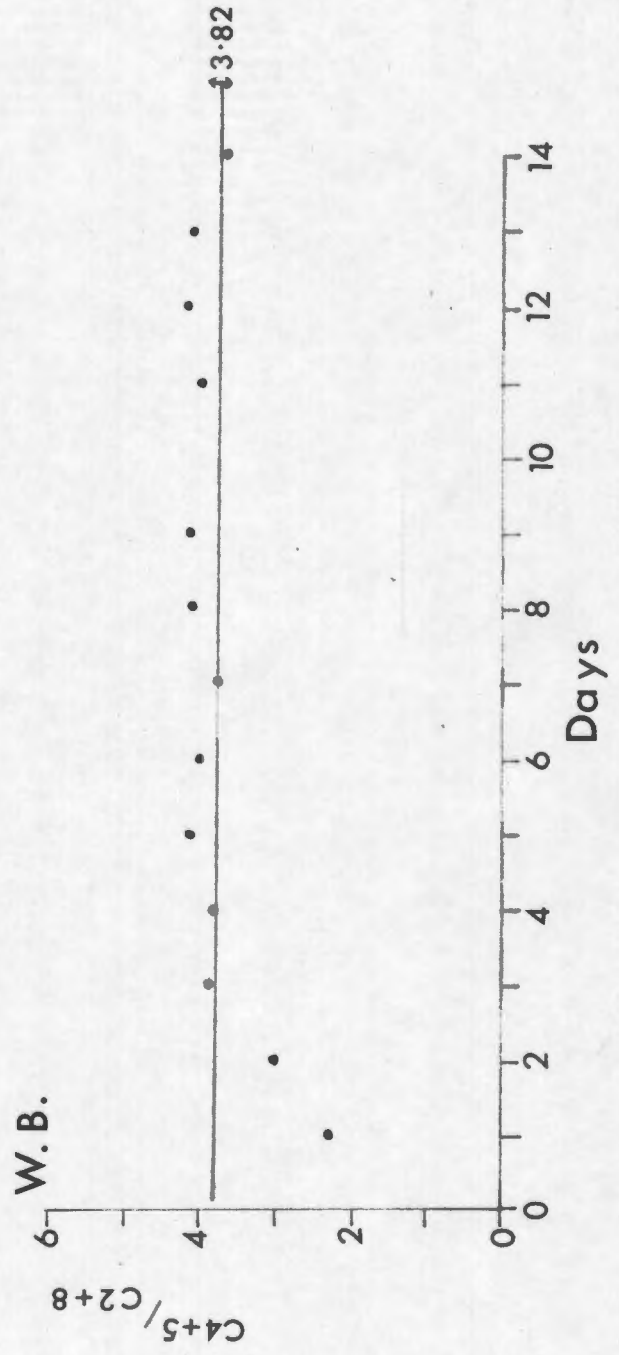
DAILY



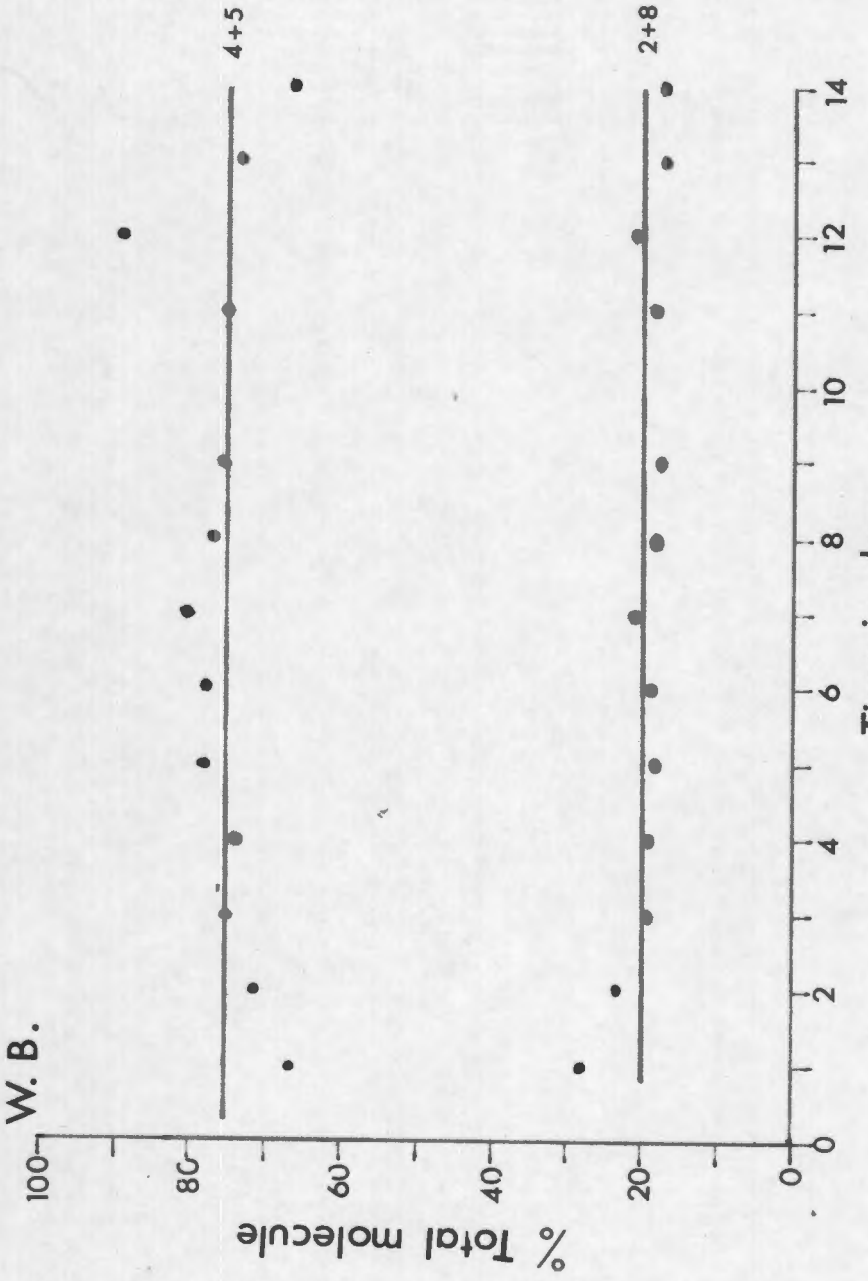
7-26: The daily incorporation of glycine-2-^{14}C into urinary uro acid, C4+5 and C2+8 in symptomatic porphyric, W.B.



7-27: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in symptomatic porphyric, W.B.



7-28: Daily C4+5: C2+8 ratio's scattered around mean ± S.E. (↕) in symptomatic porphyric, W.B.



7-29: Disposition of radioactivity within uric acid molecule, daily values around mean, in symptomatic porphyric, W.B.

urinary urate values of 491 to 688 mg/day. Usually 350 to 500 mg of uric acid were isolated, enough for counting and degradation.

Except on day 10 where a conical flask broke during the urinary urate isolation procedure, large amounts of uric acid, barium carbonate and glyoxylic acid semicarbazone were available for counting. One batch of the latter was contaminated with lead sulphate and weights for counting were erroneously high. However, adequate amounts of uric acid were available to repeat degradation procedure with dilution factors ranging from 1 to 3.89.

Thus experiment was completely acceptable.

(b) Results.

These are expressed in Table 7-8 and figs. 7-26, -27 and -28 and -29.

(c) Summary.

(i) An acceptable experiment.

(ii) Peak in urinary uric acid-C^{14} activity reached on day 2 followed by a plateau of activity (fig.7-26).

(iii) 0.231%, 0.173% and 0.046% of the administered dose was excreted as urinary uric acid-C^{14}, C4+5 and C2+8 over the 14 days of study (fig. 7-27).

(iv) The mean ± S.E. of C4+5: C2+8 ratios was 3.82 ± 0.15 whilst the average for the first 4 days was 3.3 (fig. 7-28).

(v) Over the experiment, C4+5 comprised 75.48% of the molecule's activity whilst 20.09% resided in C2+8. As in earlier experiments, 28.14% and 23.44% of the activity values considerably more than the mean, came from C2 and 8 on days 1 and 2 of the experiment (fig.7-29).

C. SOUTH AFRICAN GENETIC PORPHYRIC SUBJECTS.

(1) A. v. R.

(a) Evaluation of experimental findings.

A. v.R., a White female aged 19, only noticed skin lesions a few months before her admission. There was a possibility of her father having the same trouble. She had never experienced an acute attack and was admitted to the ward quite well, for investigation. There was no evidence of renal or liver disease. Her stool and urine porphyrin excretion pattern confirmed the diagnosis of South African genetic porphyria (S.A.G.P.) and as urinary ALA and PBG levels were nearly always within normal limits, she was in remission.

1.9756×10^8 dpm were injected on day 1 of the experiment. Over the ensuing 14 days, blood urate varied between 3.36 and 4.14 mg/100 ml (6 values) whilst 24 hour urinary urate excretion ranged between 363 mg to 665 mg. Between 2 00 and 400 mg uric acid were isolated daily allowing adequate amounts to be counted and degraded. As large amounts (250 mg to 350 mg) were degraded, "cold" carrier uric acid was added only to 5 specimens, the dilution factor lying between 1 and 2.

The yield of barium carbonate (more than 200 mg) and of glyoxylic acid semicarbazone (70 to 125 mg) was good and radioactivity in the counted samples was high.

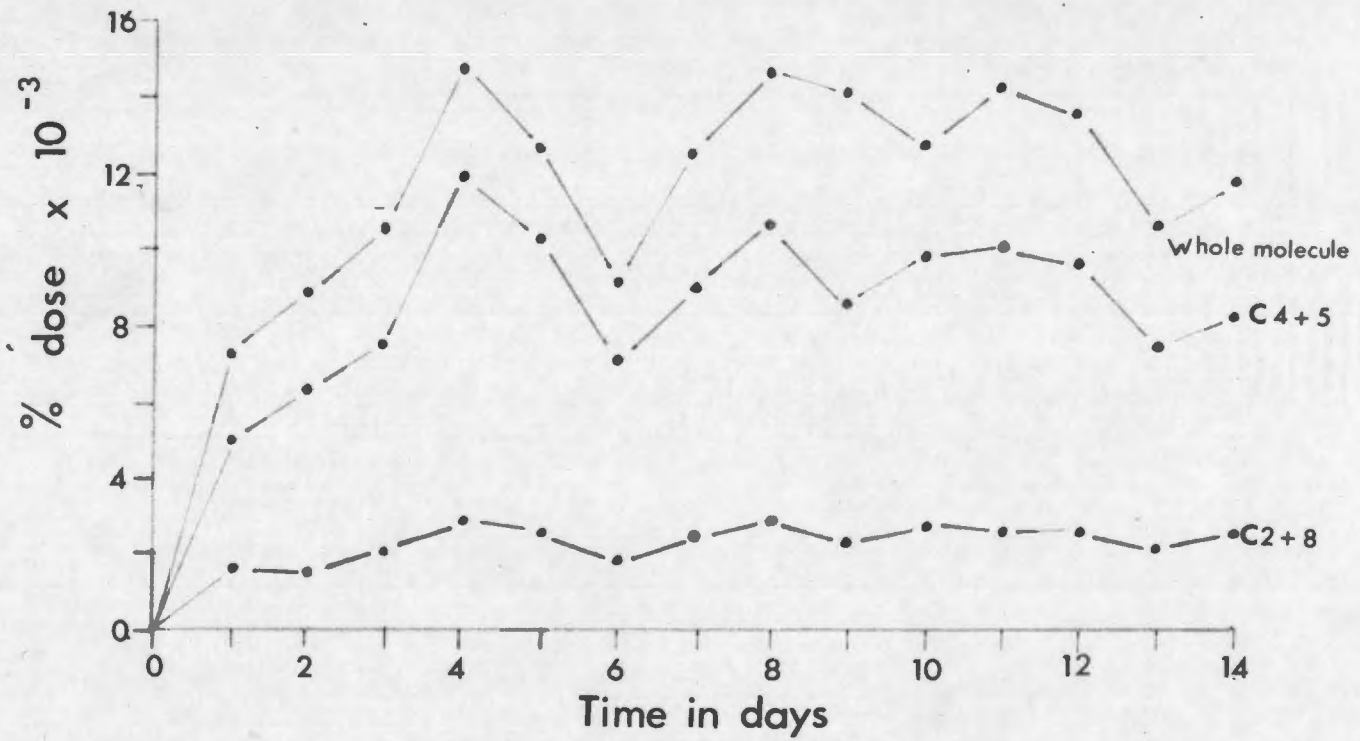
TABLE 7-9.

Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	7.282	7.282	5.140	5.140	1.710	1.710
2	8.944	16.227	6.390	11.530	1.607	3.317
3	10.624	26.851	7.588	19.118	2.068	5.385
4	14.908	41.759	12.063	31.181	2.896	8.281
5	12.776	54.536	10.339	41.520	2.673	10.954
6	9.112	63.648	7.124	48.645	1.888	12.842
7	12.633	76.281	8.983	57.628	2.453	15.295
8	14.241	90.521	10.690	68.318	2.868	18.163
9	12.853	103.374	8.615	76.932	2.393	20.556
10	14.767	118.141	9.869	86.802	2.776	23.334
11	14.226	132.367	10.171	96.972	2.701	26.035
12	13.770	146.137	9.781	106.753	2.656	28.690
13	10.640	156.778	7.491	114.245	2.173	30.864
14	11.990	166.768	8.322	122.567	2.609	33.473

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 + 8 by a South African genetic porphyric, A.V.R., studied during remission. Values in the table are expressed as percentage dose x 10^{-3}.

DAILY

A.v.R.



7-10: The daily incorporation of glycine-2-^{14}C into urinary uric acid, C4+5 and C2+8 in S.v.v. genetic porphyria in remission, A.v.R.

7-31: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in remission, A.V.R.

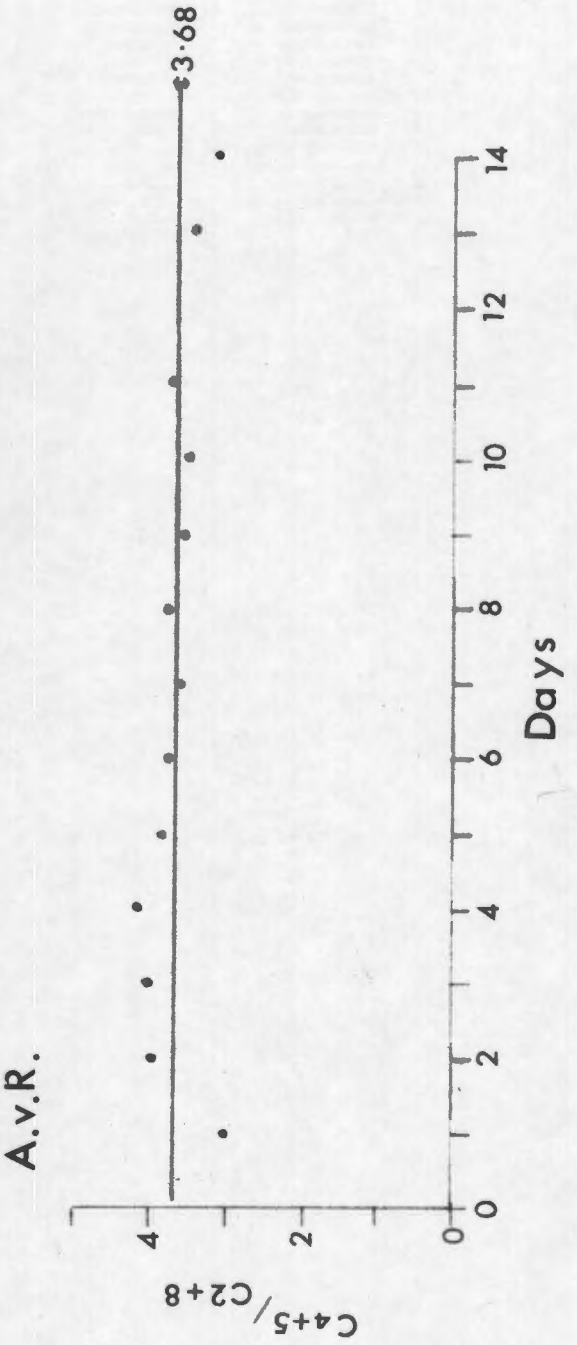
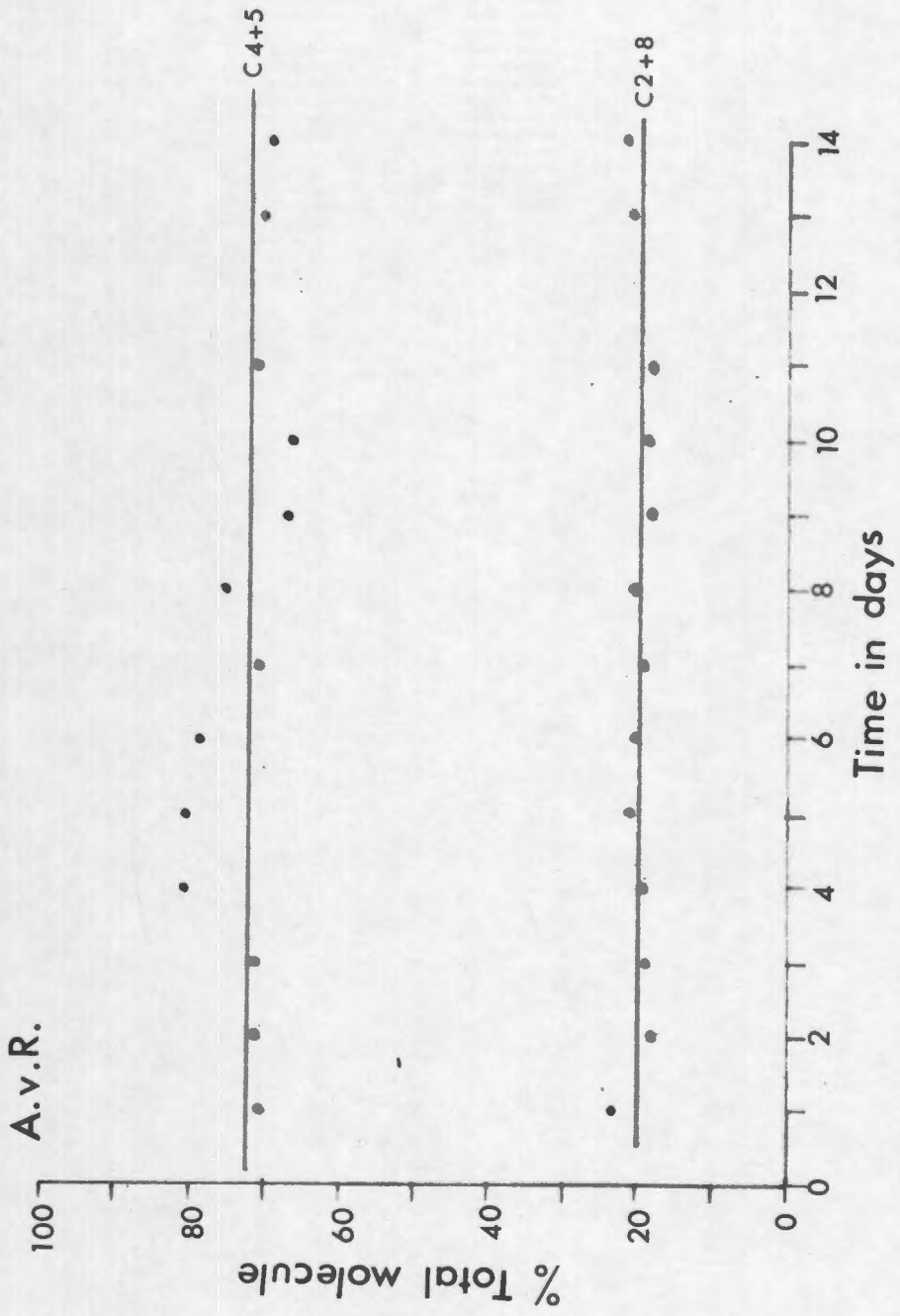


Fig. 7-32: Daily C4+5: C2+8 ratio's scattered around mean ± S.D. (‡) in S.A. genetic porphyric in remission, A.v.R.



7-33: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in remission, A.v.R.

There were no technical hitches and this was an extremely accurate experiment.

(b) Results.

See Table 7-9 and figs. 7-30, -31, -32 and -33 for results. Note how the peak activity is reached in urinary uric acid-C^{14} on the 4th day following glycine administration (fig. 7-30). Note also how there is no early hump in C2+8 activity (fig. 7-29, -32) and C4+5: C2+8 ratios vary closely around the mean ± S.E. of 3.68 ± 0.08 (fig. 7-31). In contrast to earlier experiments, the average ratio for the first 4 days is 3.8, a value higher than the mean for the experiment.

(c) Summary.

(i) An accurate experiment.

(ii) Peak in excreted urinary uric acid-C^{14} activity reached on day 4 followed by a plateau as in normal controls. In contrast to earlier experiments, there was no early hump in C2+8 activity (fig.7-30).

(iii) 0.167%, 0.123% and 0.033% of the administered dose of glycine-2-C^{14} were excreted as urinary uric acid, C4+5 and C2+8 respectively (fig.7-31).

(iv) The mean ± S.E. C4+5: C2+8 ratio is 3.68 ± 0.08. The average ratio for the first 4 days is, in contrast to previous studies, higher than the mean, viz. 3.8 (fig. 7-32).

(v) On average, 72.67% of the molecule's

activity resided in C4+5 whilst C2+8 comprised 20.1%. On day 1, 23.49% of the molecule's activity lay in carbons 2 and 8 (fig. 7-33).

(2) C.V.H.

(a) Evaluation of experimental results.

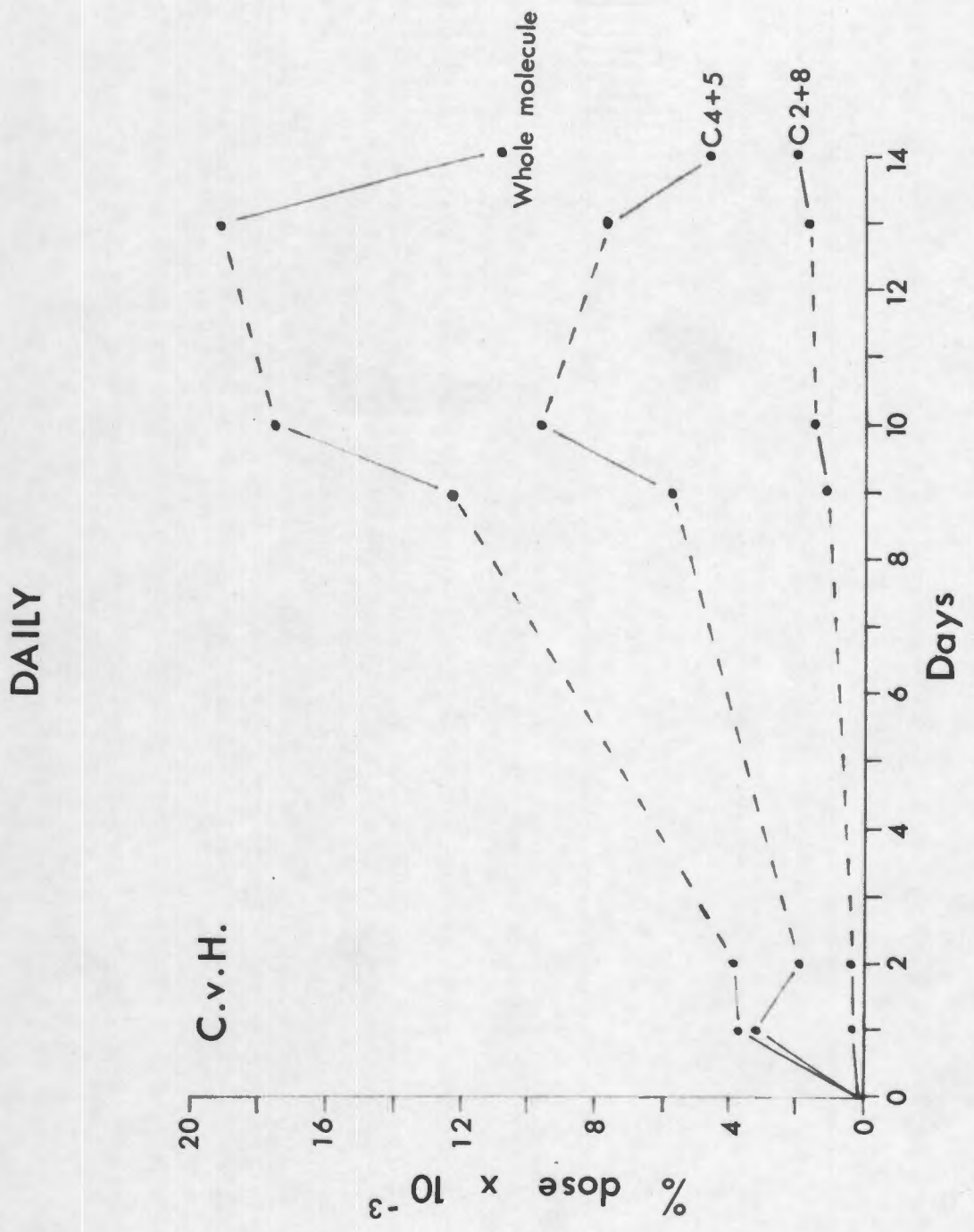
C. V. H. was a known SAGP who also suffered from chronic pyelonephritis, hypertension, uraemia and hyperuricaemia. She was admitted to the general medical ward confused with a blood urea of 179 mg/100 ml, blood pressure of 170/110 and weakness in the limbs, mainly proximally. Her urine was heavily infected. She was catherised, thus enabling accurate 24 hour urine volumes to be collected. On admission, her stool and urine porphyrin levels were grossly elevated and she excreted 51.47 mg PBG and 21.03 mg ALA per 24 hours in her urine confirming an acute attack of porphyria. These levels fell to normal over the following 8 days at which time she was clinically much improved. Her improvement also coincided with a marked fall in her blood urea. Glycine-2-C^{14} was injected on admission at the height of her illness.

Clinically there are a number of important points to bear in mind before evaluating results. In the presence of chronic renal disease with uraemia, as urate will be largely eliminated via extrarenal channels, information regarding the percentage dose excreted as uric acid in the urine is of little value. In this type of situation,

TABLE 7-10.

Days	URIC ACID	C4 + 5	C2 + 8
	Daily	Daily	Daily
1	3.816	3.383	0.292
2	3.974	1.976	0.338
3	—	—	—
4	—	—	—
5	—	—	—
6	—	—	—
7	—	—	—
8	—	—	—
9	12.171	5.722	1.019
10	17.547	9.597	1.478
11	—	—	—
12	—	—	—
13	19.161	7.647	1.592
14	10.680	4.434	1.878

The percentage dose glycine-2-C^{14} excreted in the urine daily as uric acid, C4 + 5, and C2 + 8 by a South African genetic porphyric, C.v.H., studied during an acute attack. Values in the table are expressed as percentage dose x 10^{-3}.



7-34: The daily incorporation of glycine-2-c^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack. C.v.H.

7-35: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (↕) in S.A. genetic porphyric in acute attack, C.v.H.

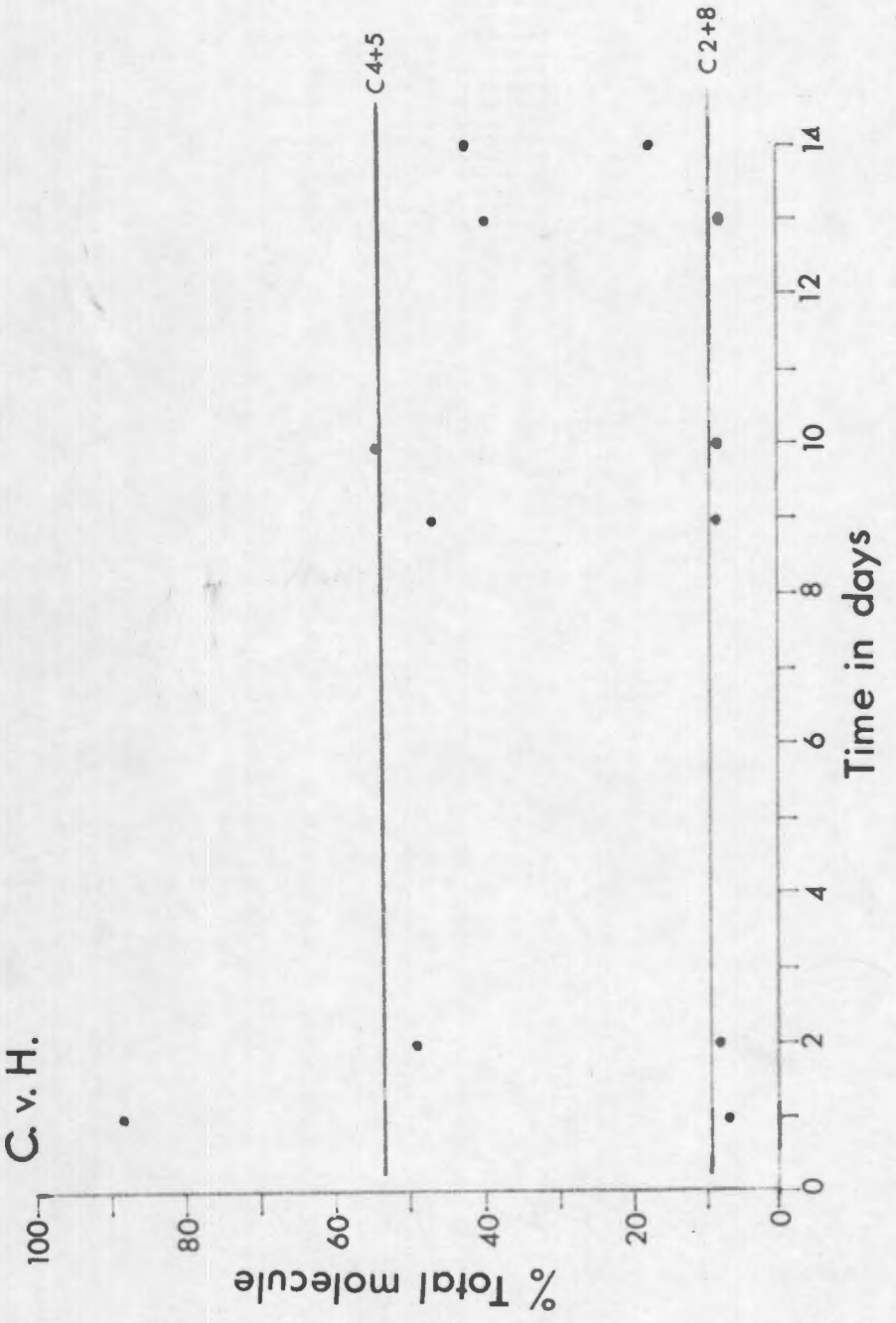


Fig. 7-36: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in acute attack, C.v.H.

it would have been ideal to simultaneously inject uric acid-N^{15} in order to calculate the urate pool size and percentage renal/extrarenal excretion along the lines described by Seegmiller et al (404). The advantages of comparing incorporation values of glycine-2-C^{14} into uric acid after correcting for extrarenal disposal have already been stressed in detail (see pages 201 and 211, Chapter IV).

The presence of hyperuricaemia in this patient implies a large miscible uric acid pool. Thus the dilution of newly formed urate will be greater, also influencing incorporation values. Applying Seegmiller's technique (404) this could also have been corrected along the lines discussed on page 211-213, Chapter IV. Thus, on both counts, incorporation values can be disregarded in this experiment.

However, none of the above arguments influence the validity of the C4+5:C2+8 ratios, nor the disposition of radioactivity within the urate moiety.

From an experimental point of view, this study was also unsatisfactory. 1.8321×10^8 dpm glycine-2-C^{14} were injected and the experiment was conducted as before for the ensuing 14 days. Serum uric acid varied from 7.9 to 11 mg/100 ml and 24 hour urinary urate from 200 to 490 mg. Extreme difficulty was encountered in trying to isolate uric acid from the urine and on bubbling H_2S through the

copper-urate solution, copper sulphide precipitate remained in suspension. As a result, no specimens were obtained on days 4, 6, 7 and 11. On other days, the weights of uric acid for counting and degradation were so small that any radioactivity in the sample was not detectable. The only results which were thought acceptable were obtained on days 1,2,9,10,13 and 14. Degradation samples were diluted by factors of 1.5 to 7 and except on day 9 where 53.8 mg of uric acid were obtained, weights of uric acid counted were under 10 mg.

(b) Results.

These are expressed in Table 7-10 and figs. 7-34, 7-35 and 7-36.

(c) Summary.

(i) Unsatisfactory experiment both technically and because of the presence of chronic renal disease with uraemia and hyperuricaemia. One cannot, therefore, get a true idea of the incorporation of glycine-2-C^{14} into uric acid and its component carbon atoms.

(ii) The C4+5: C2+8 ratios varied widely around the mean ± S.E. value of 6.12 ± 1.24. The average ratio for the first 2 days was 8.65, again above the mean as with A.v.R. These ratios are significantly higher than those observed in the normal controls, the symptomatic porphyrics and the S.A. genetic porphyric studied during remission (fig.7-35).

(iii) On average, 53.67% and 9.85% of the molecule's activity resided in C4+5 and C2+8 respectively. Note how little activity was present in carbons 2 and 8 (fig.7-36).

(3) M.N.

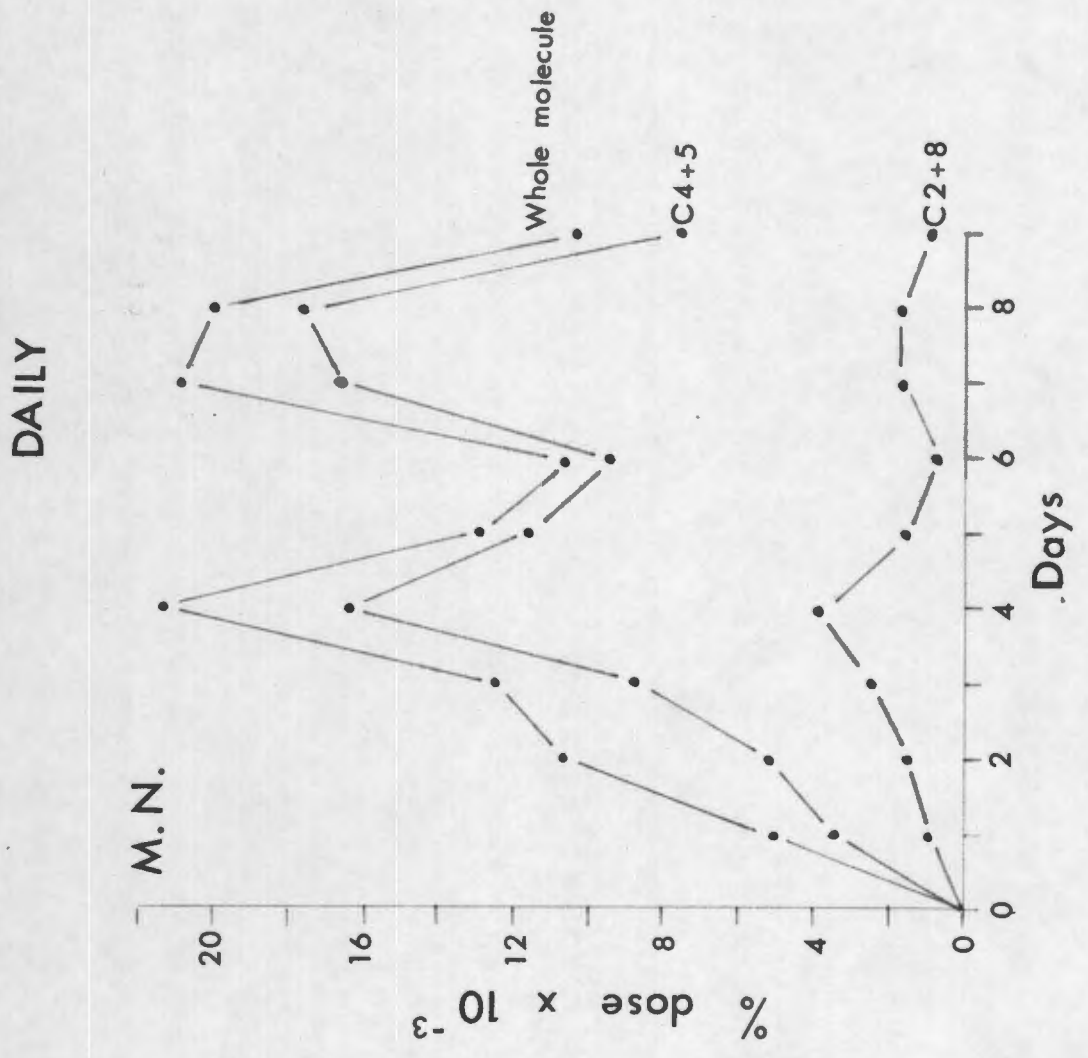
(a) Evaluation of experimental results.

M.N. White female aged 31, a known case of S.A.G.P. with positive family history, had been treated three times in the past 7 years for acute porphyria. Just prior to her present admission, she complained of severe central chest pain associated with acute diffuse cramp-like abdominal pain with no vomiting. Her urine had been dark for 2 days. On investigation, she was found to have a small reducible sliding hiatus hernia associated with a spastic body of the oesophagus. The pain improved on sparine and bed rest over a few days. She was on ward diet eating according to her appetite. During her nine-day stay in hospital, 24 hour urinary ALA levels ranged from 11.02 to 14.51 mg and PBG from 9.48 to 15.03 mg. She passed an average of about a litre of urine daily. When these values are compared with the published normal values for ALA and PBG of < 6 mg and < 2 mg/litre urine respectively (90), M.N. excreted excessive amounts of porphyrin precursors in her urine. Thus, while the hiatus hernia could have accounted for the patient's pain,

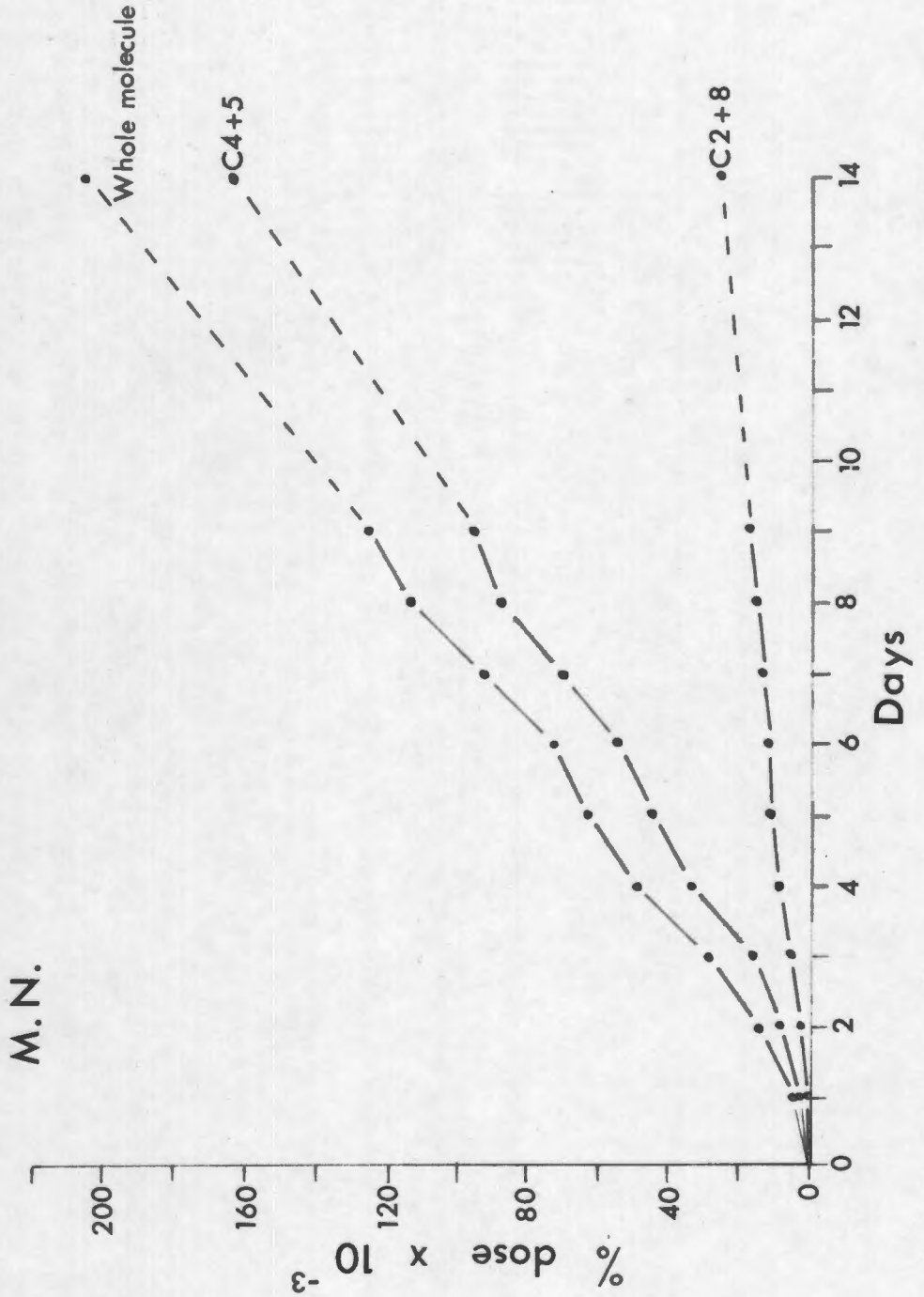
TABLE 71.

Days	URIC ACID		C 4 + 5		C 2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	5.129	5.129	3.610	3.610	1.098	1.098
2	10.775	15.904	5.263	8.873	1.792	2.890
3	12.557	28.461	8.882	17.755	2.570	5.460
4	21.457	49.918	16.403	34.158	4.014	9.474
5	12.913	62.831	10.890	45.048	1.679	11.153
6	10.614	73.445	8.732	53.780	0.914	12.067
7	20.960	94.405	16.510	70.290	1.756	13.823
8	20.116	114.521	17.837	88.127	1.698	15.521
9	10.340	124.861	7.891	96.018	0.985	16.506
14		200.000		160.000		25.000

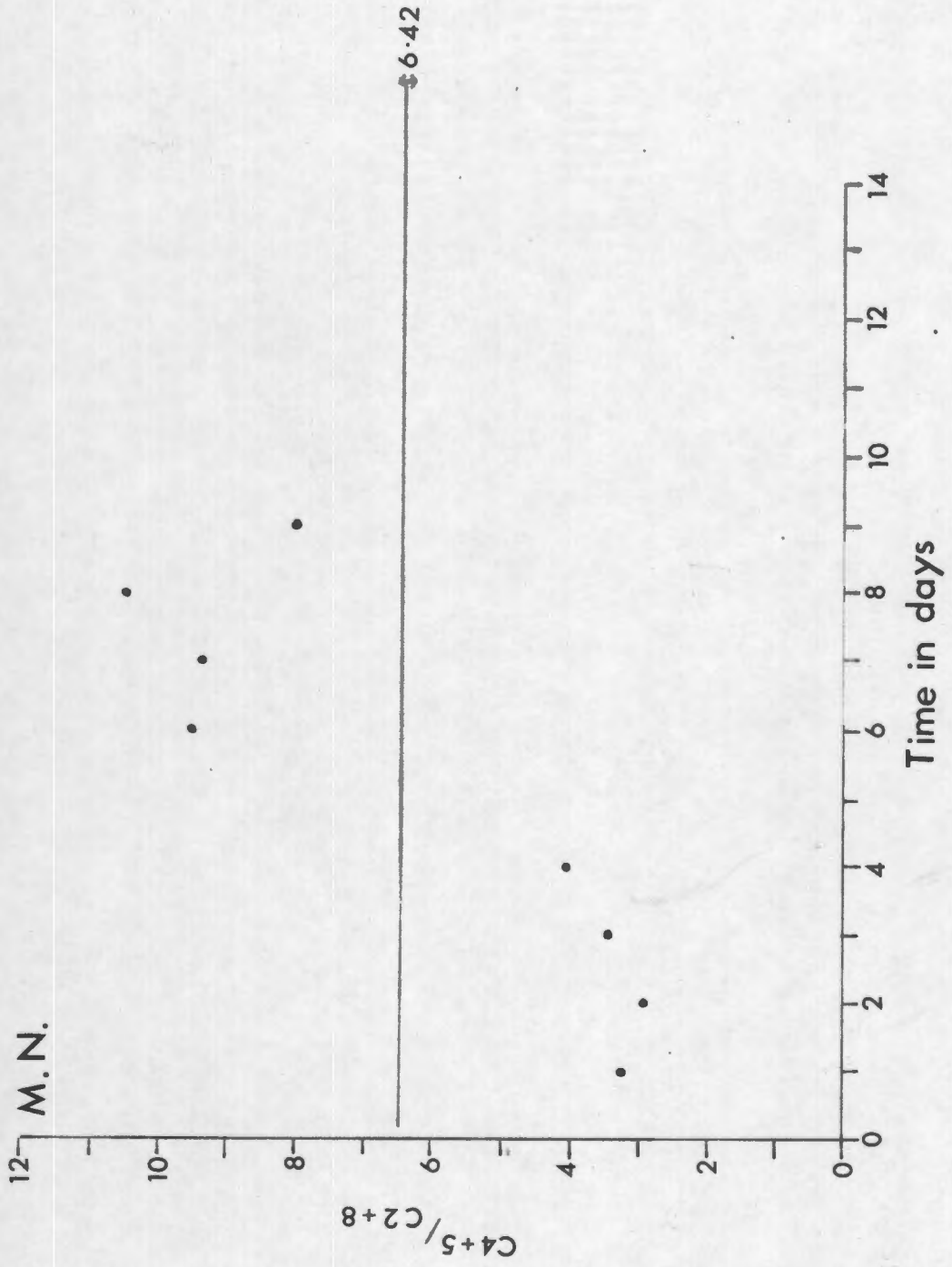
The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 and 8 by a South African genetic porphyric, M.N., studied during an acute attack. The 14 day cumulative incorporation figures have been extrapolated from the 9 values obtained. Values in the table are expressed as percentage dose x 10-3.



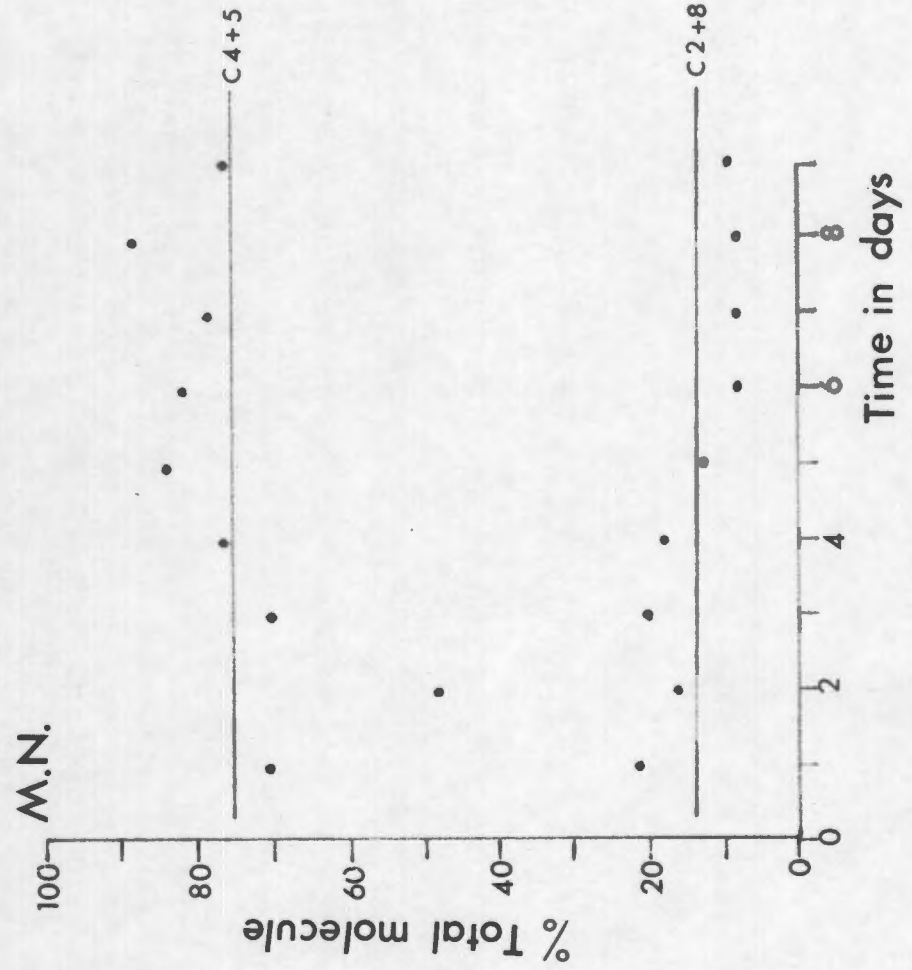
7-37: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, M.N.



7-38: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, M.N.



7-39: Daily C4+5: C2+8 ratio's scattered around mean ± S.E. (↕) in S.A. genetic porphyria in acute attack, M.N.



7-40: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in acute attack, M.M.

she has been regarded as an acute porphyric for the purposes of this thesis. She was given glycine-2-C^{14} on the day of her admission.

Her renal, hepatic and haemopoietic functions were normal. 1.7821×10^8 dpm glycine-2-C^{14} were injected on day 1. Serum urate varied between 2.50 and 5.39 (5 values) whilst 24 hour urate excretion was 279 to 539 mg over the ensuing 9 days. Usually enough urate was isolated from the urine for both degradation and counting and on days 1,2,6 and 7, "cold" carrier uric acid was added to the radioactive sample to be degraded, the dilution factor varying between 1 and 4. Over 100 mg barium carbonate and between 20 and 70 mg glyoxylic acid semicarbasone were obtained for counting. There were no technical hitches and this was a good and completely acceptable experiment.

(b) Results.

These are summarized in Table 7-11, figs. 7-37, -38, -39 and -40. Results are interesting and differ from the previously reported experiments. Note how peak values of urinary uric acid-C^{14} activity are reached on day 4 followed by a trough on days 5 and 6 and a secondary peak on days 7 and 8. On day 6, only 320 ml of urine were obtained, less than on other days raising the possibility of an incompletely collected 24 hour urine sample. There is a distinct early hump in C2+ 8 activity (fig. 7-37).

Incorporation values are shown in Table 7-11 and fig.7-38

and have been extrapolated to 14 days so that results can be compared with experimental data in other patients.

Fig. 7-39 depicts the C4+5: C2+8 ratios for the experiment. The mean value ± S.E. is 6.42 ± 1.01. Of great interest is the observation that on days 1 to 4, th e ratios are well below this mean value whilst those recorded on days 6 to 9 are well above. The mean ratio for the first 4 days is 3.45 which is comparable to ratios obtained in normal controls. As this may more nearly reflect what is happening in purine moieties made by the liver (page 239 , Chapter IV), the 4-day ratio may be more pertinent than the average for the study when interpreting experimental data.

The same information is seen from a different angle in fig. 7-40 where the disposition of radioactivity within the uric acid molecule is recorded. The activity in C4+5 does not vary much from the mean of 75.19% of the total activity within the molecule over the 9 days of study. However, C2+8 activity is much higher than the mean of 13.68% on days 1 to 4 and below this value on days 6 to 9, the average for the first 4 days being 19.3% a value comparable to those of normal controls.

(c) Summary.

(i) A valid experiment.

(ii) Peak values of urinary uric acid-C^{14} and its

component carbons were reached on day 4. There was a clear early hump in C2+8 activity, very much more pronounced than in other studies (fig. 7-37).

(iii) In the 9 days of the experiment, 0.1249%, 0.096% and 0.0165% of the administered glycine-2-C^{14} were excreted as urinary uric acid-C^{14}, C4+5 and C2+8 respectively. When corrected for 14 days, these values were increased to 0.200%, 0.160% and 0.025% respectively (fig. 7-38).

(iv) The mean ± S.E. of the C4+5:C2+8 ratios was 6.42 ± 1.01. The average ratio for the first 4 days was 3.4 (fig. 7-39).

(v) On average, 75.19% of the molecule's activity resided in C4+5 and 13.68% in C2+8. C2+8 comprised an average of 19.3% of the molecule's activity over the first 4 days of the experiment. (fig. 7-40).

(4) M. de J.

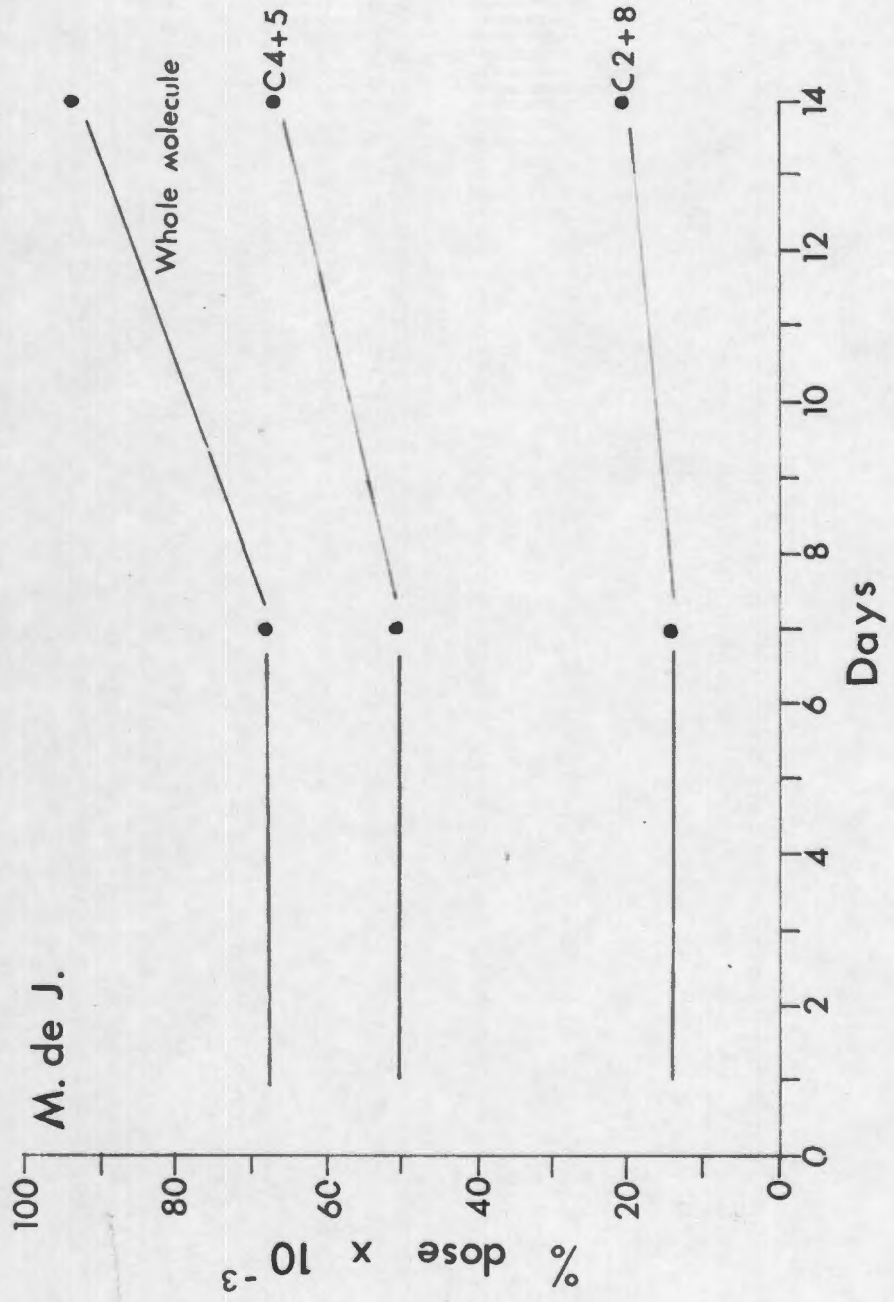
(a) Evaluation of experimental findings.

M. de J., White female aged 26, had been diagnosed as a S.A. genetic porphyric about a year and a half before her admission. Following a gynaecological operation (no thiopentone given) the patient received Sonalgin (Butobarbitone) post-operatively. Thereafter, she developed abdominal pain, numbness in her hands and feet and increasing confusion. On admission, she was treated with Sparine, Pethidine and I.V. saline. Her urinary PBG levels were 87.79 mg/day

TABLE 7-12.

Days.	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1-7	64.805	64.805	48.187	48.187	13.768	13.768
8-14	89.424	154.229	63.939	112.126	19.541	33.309

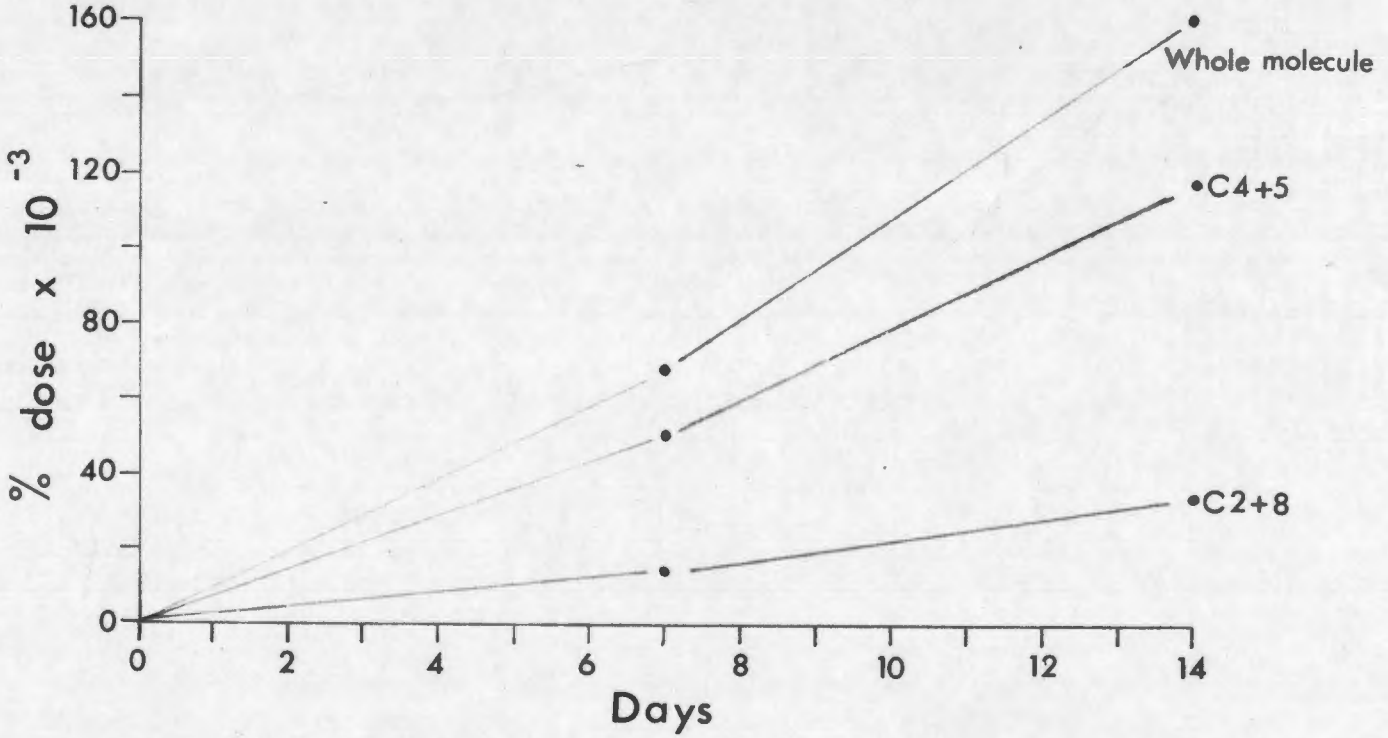
The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 + 8 by a South African genetic porphyric, M. de J., studied during an acute attack. Values in the table are expressed as percentage dose x 10^{-3}.



7-41: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyria in acute attack, M. de J.

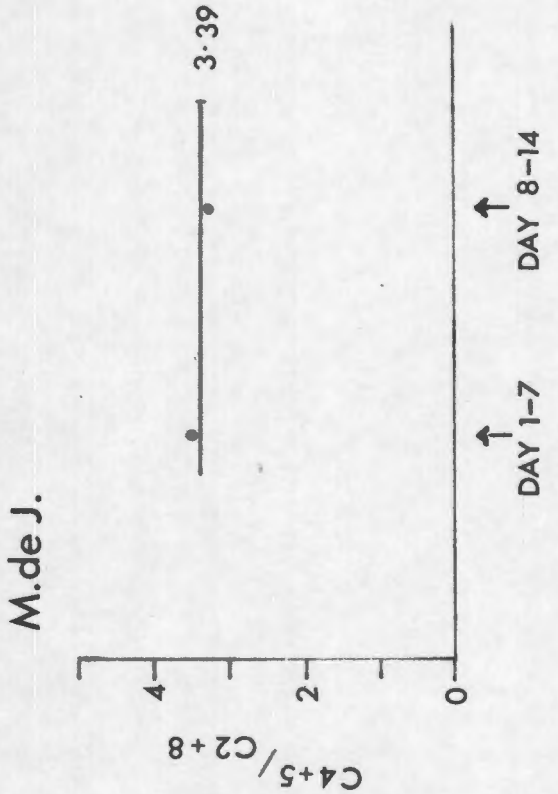
CUMULATIVE

M.de J.

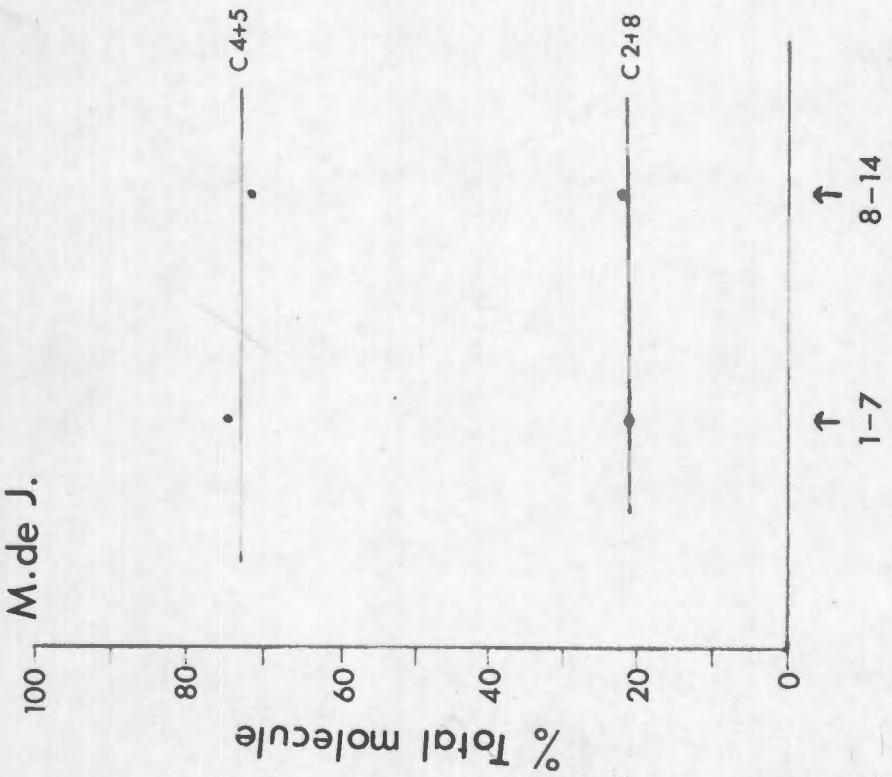


7-42: The cumulative incorporation of glycine-2-^{14}C into urinary uric acid, C4+5 and C2+8 in S.A.; genetic porphyria in acute attack. M. de J.

7-42



7-43: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (|) in S.A. genetic porphyric in acute attack, M. de J.



7-44: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in acute attack, M. de J.

and ALA, 52.58 mg/day. She was given glycine-2-C^{14} on admission and over the two weeks of the experiment, her PBG excretion dropped to 11.78 mg/day and ALA to 6.44 mg/day. She was catheterized initially allowing for accurate 24-hour urine samples to be collected. Her blood urea, 41 mg per 100 ml on admission rose to a maximum of 80 mg per 100 ml possibly on a pre-renal basis. During her convalescence, she was treated with antibiotics for urinary and gynaecological infection. Her renal, hepatic and haematological functions were normal.

1.9516×10^8 dpm glycine-2-C^{14} were injected at the beginning of the experiment. Serum urate varied between 3.32 to 5.93 mg/100 ml(5 values and 24-hour urinary urate between 240 and 583 mg. The same difficulties as with C.v.H. in isolating uric acid from the urine were encountered but in this experiment, all uric acid obtained from days 1 to 7 and days 8 to 14 were pooled and recrystallized providing adequate amounts of uric acid for accurate counting and degradation without dilution. To calculate incorporation data, the total activity excreted was calculated from the average activity of the pooled sample multiplied by the total number of millimoles uric acid excreted in the urine during the 7 day period. This is an approximation but probably represents a value in the correct order of magnitude for the experiment.

Adequate weights of uric acid, glyoxylic acid semi-carbazone and barium carbonate were obtained for counting.

Thus whilst far from ideal, there is some valid information extractable from this study.

(b) Results.

These are summarized in Table 7-12, figs. 7-41, -42, -43, and -44.

(c) Summary.

(i) Technical difficulties have made this an unsatisfactory experiment but information obtained is valid.

(ii) As an approximation, 0.15%, 0.11% and 0.03% of the administered dose was excreted in the urine as uric acid-C^{14}, C4+5 and C2+8 (fig. 7-42).

(iii) TheC4+5:C2+8 ratio for days 1 to 7 was 3.5 and that for days 8 to 14, 3.27, the average ± S.E. being 3.39 ± 0.11 (fig. 7-43).

(iv) 72.93% and 21.55% of the uric acid molecules activity resided in C4+5 and C2+8 respectively (fig. 7-44).

(5) Z.M.

(a) Evaluation of experimental findings.

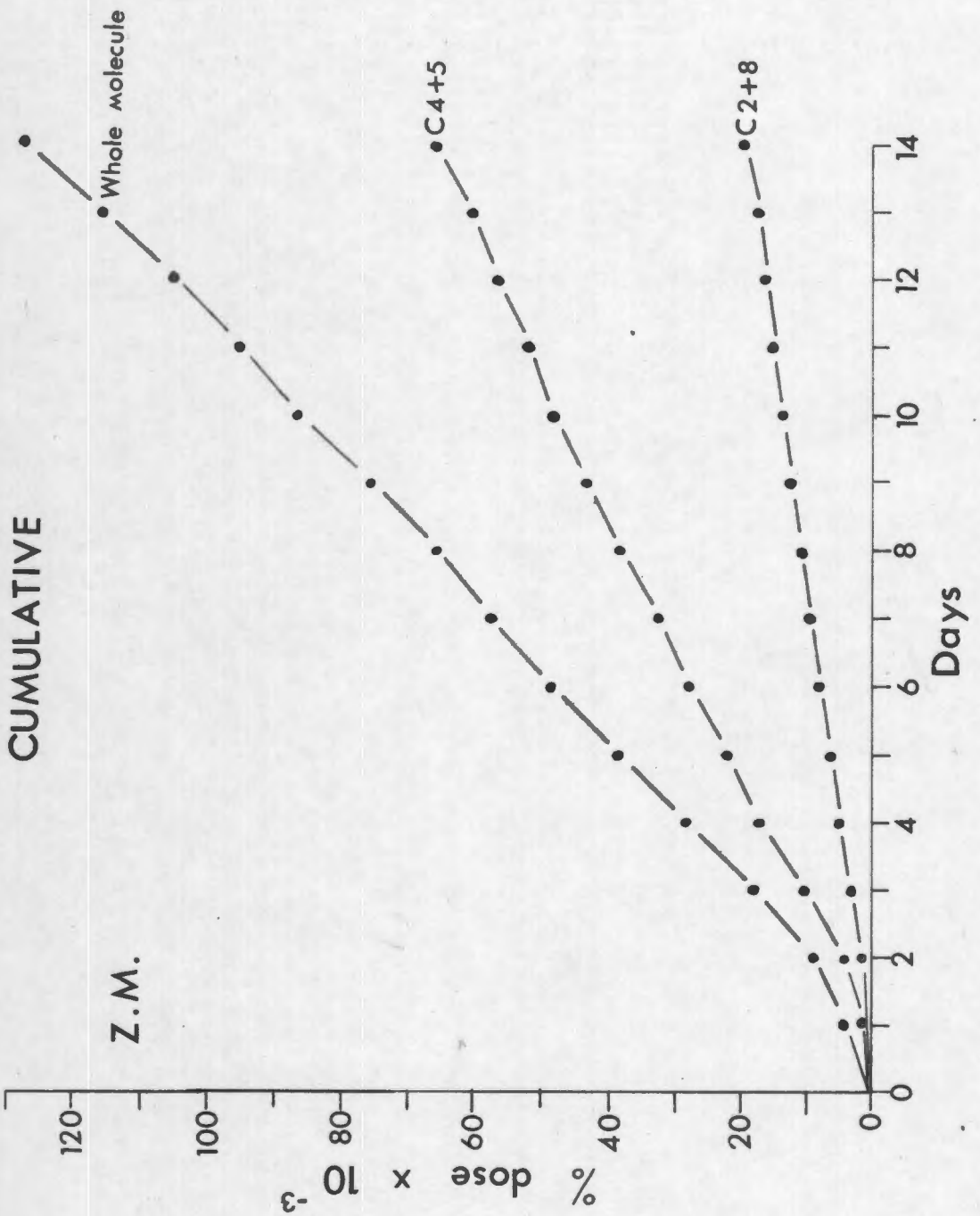
Z.M., White female, was a known hypertensive who was admitted with a one-week history of abdominal pain, drowsiness, and confusion. Her B.P. in the ward was 180/80 and shortly after admission, she became more confused and had

TABLE 7-13.

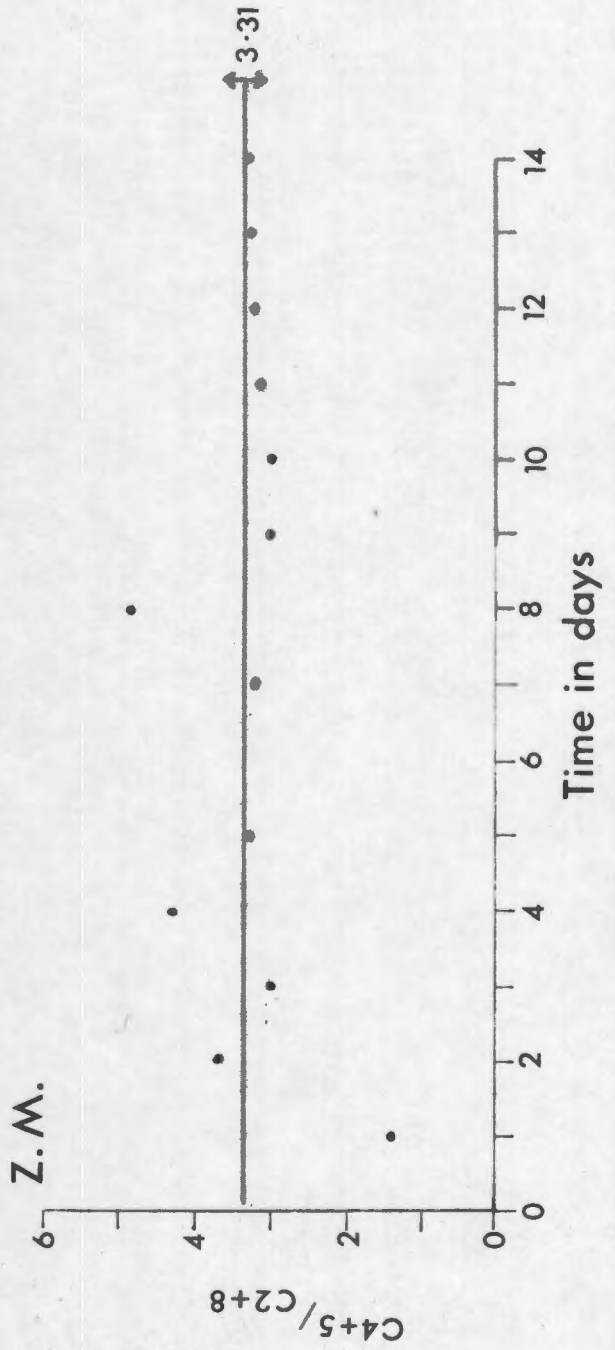
Days	URIC ACID		C4 + 5		C2 + 8	
	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	4.019	4.019	1.134	1.134	0.819	0.819
2	4.701	8.720	2.940	4.074	0.781	1.600
3	9.954	18.674	6.076	10.150	2.019	3.619
4	9.801	28.475	6.787	16.937	1.568	5.187
5	10.335	38.810	5.359	22.296	1.609	6.796
6	=10.000	48.810	= 6.000	28.296	= 1.400	8.196
7	8.825	57.635	4.749	33.045	1.469	9.665
8	7.690	65.325	5.691	38.736	1.164	10.829
9	10.126	75.451	4.849	43.585	1.608	12.437
10	10.894	86.345	4.492	48.077	1.492	13.929
11	8.697	95.042	3.841	51.918	1.224	15.153
12	10.361	105.403	4.203	56.121	1.302	16.455
13	10.564	115.967	4.429	60.550	1.359	17.814
14	11.305	127.272	5.075	65.625	1.537	19.351

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 and 8 by a South African genetic porphyric, Z.H., studied during an acute attack. The values on day 6, marked by an asterisk, are estimated from the graph of the data in this experiment. Values in the table are expressed as percentage dose x 10^{-3}.

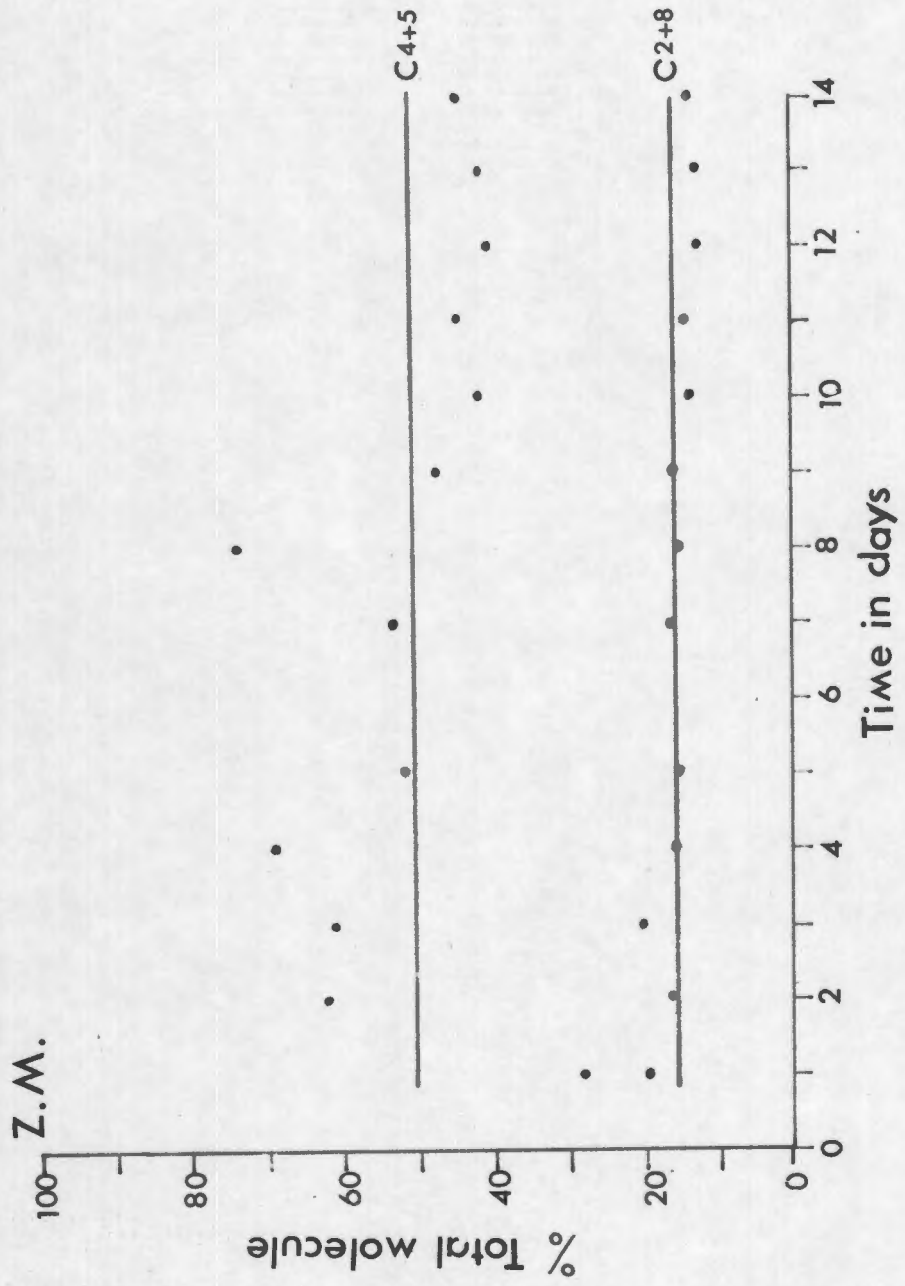
7-45: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, Z.M.



7-46: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, Z.M.



7-47: Daily C4+5: C2+8 ratios scattered around mean ± S.E. (⟺) in S.A. genetic porphyria in acute attack, Z.W.



7-48: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in acute attack. Z.M.

hallucinations. She was catheterized, ensuring accurate urine collections. Her urinary PBG and ALA on admission was 43.3 and 50.0 mg/day respectively, whilst urinary copro- and uroporphyrin was 2,460 and 22,055 ug/l respectively. Stool coproporphyrin was 860 µg/G and protoporphyrin 642 ug/G. Thus the diagnosis of acute S.A. genetic porphyrin was confirmed. She made a full clinical recovery.

1.9196 x 10^8 glycine-2-C^{14} were injected at the onset of the illness. Serum urate varied between 4.82 and 8.58 mg/100 ml (7 values) with 4 of the 7 values exceeding 7mg/100 ml. Thus her miscible urate pool was probably larger than normal and in conjunction with renal disease (hypertension and raised blood urea) it can be expected that less glycine-2-C^{14} will be incorporated into urinary uric acid. 24-hour urinary urate levels lay between 288 and 527 mg. Difficulty in isolating the uric acid from the urine was again encountered. At this stage, "salting out" the copper sulphide colloidal suspension by adding sodium chloride enabled daily counting and degradation to be performed. Generally, the dilution factor for the sample of uric acid degraded was between 1 and 2, large amounts of uric acid being degraded. On day 6, a conical flask containing the copper urate in solution broke. On days 2 and 11, dilution factors were 18.179 and 5.287 respectively.

Yields from degradation procedures were good and adequate amounts of uric acid, barium carbonate and glyoxylic acid

semicarbazone were obtained for counting.

This was therefore a valid experiment.

(b) Results.

These are expressed in Table 7-13, figs. 7-45, -46, -47 and -48.

(c) Summary.

(i) A valid experiment. There was evidence of renal disease with uraemia. In addition hyperuricaemia pointed to a larger than normal miscible uric acid pool. Thus incorporation values probably were lower on this account.

(ii) Peak values were reached on day 3 followed by a plateau of urinary uric acid-C^{14} activity (fig. 7-45).

(iii) 0.127%, 0.66% and 0.019% of the administered dose of glycine-2-C^{14} were excreted as urinary uric acid, C4+5 and C2+8 respectively (fig. 7-46).

(iv) The C4+5:C2+8 ratios had a mean ± S.E. of 3.31 ± 0.22. Average ratio for the first 4 days was 3.1 (fig. 7-47).

(v) The mean% of the molecule's activity residing in C4+5 and C2+8 was 50.88% and 15.59% respectively. On day 1, 19.79% of the total activity came from C2+8 (fig. 7-48).

(6) A.Jov.

(a) Evaluation of experimental results.

For 13 years, before admission, A. Jov., White female, had suffered from excessive skin fragility. 9 days after

hysterectomy, patient became mentally dull, confused, dysarthric and her diaphragm was noted to be paralyzed. Her urinary PBG and ALA levels on admission were 57.3 and 55.3 mg/day and the concentrations of urinary copro- and uroporphyrin were 1,719 and 4,592 ug/l. Stool coproporphyrin was 1,611 ug/G and protoporphyrin 1,446 ug/G. Thus an attack of acute S.A. genetic porphyria was confirmed.

1.9180×10^8 glycine-2-C^{14} were injected at the beginning of the experiment. Daily urinary urate ranged from 317 to 536 mg whilst serum urate ranged between 1.81 to 3.28 mg/100 ml(6 values) with 4 values under 2 mg/100 ml. This would indicate a small miscible urate pool probably tending to raise incorporation values.

Containers
Specimens were broken on days 5, 10, 12 and the experiment was of 13 day duration. Large amounts (300 to 350 mg) of uric acid were degraded, "cold" carrier uric acid was added to some of the radioactive samples, the dilution factors ranging from 1 to 3. Only in 2 samples was this factor greater, viz. 3.9 on day 4 and 4.8 on day 13.

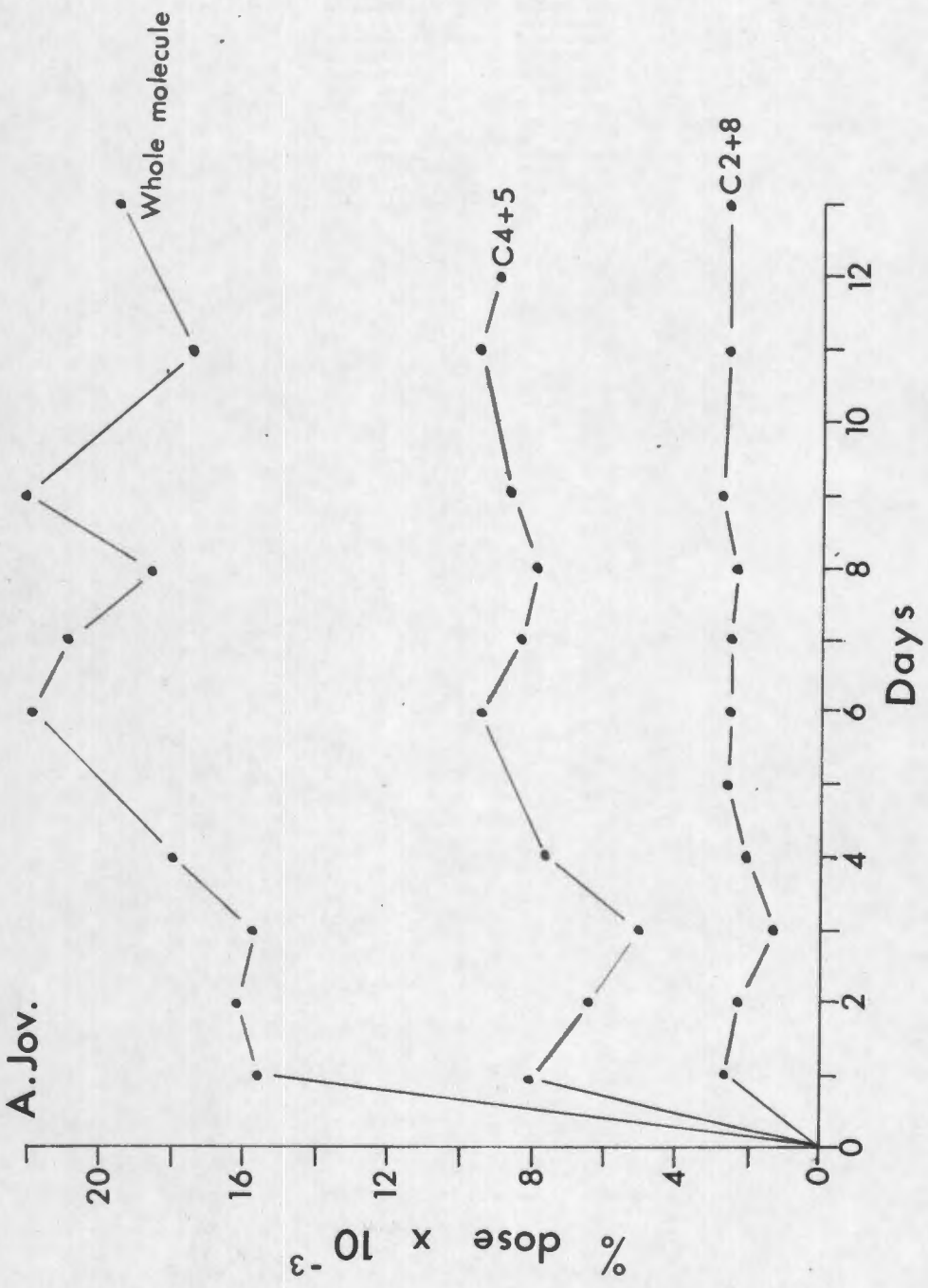
Yields from degradation were excellent and large amounts of uric acid, barium carbonate and glyoxylic acid semicarbazone were obtained for counting. Most of the glyoxylic acid samples weighed over 100 mg, ranging from 92 to 145.3 mg.

This was therefore an accurate experiment.

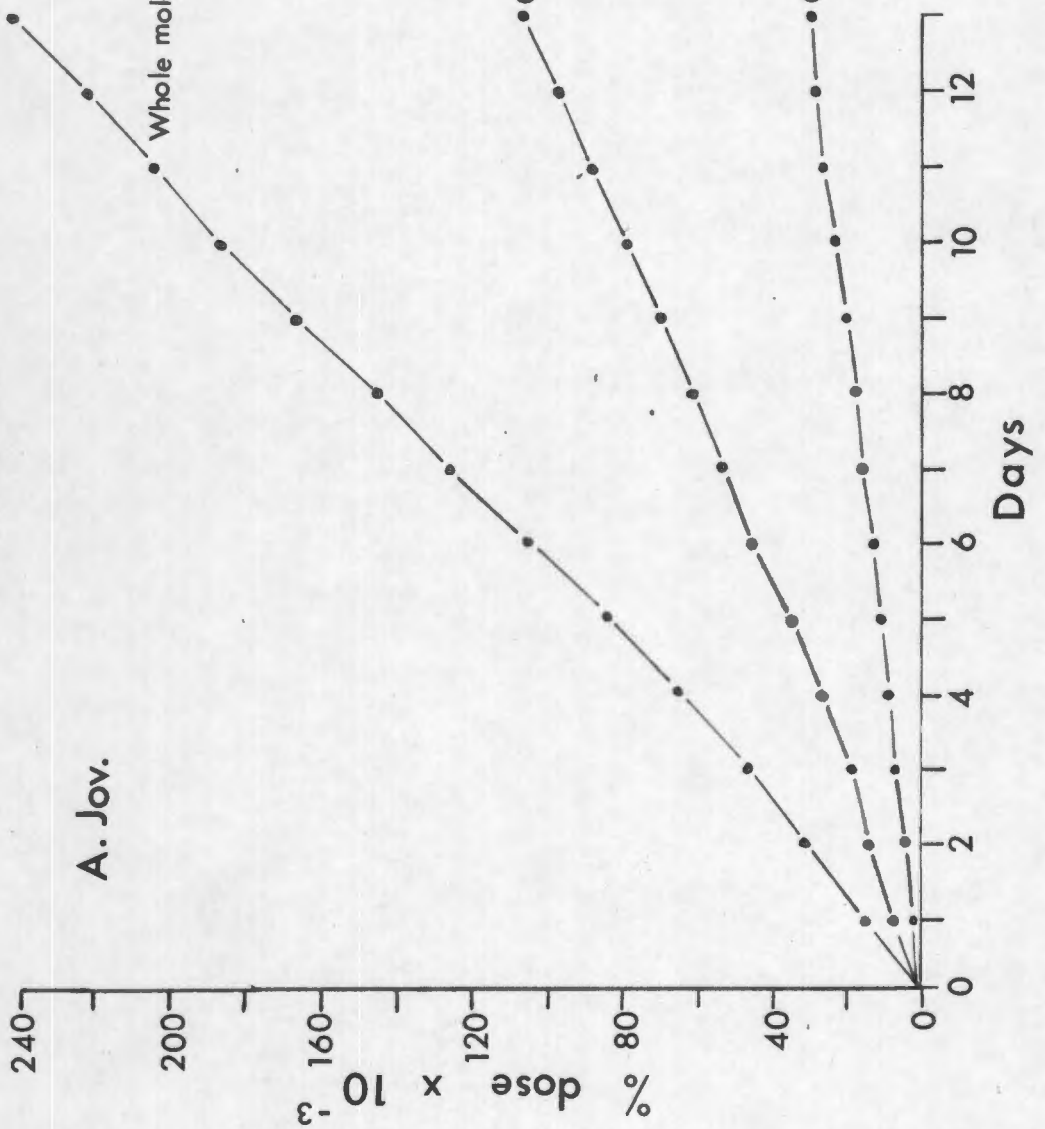
TABLE 7-14.

Days.	URIC ACID		C4 + 5		C2 + 8	
1	Daily	Cumul.	Daily	Cumul.	Daily	Cumul.
1	15.605	15.605	8.154	8.154	2.785	2.785
2	16.195	31.800	6.437	14.591	2.341	5.126
3	15.578	47.378	5.049	19.640	1.649	6.775
4	17.953	65.331	7.648	27.288	2.147	8.922
5	*18.200	83.531	*8.800	36.088	*2.400	11.322
6	21.909	105.440	9.416	45.504	2.590	13.912
7	20.813	126.253	8.212	53.716	2.586	16.498
8	18.419	144.672	7.910	61.626	2.386	18.884
9	22.035	166.707	8.621	70.247	2.676	21.560
10	*19.800	186.507	*9.000	79.247	*2.600	24.160
11	17.277	203.784	9.520	88.767	2.525	26.685
12	*19.000	222.784	*9.200	97.967	*2.530	29.215
13	19.321	242.105	8.887	106.854	2.532	31.747
14		262.000		116.000		34.00

The percentage dose glycine-2-C^{14} excreted in the urine daily and cumulatively (cumul.) as uric acid, C4 + 5 and C2 +8 by a South African genetic porphyric, A. Jov., studied during an acute attack. Values in the table are expressed as percentage dose x 10^{-3}. Those values marked by an asterisk were estimated from the corresponding graph of the above data. The 14-day cumulative incorporation figure is derived by extrapolation from other values.

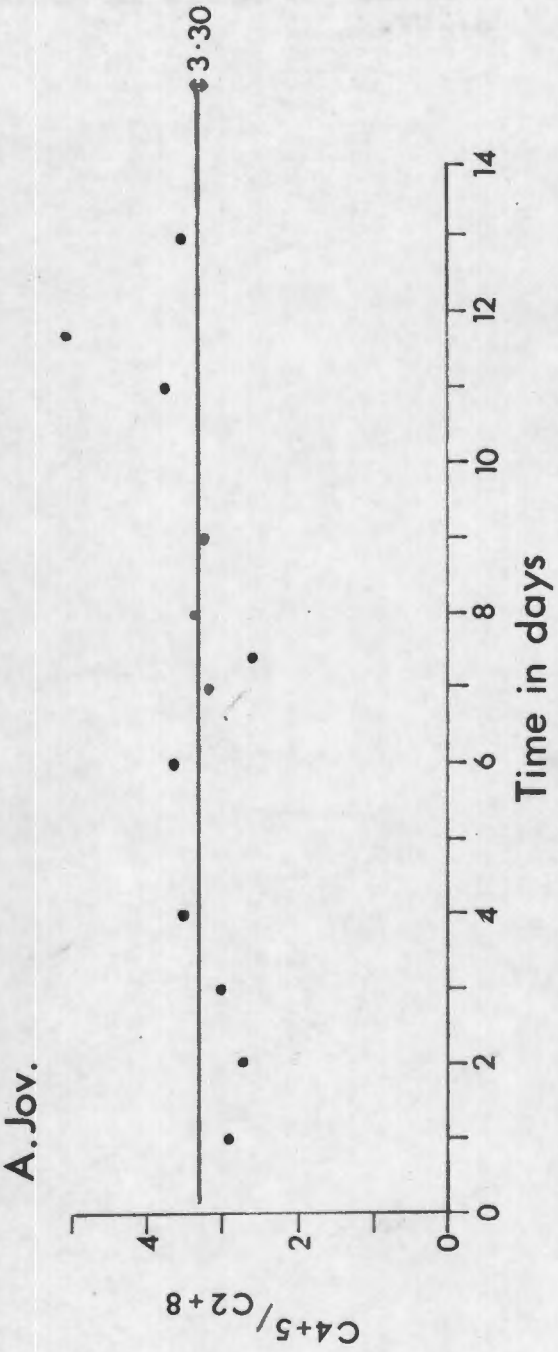


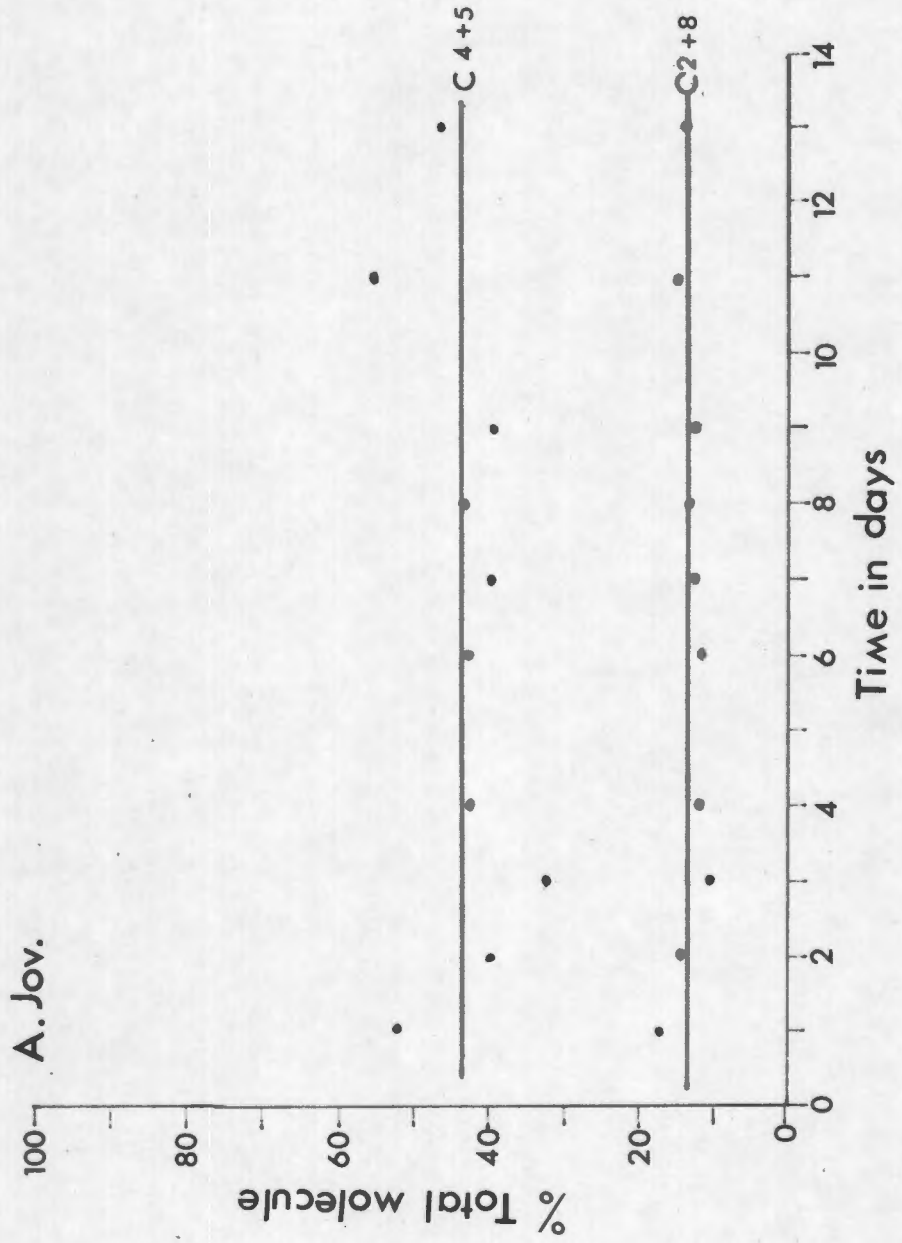
7-49: The daily incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, A. Jov.



7-50: The cumulative incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 in S.A. genetic porphyric in acute attack, A.Jov.

Fig. 7-51: P.T.O. for legend.





7-52: Disposition of radioactivity within uric acid molecule, daily values around mean, in S.A. genetic porphyric in acute attack, A. Jov.

(b) Results.

These are expressed in Table 7-14 and figs. 7-49, -50, -51 and -52.

(c) Summary.

(i) An accurate experiment. The low blood uric acid levels probably pointed to a small miscible urate pool which would tend to increase incorporation values.

(ii) Peak values were reached on day 1 but the plateau of urinary uric acid-C^{14} tended to rise to a new and higher level after 4 days (fig. 7-49).

(iii) Corrected for 14 days, 0.262%, 0.116% and 0.034% of the dose was excreted in the urine as uric acid, C4+5 and C2+8 respectively (fig. 7-50).

(iv) The mean $\pm$ S.E. C4+5 : C2+8 ratio for the experiment was 3.30 ± 0.08 whilst that of the first 4 days was 3.02 (fig. 7-51).

(v) On average for the experiment, 43.26% and 13.19% of the molecule's activity resided in C4+5 and C2+8 respectively. On day 1, C2+8 comprised 17.85% of the total activity in the uric acid moiety (fig. 7-52).

II. SERUM BILIRUBIN-C^{14} STUDIES.

Following the administration of glycine-2-C^{14}, the label appears in serum bilirubin in three distinct peaks of activity, the first within hours, the second at about 3 to 5 days and the third about 100 to 140 days after giving the isotope (205,207,498). The latter represents catabolism of haem derived from haemoglobin of senescent red cells. The former two constitute what is known as early-labelled or shunt bilirubin, the nature of which has been discussed in great detail in Chapter III, pages 117-131.

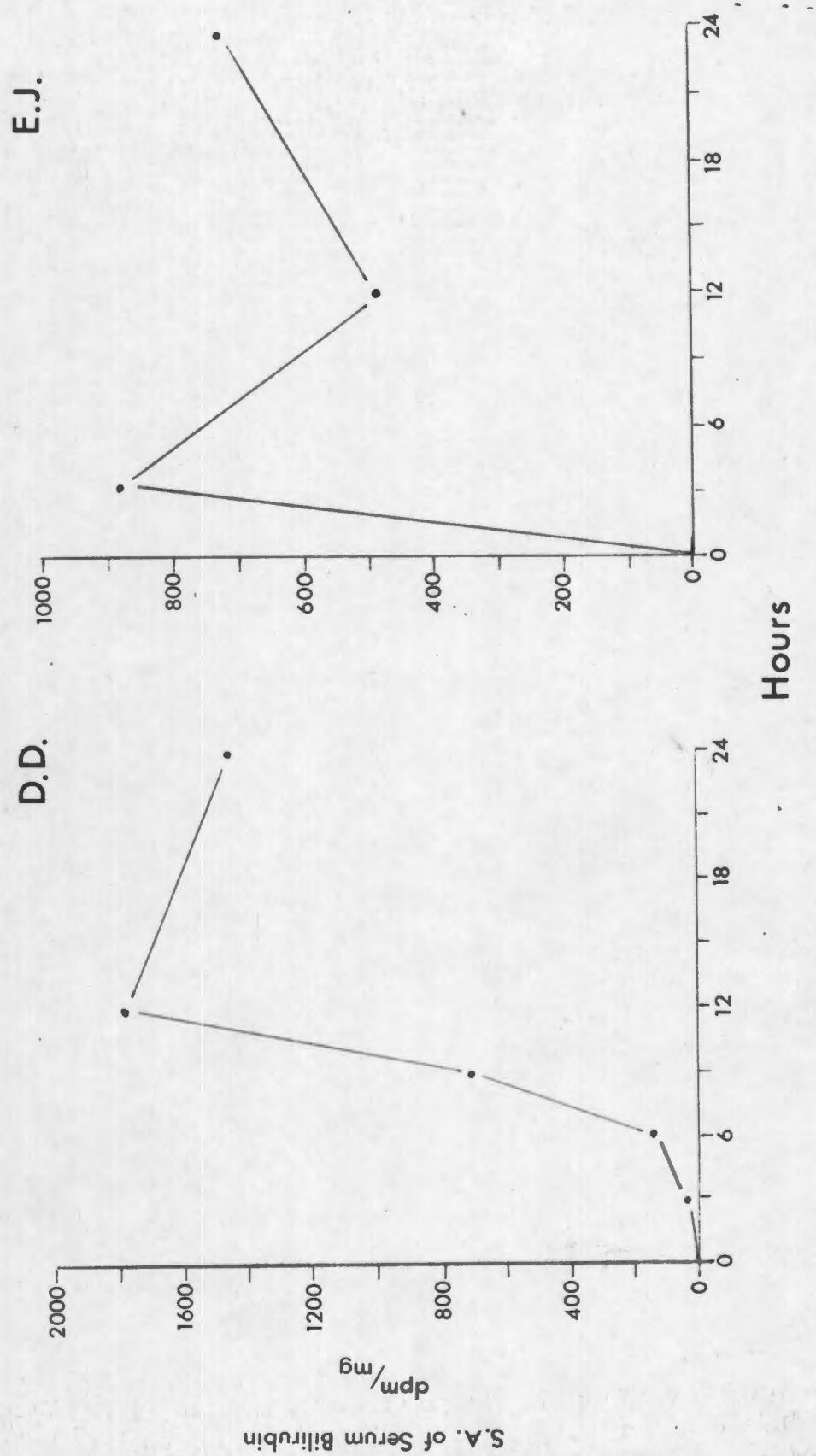
The first peak is thought to be derived from non-haemoglobin haem, mainly from the liver. The second is felt to reflect ineffective erythropoiesis by the marrow. As the hepatic porphyrias, e.g. S.A. genetic and symptomatic porphyria, are disorders of liver porphyrin metabolism, this section of study aims to record what is happening to liver haem by observing the first peak of early-labelled serum bilirubin in normal and porphyric human subjects.

Following the administration of glycine-2-C^{14}, about 40 ml of heparinized blood was taken at varying time intervals for the following 5 to 7 days. Determination of the specific activity of serum bilirubin was performed along the lines discussed at length in Chapter VI, pages 311-326. Simultaneously, haemin was isolated from red cells daily by methods described on page 327 and both the bilirubin

and haemin crystals were combusted and counted as barium carbonate, as described on page 306 . Two methods of isolating bilirubin from serum were employed, one derived from work of Ostrow, Hammaker and Schmid (331) and the second from Israels et al (208,498). The merits and demerits of these procedures have been commented on in Chapter VI, Pg.319-332. In the 11 patients studied, isolation procedure of Israels was used in only 3, viz. D.D., a normal, R.N., a symptomatic porphyric and T.M., a S.A. ~~genetic porphyric~~ symptomatic subject.

A drawback of any study on serum bilirubin-C^{14} activity following glycine-2-C^{14} is that activity in bilirubin even at peak values is low and small amounts (0.015 to 0.030 mg) of bilirubin are available for counting. This means that at peak values, the specific activity of bilirubin samples is only 2 to 4 cpm above background. To minimise errors involved in measuring such low radioactivity, samples have been counted for 200 to 400 minutes.

Serum bilirubin-C^{14} and haem-C^{14} activity has been determined in 5 normal, 3 symptomatic porphyric and 3 S.A. genetic porphyric subjects. All had normal haemopoietic function except one normal control, E.J., who had a haemolytic anaemia. Unless specifically indicated, liver function in the normal and S.A. genetic porphyric subjects was normal. Neither of the three symptomatic porphyric subjects was jaundiced.



7-53: The incorporation of glycine-2-C^{14} into the first peak of early-labelled serum bilirubin in two subjects, D.D. a normal and E.J., a subject with haemolytic anaemia.

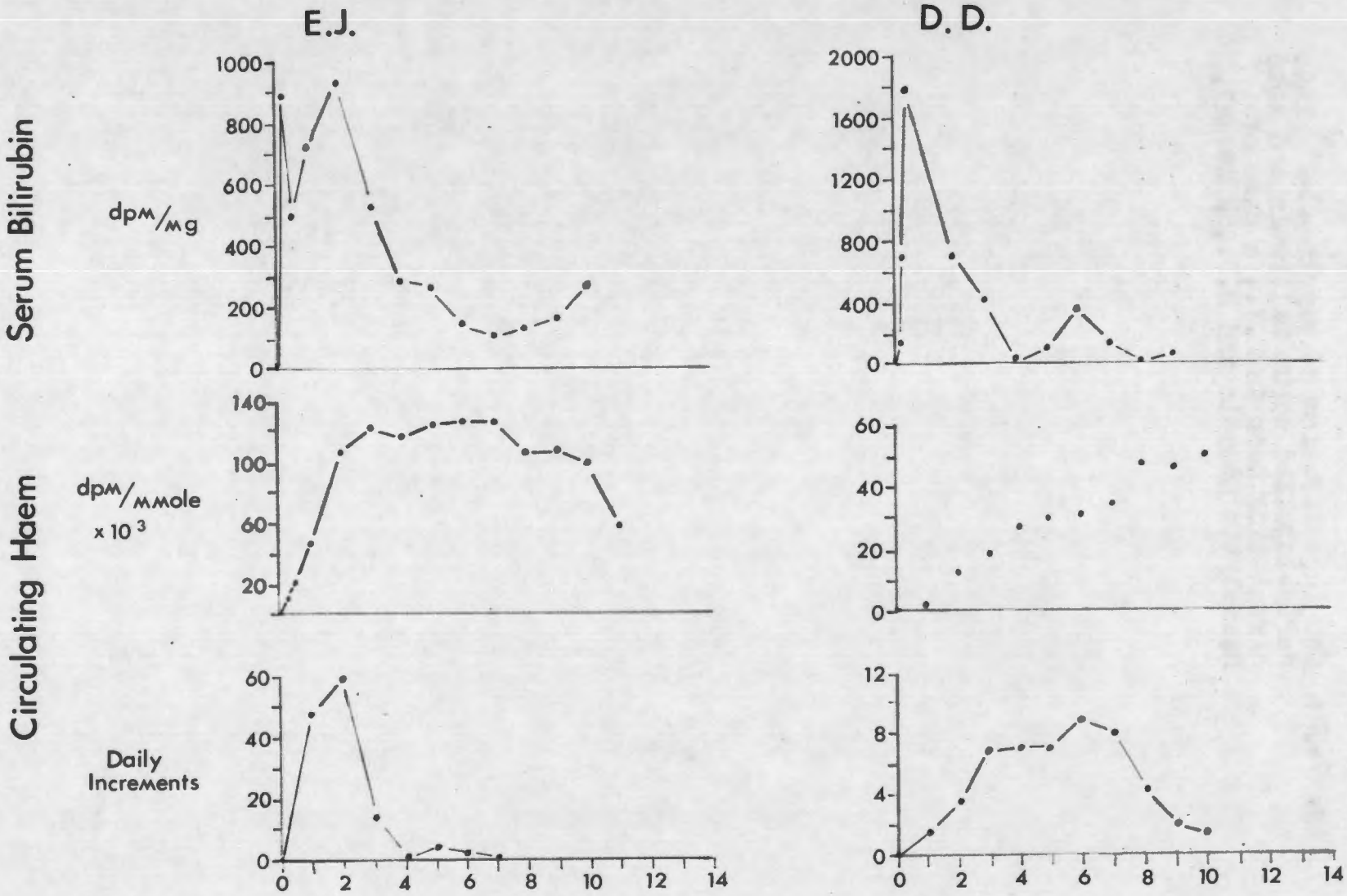


Fig. 7-9: P.T.O. for legend.

A. NORMAL SUBJECTS.

(1) D.D.

D.D., a Coloured male, following treatment ~~treated~~ for neurosyphilis was given 1.48220×10^8 dpm glycine-2-C^{14} on day 1. Results are expressed in Table 7-15 and figs. 7-53 and 7-54.

In fig. 7-53, note that the early peak occurs at 12 hours, which is in accordance with the data of Israels et al (205,207,498). At peak value, specific activity of serum bilirubin is 1,787 dpm/mg.

In fig. 7-54, the relationship between haem increments and the 2nd peak of shunt bilirubin is clearly seen, whilst the 1st peak occurs long before there is appreciable activity in circulating haem. These findings confirm the observations of Israels et al (205,207,498) and support the contention that the early peak is derived from non-haemoglobin haem. Note how the magnitude of the first peak is greater than that of the 2nd.

(2) E.J.

E.J., a Coloured female aged 18, was admitted with a 3-week history of dizziness and lassitude. She was shown to have an haemolytic anaemia with, on admission, a haemoglobin of 3.3 , reticulocyte count of 7.4% and a serum bilirubin of 2.3, conjugated 1.9 mg/100 ml. Liver biopsy was normal.

Patient was treated with repeated blood transfusions and was discharged on Prednisone. Despite extensive investigation, the cause for her anaemia was not established. The glycine-2-C^{14} was injected after full treatment had been instituted.

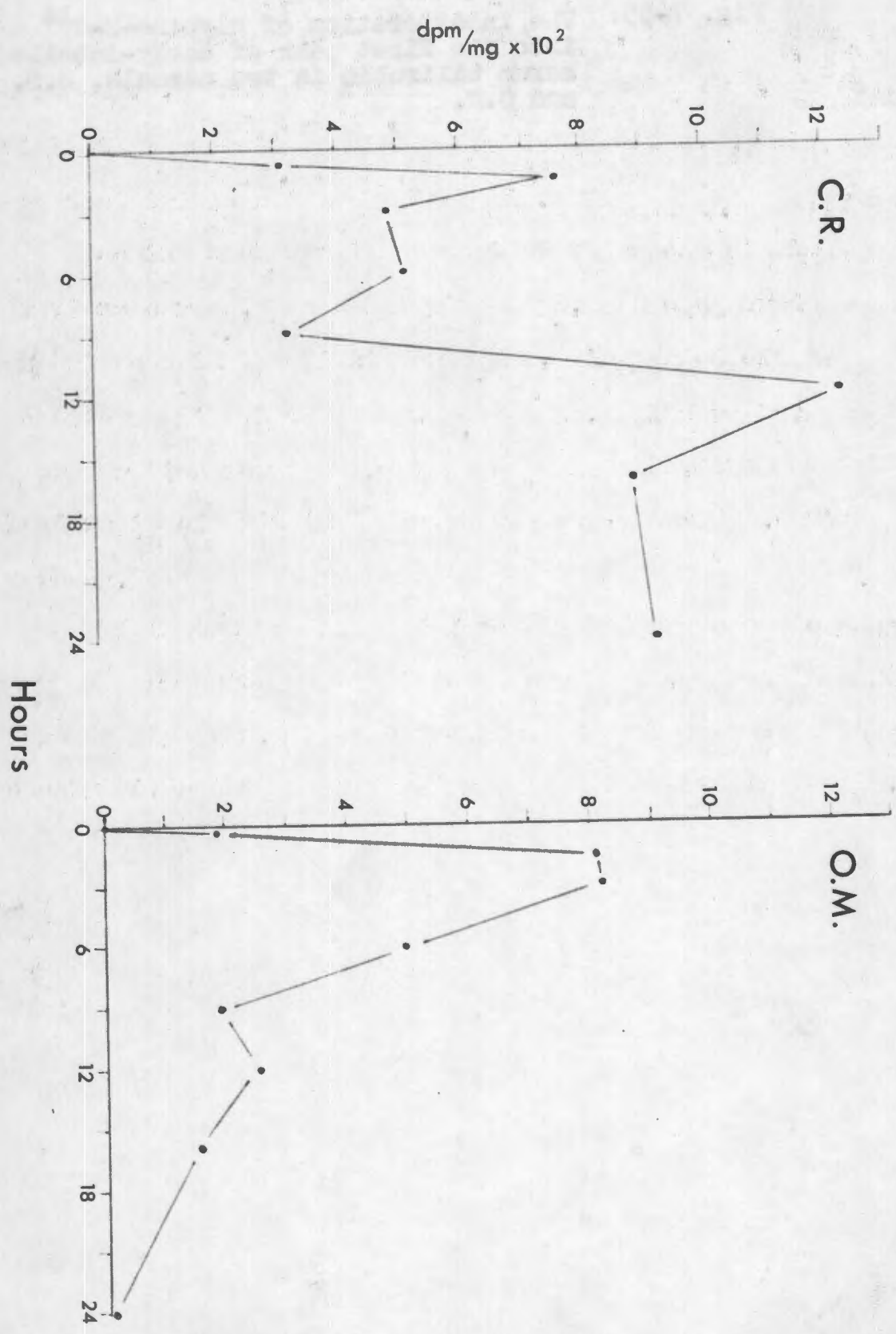
This case of haemolytic anaemia was chosen to see what effects haemolysis and a hyperplastic marrow would have on the early-labelled serum bilirubin-C^{14} pattern.

1.75107×10^8 dpm glycine-2-C^{14} were injected I.V. and the blood samples were collected over the following 10 days. There were no technical hitches.

Only 3 samples were tested for bilirubin-C^{14} activity in the first 24 hours (see fig.7-53) and of the 3, maximal activity was apparent at 3 hours.

Some exceedingly interesting data have been graphically set out in fig. 7-54. There are three distinct peaks in serum bilirubin-C^{14} activity. The first occurs before the label has entered circulating haem presumably reflecting liver haem turnover. The 2nd occurs between 1 and 3 days matching in time sequence maximum haem increments. The second peak is a broad one and presumably is the result of ineffective erythropoiesis by an overworking marrow. The bilirubin-C^{14} activity definitely tends to rise again after the 7th day at a time when there are no increments in circulating haem activity. In fact, instead of the usual plateau of activity occurring by the 10th to 14th day

Fig. 7-55: I.T.C for legend.



6-56: The incorporation of glycine-2-C^{14} into early-labelled serum bilirubin and into circulating haem in two normals, O.M. and C.R.

and lasting many weeks, after the 7th day, activity in circulating haem falls off rapidly, the result no doubt of the haemolytic process. This premature destruction of circulating red cells is clearly reflected in the serum bilirubin-C^{14} activity.

This experiment provides valuable assurance that methods with their drawbacks, do portray an accurate picture of changes in serum bilirubin-C^{14} activity following glycine-2-C^{14}.

(3) O.M.

1.72088×10^8 dpm glycine-2-C^{14} were given to this normal control and blood was collected for the following 6 days.

In fig. 7-55 note how the early peak occurs at three hours with a little kick at 12 hours and virtually no activity by 24 hours.

The 2nd peak follows closely the ascending limb of the marrow/circulating haem increment curve (fig. 7-56) again emphasizing the relationship between the two.

(4) G.R.

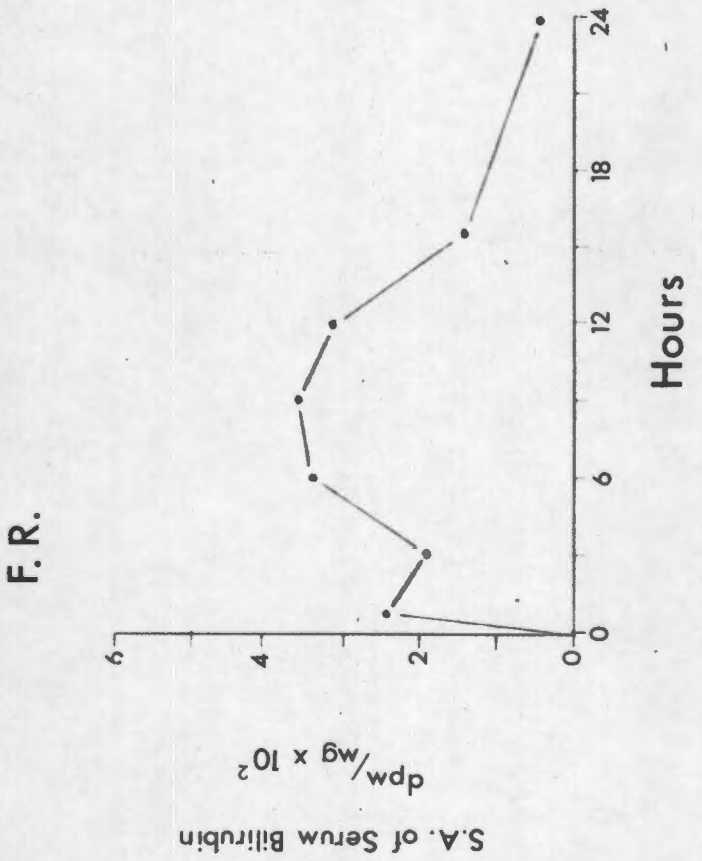
This normal control received 1.92402×10^8 dpm glycine-2-C^{14}. In fig. 7-55, note the distinct double peak in the 1st 24 hours, the first at 3 hours, the second component at 12 hours. There is still appreciable activity at 24 hours.

which, as it occurs whilst there is little activity in circulating haem (fig. 7-56) clearly depicts bilirubin from non-haemoglobin haem source. The slowly rising activity in serum bilirubin-C^{14} activity occurs at atime when increments in circulating haem-C^{14} activity are increasing.

(5) P.R.

Following the injection of 1.68939×10^8 dpm glycine-2-C^{14}, the label again appeared in peak 1 of early labelled bilirubin with two maxima, the first at 30 minutes, the second at 9 hours (fig. 7-57). The significance of the first component will be discussed in the following chapter.

The clear relationship between peak 2 and haem increments is again shown in fig. 7-58.



7-57: The incorporation of glycine-2-C^{14} into the first peak of early-labelled serum bilirubin in a normal, F.R.

7-58: The incorporation of glycine-2-^{14}C into early-labelled serum bilirubin and circulating haem of a normal, F.R.

B. SYMPTOMATIC PORPHYRIC SUBJECTS.

(1) R.N.

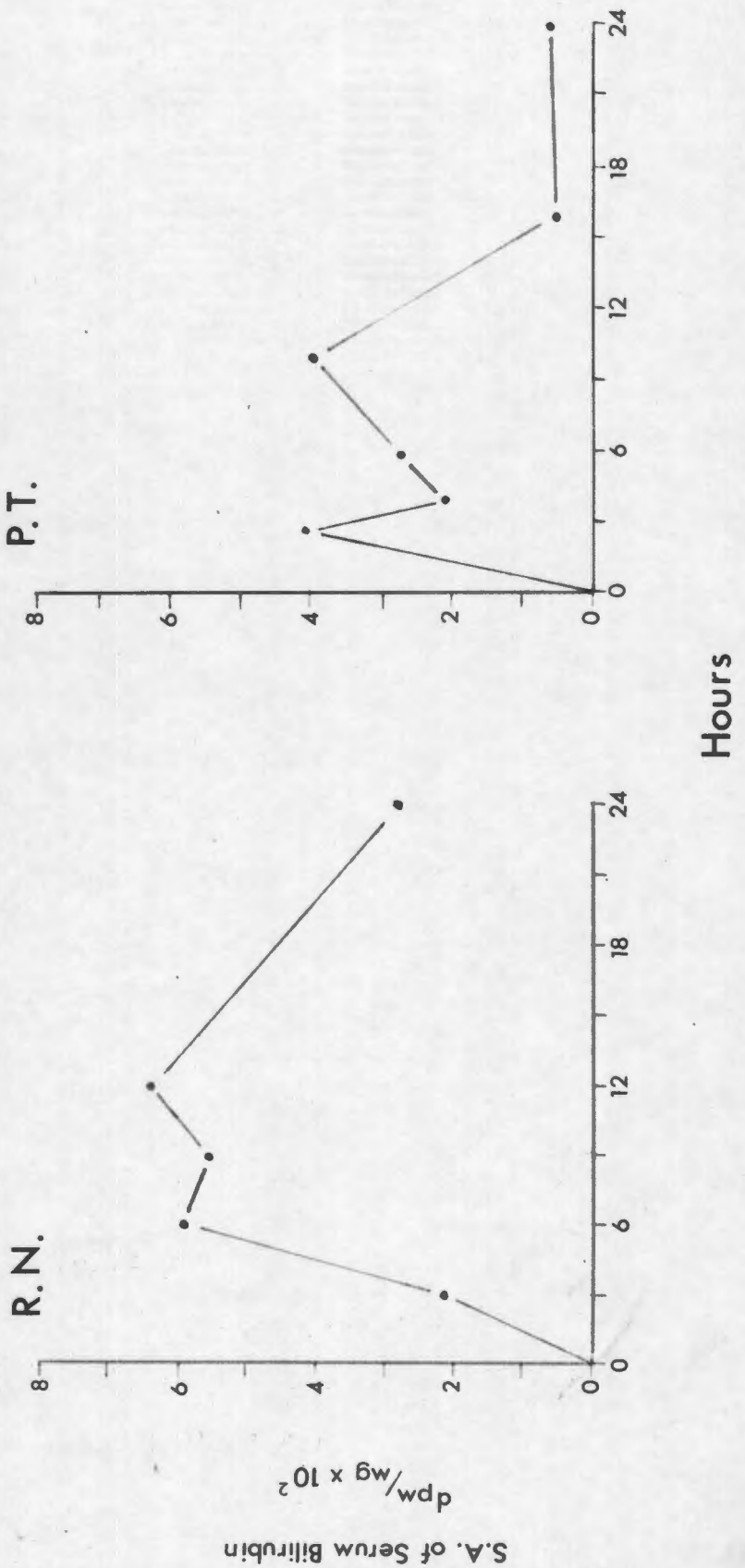
R.N., an African subject had increased skin pigmentation and typical skin lesions of porphyria. He was a heavy drinker and had clinical and biochemical evidence of liver disease. His serum bilirubin averaged 0.78/mg/100 ml (14 values) and his plasma volume was 1.451 litres (measured with chromium-labelled red cells). PCV ranged from 41% to 44%. Laboratory tests confirmed the clinical diagnosis of symptomatic porphyria.

Following the injection of 1.51500 x 10^8 dpm glycine-2-C^{14}, activity appeared in early labelled bilirubin in two peaks. The first (see fig. 7-59) reached a maximum at 12 hours falling thereafter. The second peak with maxima on days 3 and 6 virtually parellelled haem increments. This may be fortuitous rather than physiological, but again the obvious correlation between the 2nd fraction of shunt bilirubin and the maximum increment in circulating haem activity is well shown (fig. 7-60).

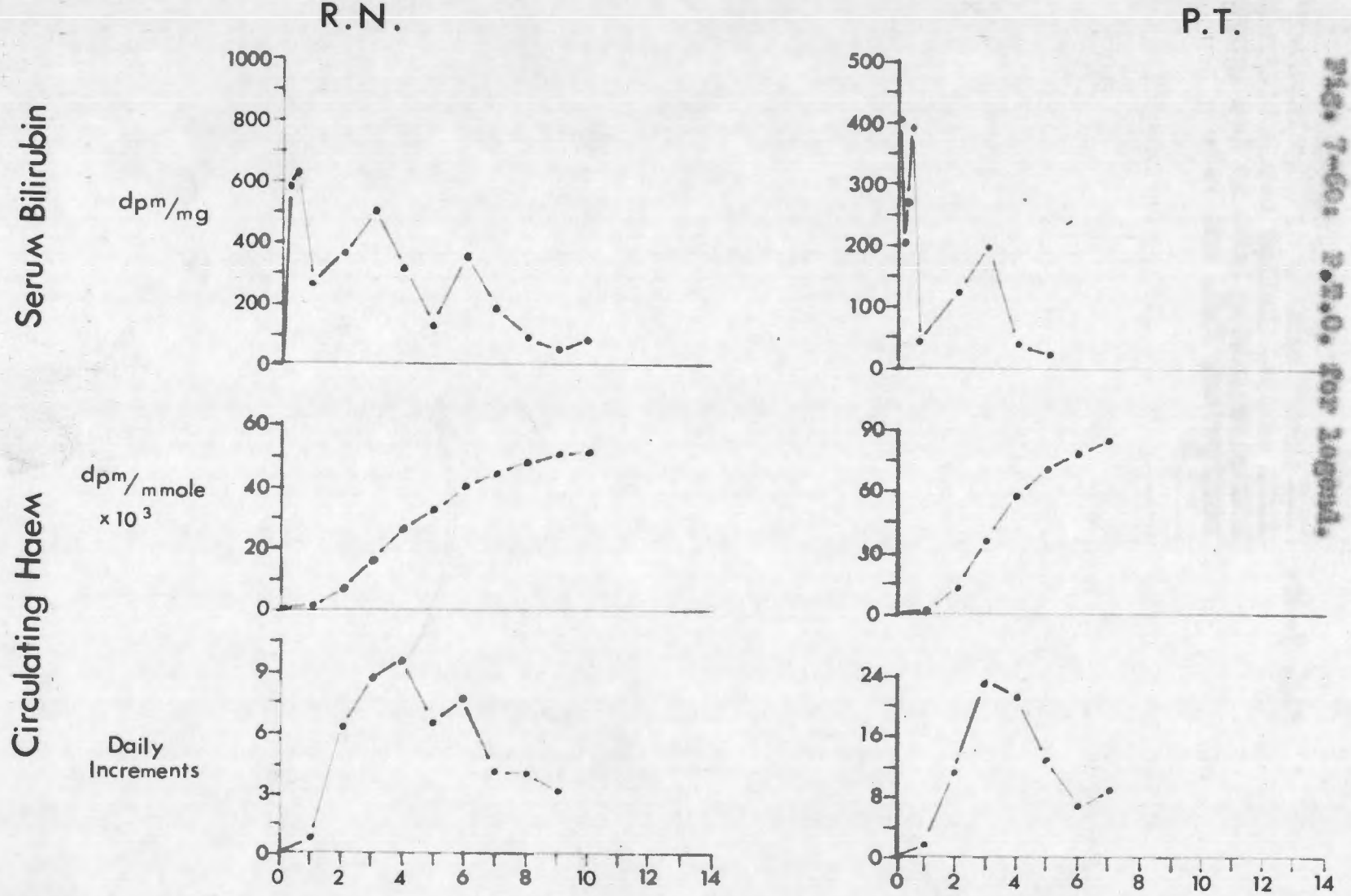
(2) P.T.

P.T., an African male with symptomatic porphyria, was given 1.69562 x 10^8 dpm glycine-2-C^{14} I.V. His results are shown in fig. 7-59 and 7-60.

Quite striking is the bifid 1st peak with maxima at



7-59: The incorporation of glycine-2-C^{14} into the first peak of early-labelled serum bilirubin in two symptomatic porphyrics, R.N. and P.T.


R.N.
P.T.
Serum Bilirubin
dpm/mg
1000
800
600
400
200
0
500
400
300
200
100
0
Circulating Haem
dpm/mmole
x 10 3
60
40
20
0
90
60
30
0
Daily
Increments
9
6
3
0
24
16
8
0
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12 14
Time in days

3 hours and again at 10 hours. Activity falls sharply by 16 hours. (fig. 7-59).

The clear correlation between increments in circulating haem-C^{14} activity and the 2nd fraction of shunt bilirubin is beautifully shown in this experiment (fig.7-60).

(3) T.M.

Patient, T.M., White male aged 46, had suffered from typical porphyric skin lesions for 3 years prior to admission. His alcoholic intake was minimal and he never had any features in the past of acute porphyria. There was no family history of porphyria. On examination, liver edge was palpable but not abnormal. Liver biopsy showed fatty change and patchy mild siderosis. Serum iron was in the normal range (121 ug/100 ml). Bromsulphthalein excretion test, no dye was detected at 45 minutes. Serum bilirubin, proteins and turbidity tests, were normal.

Urinary ALA and PBG levels were 3.2 mg and 1.2 mg/day respectively, concentration of coproporphyrin and uroporphyrin was 586 and 2,232 ug/l respectively. Stool coproporphyrin was 30 ug/G and protoporphyrin 41 ug/G. Repeated tests revealed total stool porphyrin of less than 150 ug/G usually less than 100 ug/G. Thus this patient was a fairly typical case of symptomatic porphyria.

He was given 1.44952 x 10^8 dpm glycine-2-C^{14} and the changes in serum bilirubin-C^{14} were quite remarkable.

Note in fig. 7-61 the massive extent of the first fraction of early-labelled bilirubin. Whereas the specific activity of serum bilirubin at the first peak ranged from 300 to 1,800 dpm/mg in the other patients studied, T.M. displays a specific activity maximum of 6,219 dpm/mg bilirubin within the first 24 hours following glycine administration. Not only is the peak much higher than in other studies but it is broader. An early maximum is noted at 3½ hours, activity then falls slightly and rises to a plateau at about 6,000 dpm/mg lasting from 12 hours to 24 hours. Thus considerable activity is excreted as bilirubin-C^{14} in the first 24 hours.

Another difference from other bilirubin experiments is the apparent absence of a 2nd peak of serum bilirubin-C^{14} activity. The reason is self-evident when studying figs. 7-62 and 7-63. The maximum increment in circulating haem activity occurs early in this patient on days 2 and 3 (fig. 7-62). Newly formed haemoglobin is diluted in the total circulating haemoglobin pool. Assuming a life span of 120 days in the absence of haemolysis, the haem increment multiplied by 120 should give approximately the specific activity of the newly formed circulating haem moiety undiluted. This has been worked out in every experiment and it has been observed that whilst the 2nd fraction of early-labelled serum bilirubin roughly parallels the haem increment, the latter is about 2 to 20 times as active. (See R.N.

fig. 7-63). Thus by knowing the increments in undiluted circulating haem-C^{14} activity, one can visualize the order of magnitude of peak 2 of shunt bilirubin.

Fig. 7-63 expresses all activity as dpm/millimole porphyrin moiety. Note how in T.M. (in contrast to R.N.), the activity in serum bilirubin-C^{14} in the first 24 hours is far in excess to that of the newly formed haem moiety. If one takes approximately 1/3 or less of the haem increment curve which would reflect the 2nd fraction of early-labelled bilirubin, it can be seen that this 2nd peak in T.M. will be completely buried in the 1st component of serum-bilirubin-C^{14}.

Thus, urinary uric acid degradation and serum bilirubin-C^{14} studies have been performed on normals and porphyrics with widely differing results, the significance of which will be discussed in the following chapter.

7-61: The incorporation of glycine-2-^{14}C into the first peak of early-labelled serumbilirubin in M.S., an acute S.A. genetic porphyric and T.M., a symptomatic porphyric.

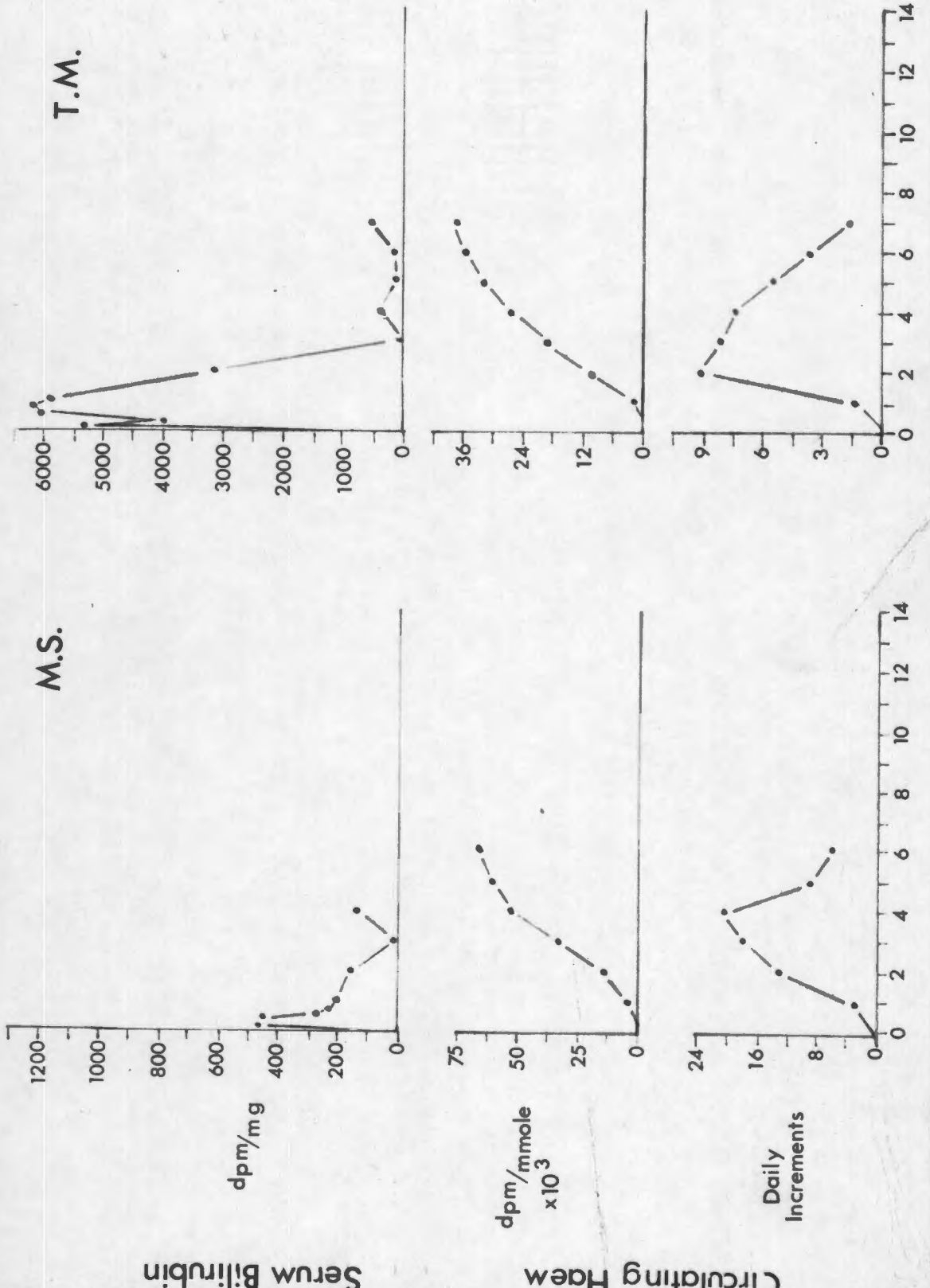


Fig. 7-62: The incorporation of glycine-2-C^{14} into early-labelled serum bilirubin and circulating haem in M.S. an acute S.A. genetic porphyric and T.M., a symptomatic porphyric.

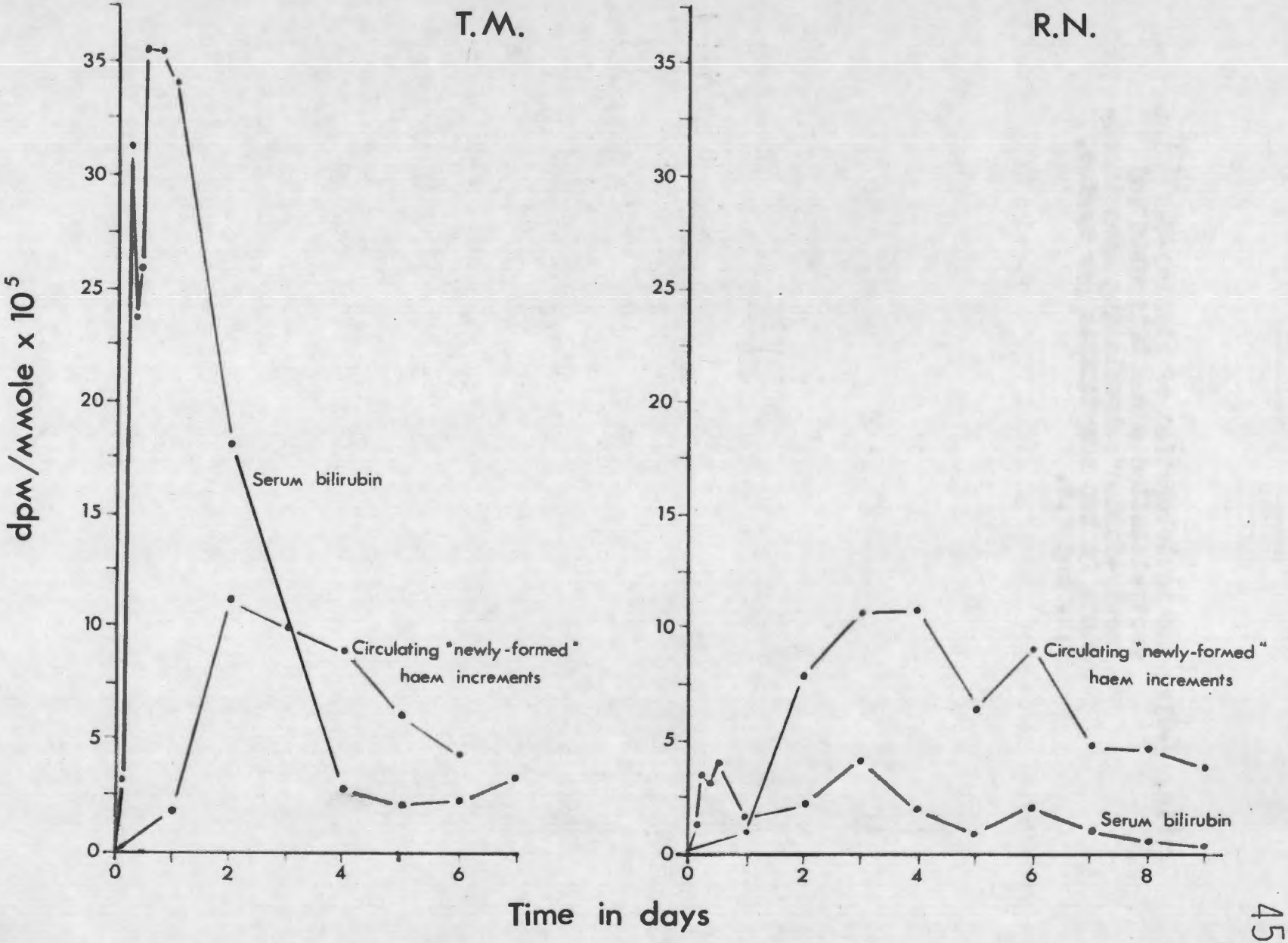


Fig. 7-631 P.T.O. for legend.

C. SOUTH AFRICAN GENETIC PORPHYRIC SUBJECTS.

(1) M.S.

For 20 years, M.S., a White female, had a fragile skin. 3 weeks prior to admission new blisters were noted on the hand and skin and shortly before admission, patient had abdominal pain and passed red urine. On examination, she was ill with upper abdominal pain and had typical porphyric skin lesions. She became disorientated, confused and had hallucinations. At this stage, serum sodium levels were low.

The Urine had large quantities of porphobilinogen (qualitatively) and urinary levels of coproporphyrin and uroporphyrin were 159 ug and 4, 178 ug/L. Stool copro- and protoporphyrin concentrations were 554 and 926 ug/G dry stool. These figures confirmed the diagnosis of acute S.A. genetic porphyria.

When better, liver biopsy was performed and the histology was normal.

This S.A. genetic porphyric received 1.58845×10^8 dpm glycine-2-C^{14} I.V. Peak activity in the 1st fraction of early-labelled bilirubin occurs from 3 to 9 hours (fig. 7-61) and whether there is a confluence of two peaks or not cannot be determined by the experimental techniques.

The 2nd fraction of shunt bilirubin is poorly shown in this experiment but the late kick in serum bilirubin-C^{14}

activity on the 4th day does correspond to the maximum increment in circulating haem-C^{14} activity (fig. 7-62).

(2) A. Jov.

A. Jov has had urinary uric acid degradation and serum bilirubin-C^{14} studies performed during an acute attack. Clinical details have already been presented earlier this chapter (page423).

1.9180 x 10^8 dpm glycine-2-C^{14} were injected and the appearance of the label in serum bilirubin and circulating haem was followed.

The first peak of activity in serum bilirubin-C^{14} occurs at 6 hours, dropping rapidly thereafter (fig. 7-64). Circulating haem-C^{14} activity reaches a plateau after 12 to 14 days and increments in activity correlate well with the 2nd fraction of early labelled bilirubin (fig. 7-65).

(3) Z.M.

Z.M. has also had uric acid and serum bilirubin-C^{14} studies performed during her acute attack of porphyria and details are available earlier this chapter (pg 416).

1.9196 x 10^8 dpm glycine-2-C^{14} were injected I.V. and it can be seen in fig. 7-64 that maximal activity in the first fraction of shunt bilirubin occurs at 3 hours. Thereafter, activity falls slowly to 12 hours and then more rapidly reaching a nadir at 1 to 3 days. Activity starts rising from the 4th to 6th day (fig. 7-65) matching

the ascending limb of the circulating haem-C^{14} increment graph. Once again, the plateau of circulating haem-C^{14} activity occurring by the 10th day is well shown (fig. 7-65).

Fig. 7-64: The incorporation of glycine-2-C14 into the first peak of early-labelled serum bilirubin in two acute S.A. genetic porphyrias, A.J. and Z.M.

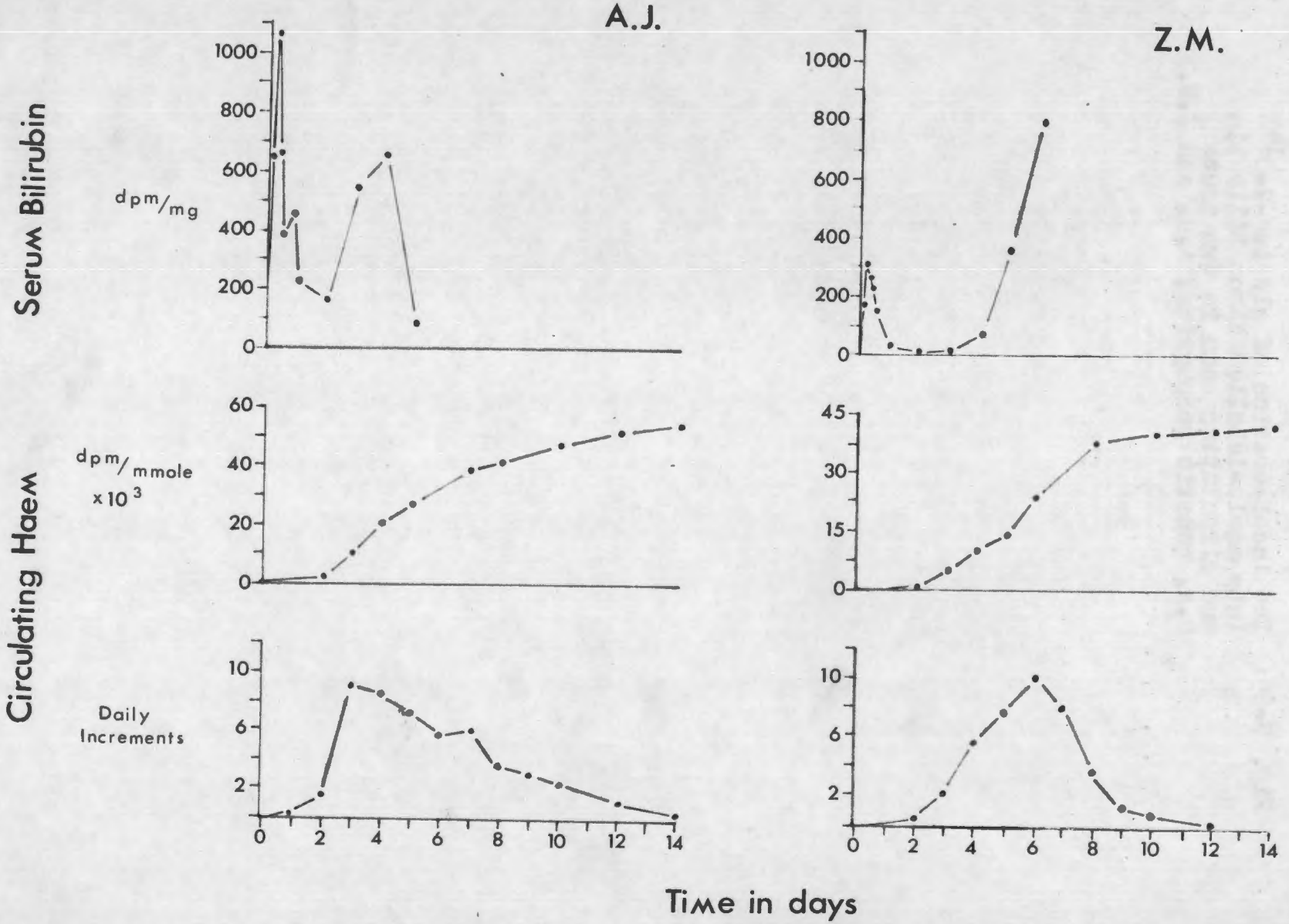


Fig. 7-65. P.T.O. for legend.

TABLE 8-1.

PATIENTS	% INCORPORATION.		
	URIC ACID	C4 + 5	C2 + 8
Normals.			
H.J.	0.210	0.137	0.035
A.J.	0.187	0.124	0.033
A.M.	0.119	0.084	0.020
Mean	0.172	0.115	0.029
Symptom. Porph.			
A.A.	0.239	0.164	0.049
B.P.	0.216	0.161	0.039
W.B.	0.231	0.173	0.046
Mean	0.229	0.166	0.045
S.A.C.P.			
In remission			
A.v.R.	0.167	0.123	0.033
Acute attack			
M.N.	0.200	0.160	0.025
M. de J.	0.154	0.112	0.033
Z.M.	0.121	0.066	0.019
A. Jov.	0.262	0.116	0.034
Mean	0.186	0.114	0.027

Percentage total dose excreted in 14 days in the urine as uric acid, C4 + 5 and C2 + 8.

CHAPTER VIII.

CORRELATION AND INTERPRETATION OF RESULTS.

I. URIC ACID DEGRADATION STUDIES.

The results of these studies have been analyzed with a view to demonstrating differences between normals and porphyrics with regard to:

(a) Percentage dose incorporated in various positions
(b) ratio of C4+5 : C2+8
(c) disposition of radioactivity within the uric acid molecule and
(d) time relations of these three parameters.

A. PERCENTAGE DOSE INCORPORATED IN VARIOUS POSITIONS.

(1) Correlation of data.

Fig. 8-1 and table 8-1 show the percentage total dose excreted in the 14-day period in the urine as uric acid, C4+5 and C2+8 in normals, symptomatic porphyrics and S.A. genetic porphyrics in remission and during an acute attack.

The experimental data from R.B., a normal, L.M., a symptomatic porphyric and C.v.H. a S.A. genetic porphyric in an acute attack, have been rejected for reasons outlined in the previous chapter (see pages 360, 372, 400).

When comparing mean values for each clinical group studied (fig. 8-1), the striking feature is the excessive incorporation of glycine-2-C^{14} into urinary uric acid and

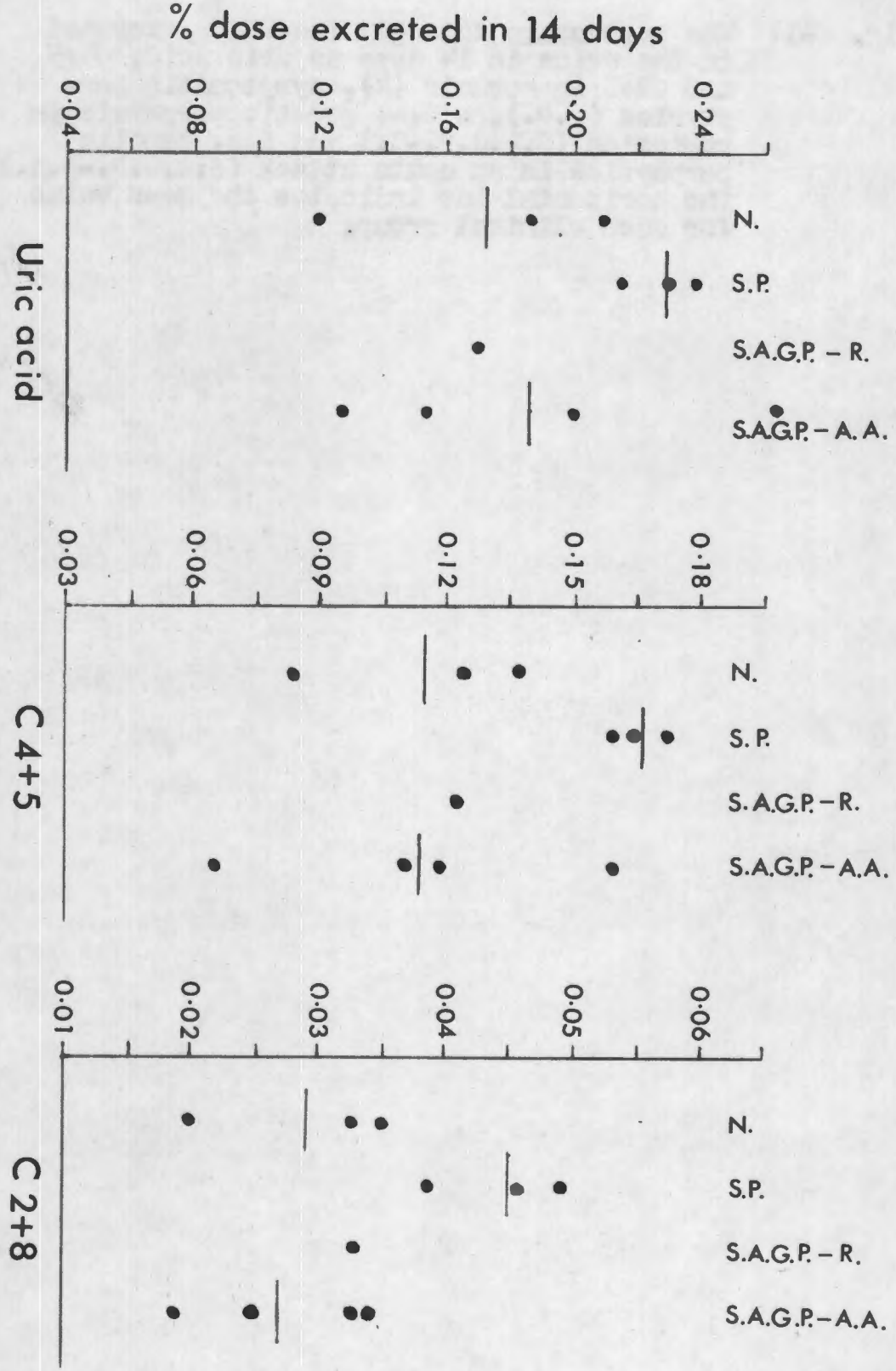


Fig. 8-1: P.T.O. for legend.

its component carbons in the symptomatic porphyrics studied. More relevant, when considering the aims of this thesis, are the findings that differences between normals and S.A. genetic porphyrics are not significant and it is evident that acute porphyrics excrete just as much activity in the urine in C2+8 of uric acid, as do normals. The significance of these observations will now be discussed.

(2) Interpretation.

If the hypothesis of Talman et al (234,437) is tenable in acute human porphyria, one would expect a significantly smaller portion of the administered glycine-2-C^{14} to enter C2+8 of purine moieties synthesized by the liver.

All one is measuring in this thesis is the percentage dose excreted in urinary uric acid and its constituent carbons over 14 days. There are limitations as to what one can infer from these incorporation studies and these have been discussed in detail in Chapter IV, pages 211-213. Two important variables to bear in mind are the relative amounts of urate eliminated via renal and extra-renal channels and the size and rate of turnover of the miscible urate pool. By administering uric acid-N^{15} along with glycine-2-C^{14}, one can allow for these variables along the lines suggested by Seegmiller et al (404). A formula has been devised (see page 213) whereby with this extra information, the corrected glycine incorporation values can be determined.

The experiment on C.v.H., a S.A. genetic porphyric studied during an acute attack, clearly illustrates the difficulties involved in interpreting results. Although an experiment fraught with technical difficulties (see pages 399-400), 6 valid points have been obtained from which one can calculate that over 14 days, approximately 0.014% of the administered dose would have been excreted as C2+8 of urinary uric acid. This value is significantly lower than the mean of 0.025% for this clinical group and the means of 0.029% (normals) and 0.042% in symptomatic porphyrics (see fig. 8-1 and Table 8-1). Does this imply difficulty in incorporating glycine-2-C^{14} into the ureide carbons of purines? Unfortunately, this cannot be inferred as owing to the presence of chronic renal disease with uraemia, significantly less urate will be excreted by the kidney, the bulk probably eliminated via extra renal channels. Secondly, the presence of hyperuricaemia implies a large miscible urate pool, a result of impaired renal excretion of uric acid. With a normal production of urate by the body, the rate of turnover will be low and any newly formed isotopic urate will be considerably diluted. On both counts, the excretion of C2+8 of urinary uric acid will be low without invoking a specific impairment of purine synthesis. Similar arguments could be used (but to a lesser degree) to explain the

lower incorporation values observed in Z.M. (See previous chapter).

The above case exemplifies the importance of knowing what percentage of the total urate eliminated, is excreted by the kidney and what the rate of turnover of the urate pool is at the time of study.

It is not known why symptomatic porphyrics incorporate a greater portion of the administered glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8. It is likely that owing to an intrahepatic obstructive difficulty in excretion of uric acid via the bile in symptomatic porphyria, relatively more urate is eliminated via renal channels, thus accounting for the higher values. This again emphasizes the importance of correcting for extrarenal urate excretion in isotopic glycine incorporation studies.

Even if the corrected incorporation of glycine-2-C^{14} into uric acid, C4+5 and C2+8 were known, there is still the possibility that any changes due to a disorder of liver purine synthesis in acute human porphyria may be masked by normal purine metabolism in other tissues. The importance of the liver in body purine metabolism as a whole has been repeatedly stressed in earlier chapters (see Chapter IV, section C3, pages 166 section E3a, pages 178 to 186; section H1(b), page 207). Basically the liver is rich in nucleotides and nucleic acids with a rapid rate of turnover

and in addition, it elaborates the precursor purine moieties for those peripheral tissues e.g. Bone marrow, dependent on salvage pathways for purine nucleotide synthesis. Work by Dowdle et al (78) on ALA-5-C^{14} has been referred to in Chapter IV, section I(4), pages 234-239. If data from ALA-5-C^{14} experiments do reflect in the main, hepatic purine metabolism, then after the 3rd or 4th day, very little isotopic uric acid is made by the liver. One can infer that following glycine-2-C^{14}, the persistence of a plateau of activity is due, after the 3rd day, to breakdown of purine moieties made in tissues. Other than the liver and that any structural difference in purine moieties made by a porphyric liver may thereby be masked, unless gross.

In an attempt to pick up differences which may otherwise have been masked, Table 8-2 and Fig. 8-2 compare the incorporation values noted 4 days after giving glycine-2-C^{14}. As with 14-day studies, a greater percentage of the administered isotope glycine is excreted in urinary uric acid-C^{14} and its component carbons in the symptomatic porphyric group. There is no significant difference between the mean values of normals and S.A. genetic porphyrics and all one can conclude is that, bearing in mind the variables that can distort and mask changes in incorporation values, no defect in the incorporation of

TABLE 8-2.

PATIENTS	% INCORPORATION.		
	Uric Acid	C4 + 5	C2 + 8
Normals.			
H.J.	0.061	0.036	0.012
A.J.	0.042	0.022	0.007
A.M.	0.026	0.019	0.005
Mean	0.043	0.026	0.008
Sympt.Porph.			
A.A.	0.080	0.051	0.018
B.P.	0.059	0.044	0.012
W.B.	0.068	0.049	0.015
Mean	0.069	0.048	0.015
S.A.G.P.			
In remission			
A.v.R.	0.042	0.031	0.008
Acute attack			
M.N.	0.050	0.034	0.009
Z.M.	0.028	0.017	0.005
A.Jov.	0.065	0.027	0.009
Mean	0.046	0.027	0.008

Percentage total dose excreted in 4 days in the urine as uric acid, C4 + 5 and C2 + 8.

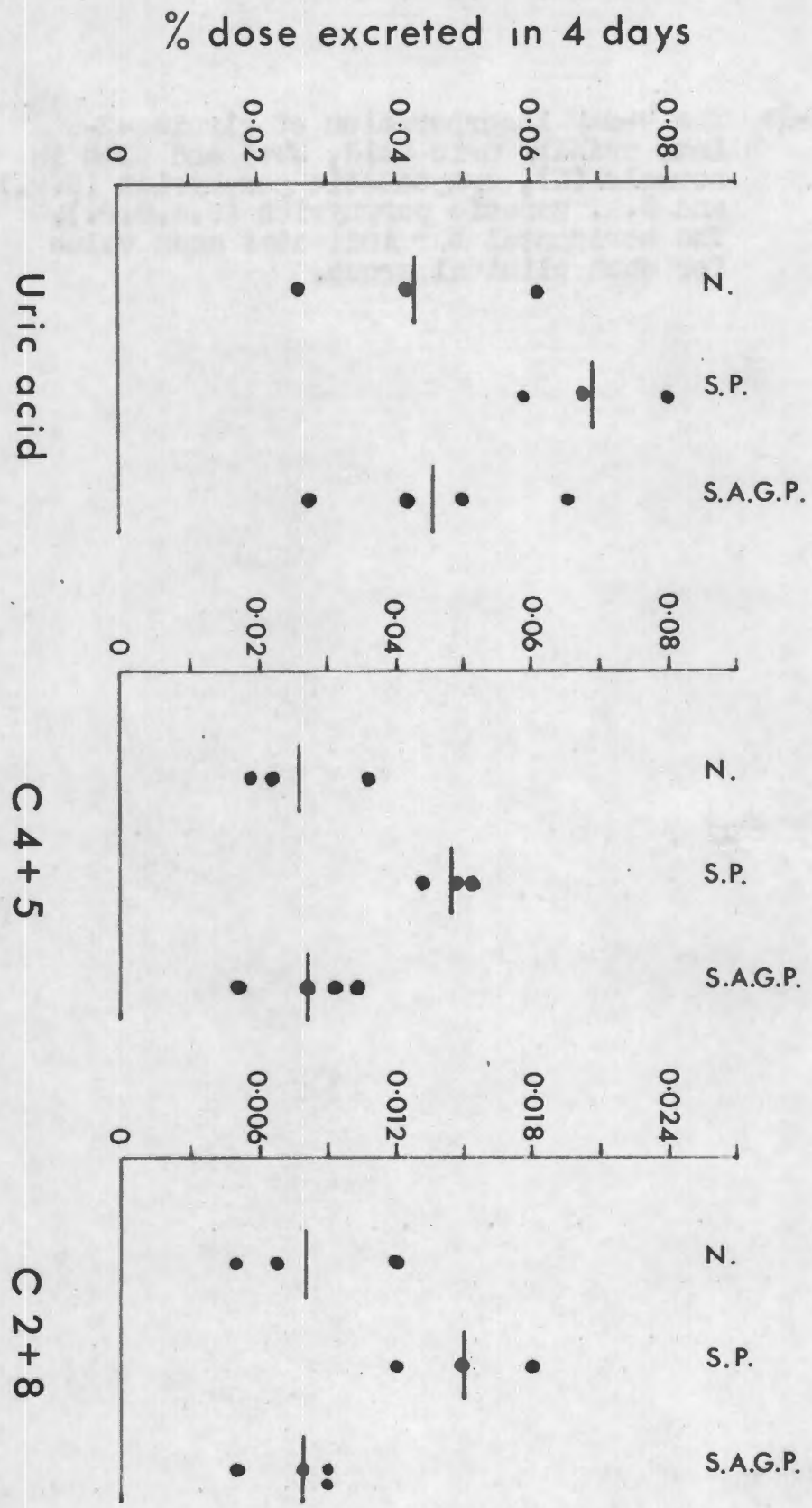


Fig. 8-2: P.T.O. for legend.

glycine-2-C^{14} into uric acid. C4+5 or C2+8 was noted in the S.A. genetic porphyric subjects studied during an acute attack.

B. RATIO OF C4+5 : C2+8.

(1) Correlation of data.

The second parameter of this study is probably the most important, i.e. the ratio of activity in C4+5 to that in C2+8 in the purine moieties of the excreted urinary uric acid.

Fig. 8-3 and table 8-3 show the C4+5 : C2+8 ratios in 13 of the 14 patients studied. No ratios could be calculated from the data of R.B., a normal, as all samples of barium carbonate were contaminated in this experiment (see Chapter VII, page 360).

The black dot in fig. 8-3 represents the average of the daily ratio ± S.E., whilst the horizontal bar in the circle refers to the mean value within each clinical group.

In normals, the mean value is 4 and there is little scatter. In symptomatic porphyrics, the mean value is 3.75 and again there is little variation. The S.A. genetic porphyric studied during remission has a ratio of 3.68. The mean value for the S.A. genetic porphyrics studied during an acute attack is 4.51. However, there is a wide scatter in this group, with M.N. and C. v.H. having high values of more than 6 and Z.M., A.J., and M. de J. having ratios of less than 3.5.

(2) Interpretation of findings.

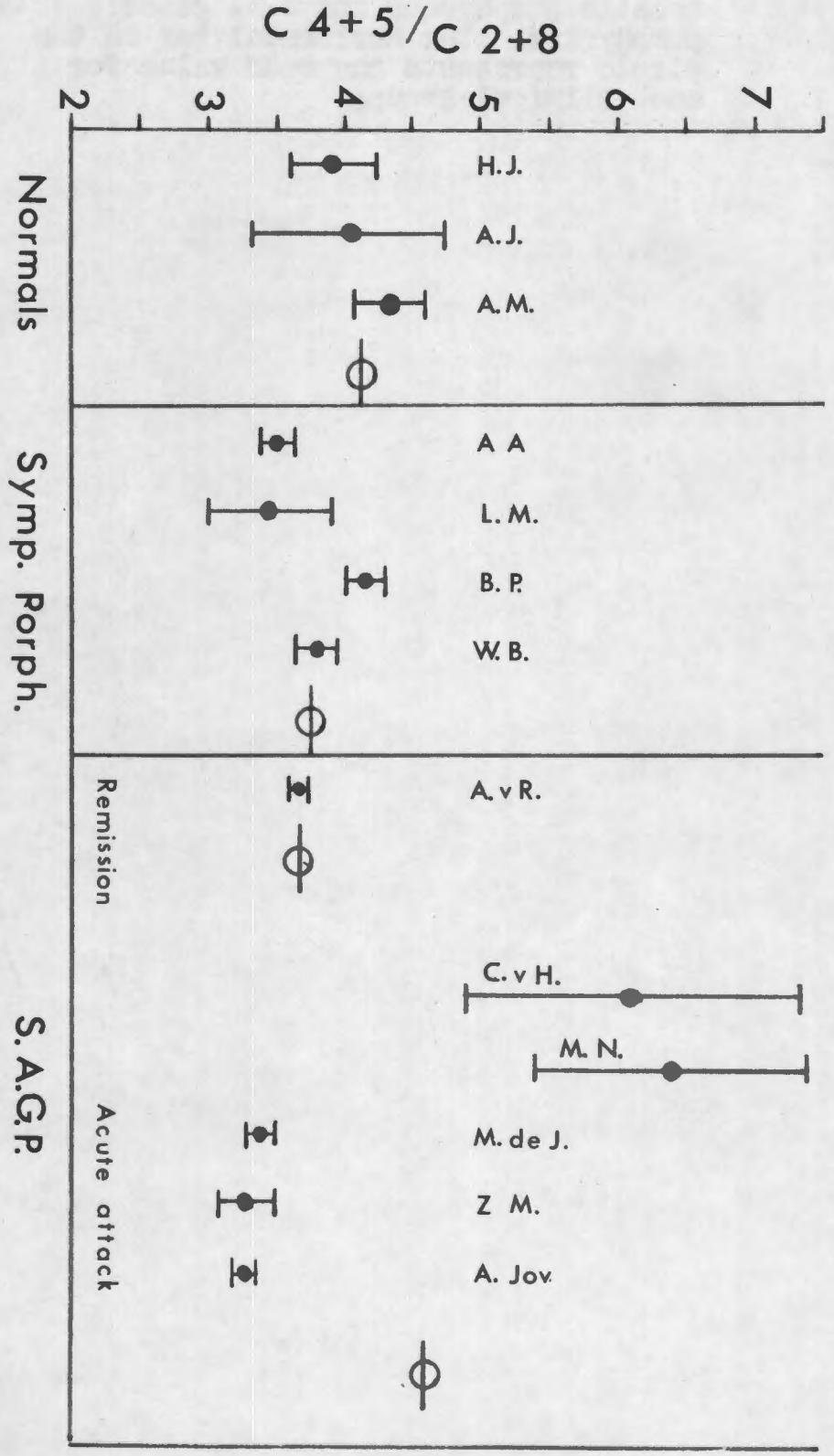
The C4+5 : C2+ 8 ratio is probably the best parameter

TABLE 8-3.

PATIENTS	C4 + S/ C2 + 8	
	Mean for experiment ± S.E. of mean	Mean value for first 4 days of experiment
Normals.		
H.J.	3.91 ± 0.30	3.1
A.J.	4.04 ± 0.72	2.6
A.M.	4.32 ± 0.26	3.9
Mean	4.09	3.2
Sympt.Porph.		
A.A.	3.51 ± 0.15	3.02
L.M.	3.49 ± 0.45	—
B.P.	4.17 ± 0.17	3.5
W.B.	3.82 ± 0.15	3.3
Mean	3.75	3.27
S.A.G.P.		
In remission		
A.V.R.	3.68 ± 0.08	3.8
Acute attack		
C.v.H.	6.12 ± 1.24	8.65 (first 2 days)
M.N.	6.42 ± 1.01	3.4
Mlle J.	3.39 ± 0.11	—
Z.M.	3.31 ± 0.22	3.1
A. Jov.	3.30 ± 0.08	3.02
Mean	4.51	4.54

Ratio of C4 + S/02 + 8.

Fig. 8-3: P.T.O. for legend.



of study as if there is any difficulty in the incorporation of the ALA-5-C into purines, relatively less activity will reside in C2 and 8 and the ratio will be high.

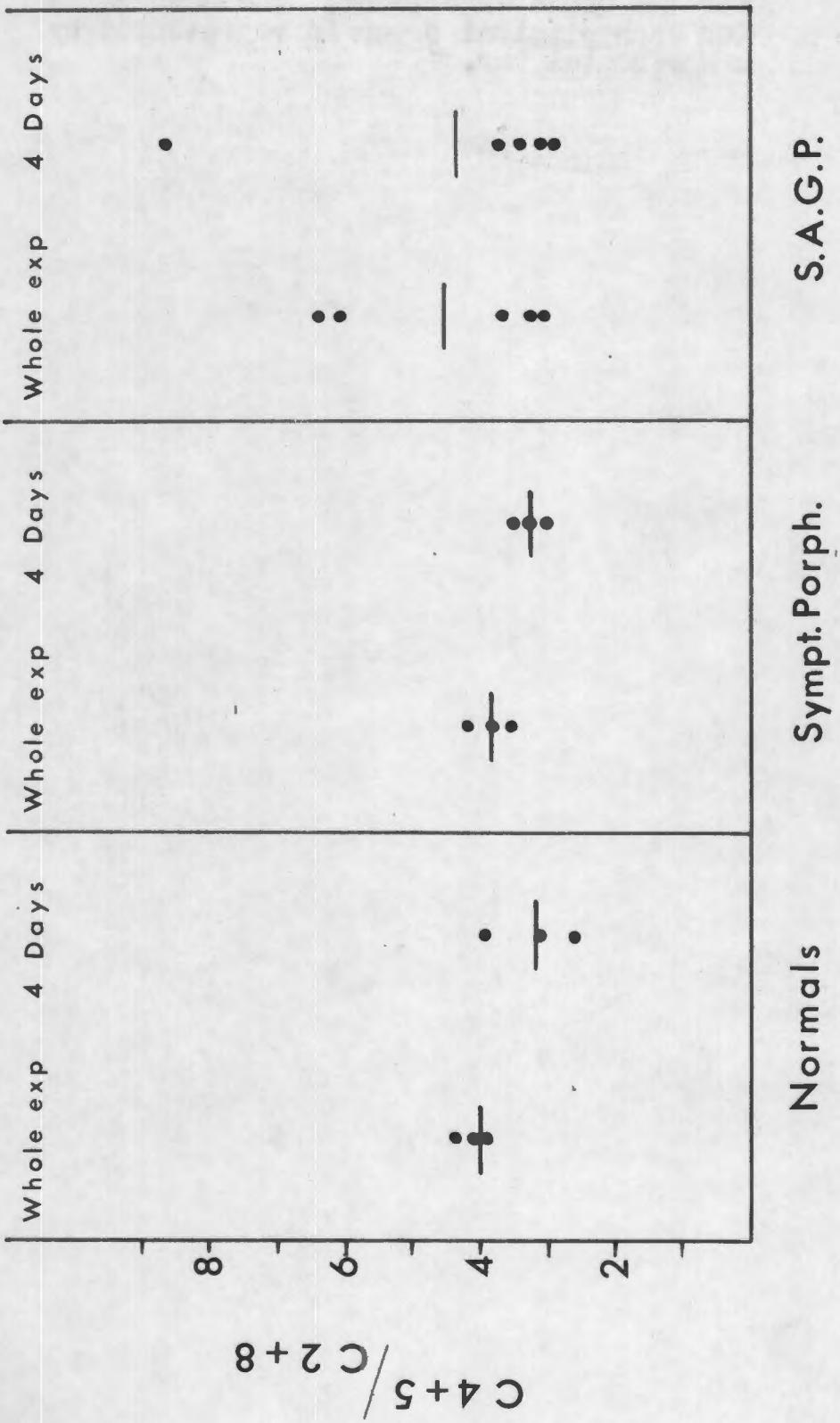
Thus it does not matter whether the urate pool turnover rate is high or low; nor will impaired renal or extrarenal excretion of uric acid influence the relative not absolute activity excreted as C2+8.

The only variable to contend with is to decide how much uric acid examined is derived from purine moieties made in the liver and how much comes from catabolism of purine compounds elaborated elsewhere. Presumably, if the porphyric liver cannot incorporate the δ carbon of ALA into purines, only the former source of uric acid will have C4+5 : C2+8 ratios which are high.

Interpreting 14 day ratios, there is no significant difference between mean values within each group. However, whilst with a wide variation in the acute porphyric group, no consistent abnormality emerged to support the hypothesis of Talman, Gajdos and their co-workers, the ratios of M.E. and C.v.H. are high and warrant explanation.

I can find no way of explaining the high ratios observed in C.v.H. It was a poor experiment (see pages 394-491) and one may venture technical inaccuracies as the cause but the 6 degradation experiments accepted were thought to be valid and in 5 of the 6, the ratios were significantly high (see fig. 7-34). It must be recorded

Fig. 8-4: P.T.O. for legend.



as an observation which is not consistently present in the other acute porphyrics studied.

The C4+5 : C2+8 ratios of M.N. (fig. 7-39) are interesting. In the first 4 days they are well below the mean averaging 3.45 and the last 4 days they are higher. This reflects relatively more activity in C2+8 early on in the experiment, an observation that will be discussed later this chapter.

Which of the ratios must one accept? The mean for the first 4 days is in the normal range; the mean over 9 days is significantly high.

It is pertinent to refer to the discussion on the ALA-5-C^{14} experiments of Dowdle et al (78) on pages 235 to 239, Chapter IV. If it is valid to assume that a major difference between ALA-5-C^{14} and glycine-2-C^{14} data is that the former reflects liver purine metabolism and the latter purine metabolism in all tissues of the body, then after 4 days, the liver is not a major source of isotopic uric acid following glycine-2-C^{14}. Thus ratios observed over the first 4 days appear more pertinent to the present discussion than mean values for the whole experiment.

4-day mean ratios for 11 patients studied (table 8-3) are set out graphically in fig.8-4. C.v.H. (2 values) still has high ratios whilst M.N. is now well within the normal range.

One can conclude that acute porphyrics show no difficulty in labelling C2+8 relative to the facility with which C4+5 atoms are labelled following the administration of glycine-2-C^{14}.

I feel that this is reasonably good evidence that in acute human porphyria, there is no specific defect in the incorporation of the δ carbon of ALA into purine moieties elaborated by the liver.

C. DISPOSITION OF RADIOACTIVITY WITHIN THE URIC ACID MOLECULE.

(1) Correlation and interpretation of findings.

Fig. 8-5 and table 8-4 show how much activity within the uric acid molecule comes from C2+8 and how much from C4+5 in normal and porphyric subjects.

In normals, an average of 17.68% of the total activity resides in C2+8 and 68.95% in C4+5 again emphasizing the approximately 4:1 ratio discussed previously. Similar observations can be made in the other cases studied.

While no significant difference exists between mean values of normals and porphyrics, it is interesting to note that in C.v.H., only 9.5% of her molecule's activity resides in C2+8. This ties up with her high C4+5 : C2+8 ratio of over 6, which has already been discussed in section B, this chapter.

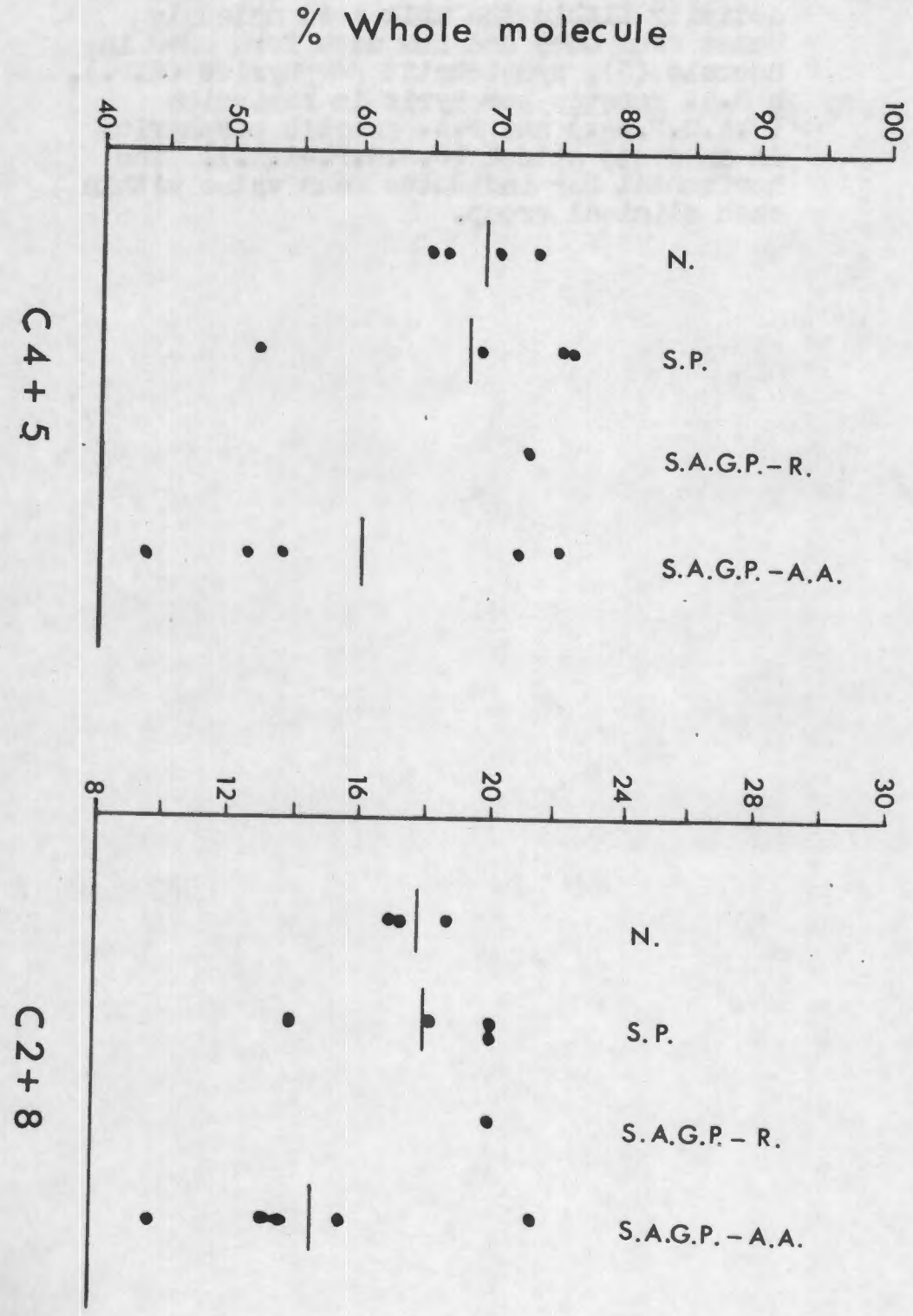
The only extra information arising from a study on the disposition of radioactivity within the molecule is that activity not accounted for by C2+4+5+8, presumably in C6, comprises about 15% of the activity in normals and symptomatic porphyrics and about 25% in the acute porphyrics studied. The significance of this finding is uncertain. Dowdle et al (76,77) have not shown any increased production of isotopic respiratory CO_2 following the administration of glycine-2-C^{14} to S.A. genetic porphyric subjects in an acute attack.

TABLE 8-4.

PATIENTS	Percentage total molecule's activity.	
	C4+5	C2+8
Normals		
H.J.	66.40	16.88
A.J.	65.38	18.80
A.M.	70.60	17.37
R.B.	73.40	—
Mean	68.95	17.68
Sympt.Porph.		
A.A.	69.28	20.11
L.M.	52.12	13.93
B.P.	74.80	18.31
V.B.	75.48	20.09
Mean	67.92	18.11
S.A.G.P.		
In Remission		
A.v.R.	72.67	20.10
Acute Attack		
C.v.H.	53.67	9.85
M.N.	75.19	13.68
M.de J.	72.93	21.55
Z.M.	50.88	15.59
A.Jov.	43.26	13.19
Mean	59.19	14.77

Disposition of radioactivity within the uric acid molecule. The values above are the mean of the daily values for the whole experiment.

Fig. 8-5: P.T.O. for legend.



D. TIME RELATIONS OF PARAMETERS A, B and C.

(1) Correlation of data.

If one refers back to the previous chapter in which the percentage dose excreted daily and cumulatively as uric acid and its component carbons was presented graphically, a fairly constant set of excretory patterns emerge.

In the main, the patterns observed in this thesis compare closely with the reported data of Gutman et al (165,168,170), Wyngaarden et al (490,492), Dowdle et al (76,77), Seegmiller et al (404), Benedict et al (18,19) and others (30,308). All have observed a rapid incorporation of isotopic glycine into uric acid reaching peak values 1 to 3 days after administration followed by a slowly declining plateau of activity over the ensuing few weeks. Expressed cumulatively, activity rose progressively over their experiments with no tendency for graph to flatten out as after ALA-5-C^{14} administration (78). Similar results have been obtained following ammonia-N^{15} (168) and formate-C^{14} (230,430,451) but not with ALA-5-C^{14} (78) and AIC-4-C^{14} (403,493).

The genesis of promptly-labelled uric acid and the plateau of excreted activity that follows has been discussed at length in Chapter IV, pages 207 to 210 and is very relevant to the present discussion. Also important are the observations of Dowdle et al (78) that after ALA-5-C^{14}, only prompt labelling occurs with no succeeding plateau

Chapter IV, pages 234-239). All through previous chapters and in earlier sections this chapter, it has been stressed that the liver, being the seat of rapidly turning over purine nucleotides and ribonucleic acids, is probably not the source of the bulk of isotopic purine moieties oxidized to uric acid after 4 days of giving a labelled precursor. Consequently, data obtained during the first few days after glycine-2-C^{14} administration may reflect precisely what this thesis aims to study, i.e. uric acid derived from those purine moieties elaborated de novo by the liver.

In the 14 cases studied, the expected pattern of excretion of urinary uric acid-C^{14} was observed. In all the cases, the early peak was noted to occur 1 to 3 days after injecting glycine -2-C^{14} except in A.v.R. and M.N. where the plateau was only reached on day 4. There was no significant difference between normals and porphyrics.

In AJ., A.M. and A.A. (figs. 7-5, -9 and -15 respectively, Chapter VII), a secondary peak of excreted urinary uric acid-C^{14} activity was observed after the 9th day. This secondary "hump" was reported in proliferative haemopoietic disorders (248,428,492,501) and thought to be due to increased nucleic acid turnover by the abnormal cells (Chapter IV, pages 207/210). There was no erythroid or myeloid overactivity in the above 3 subjects, two of whom

were normal, and one (A.A.) a symptomatic porphyric.

An unexpected and interesting observation was that whilst the activity excreted as C4+5 both daily and cumulatively roughly paralleled the uric acid-C^{14} curve of excretion, there was a distinct early hump in C2+8 activity, usually during the first 3 days of giving glycine-2-C^{14} and most marked in the S.A. genetic porphyric, M.N. (fig. 7-37). This accounted for the significantly lower C4+5 : C2+8 ratios over the first 4 days in the subjects studied compared to the mean ratios for each of the experiments (fig. 8-4) and for the relatively higher C2+8 incorporation values after 4 days (fig. 8-2) than after 14 (fig. 8-1).

This disproportionate early enrichment of C2+8 was not observed with C4+5 as is clearly shown in table 8-5 and fig. 8-6, 8-7. Note how the disposition of radioactivity within the uric acid molecule differs on day 1 from mean values for the experiment. Whilst no difference is noted between day 1 and mean values for C4+5 (fig.8-6) a consistently greater portion of the uric acid-C^{14} activity resides in C2+8 on day 1 than average for the study (fig. 8-7).

(2) Interpretation of findings.

Why is there disproportionate enrichment of carbons 2 and 8 of the uric acid-C^{14} moiety during the first few days of giving glycine-2-C^{14}?

TABLE 8-5.

PATIENTS	Percentage total molecules activity.			
	C4+5		C2+6	
	Mean for experiment	Day 1	Mean for experiment	Day 1
Normals				
H.J.	66.40	63.97	16.88	23.57
A.J.	65.38	61.84	18.80	32.56
A.M.	70.60	73.82	17.37	22.06
Mean	67.46	66.54	17.68	26.06
Sympt.Porph.				
A.A.	69.28	78.92	20.11	26.94
B.P.	74.80	68.13	18.31	24.67
W.B.	75.48	66.80	20.09	28.14
Mean	73.19	71.28	19.50	26.58
S.A.C.P.				
A.V.R.	72.67	70.59	20.10	23.49
C.V.H.	53.67	88.67	9.85	7.66
M.N.	75.19	70.38	13.68	21.40
Z.M.	50.88	28.22	15.59	19.79
A.Jov.	43.26	52.25	13.19	17.85
Mean	59.13	62.02	14.48	18.04

Disposition of radioactivity within uric acid molecule. A comparison of values on day 1 with mean values for each experiment.

Fig. 8-6: P.T.O. for legend.

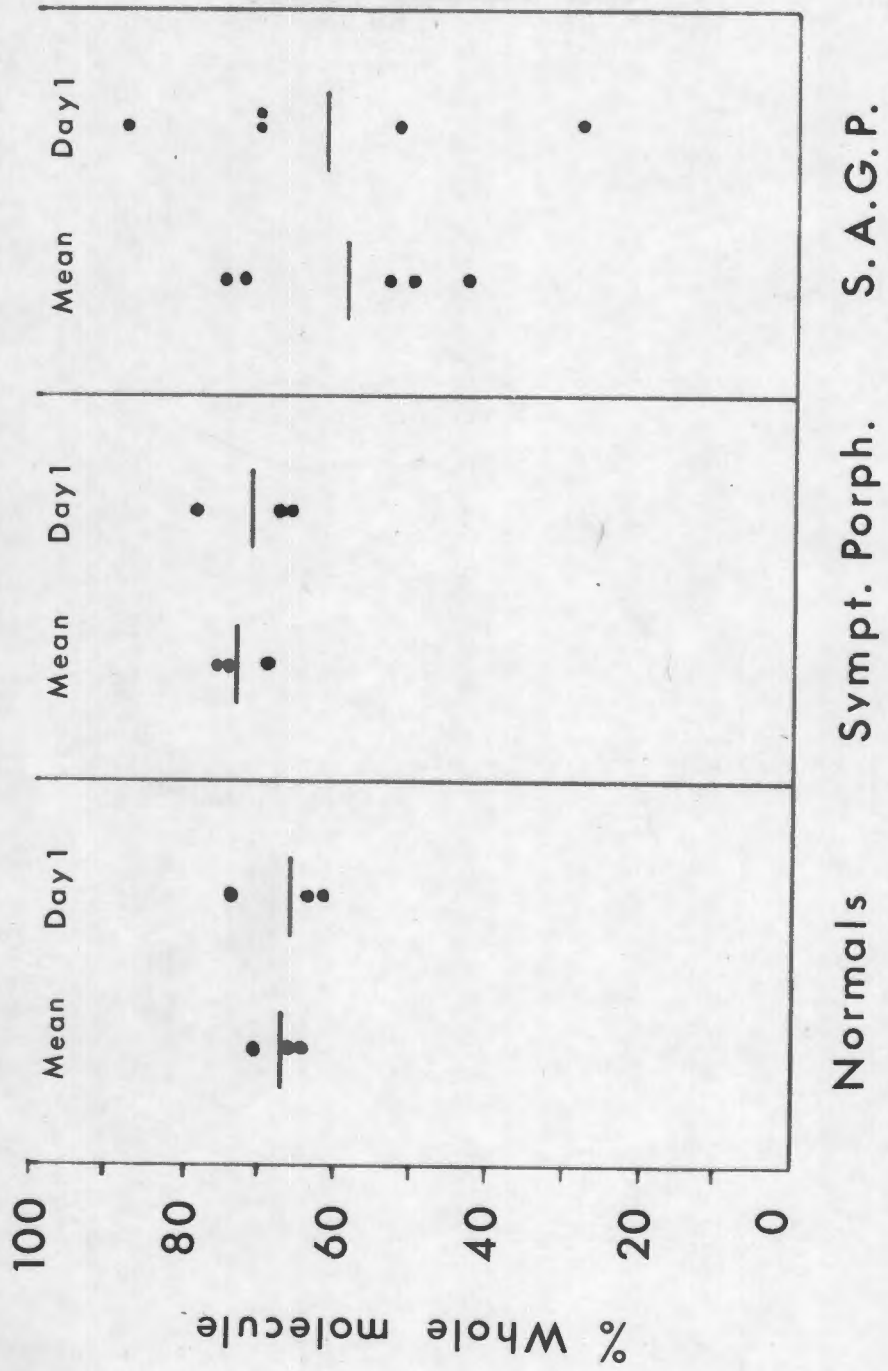
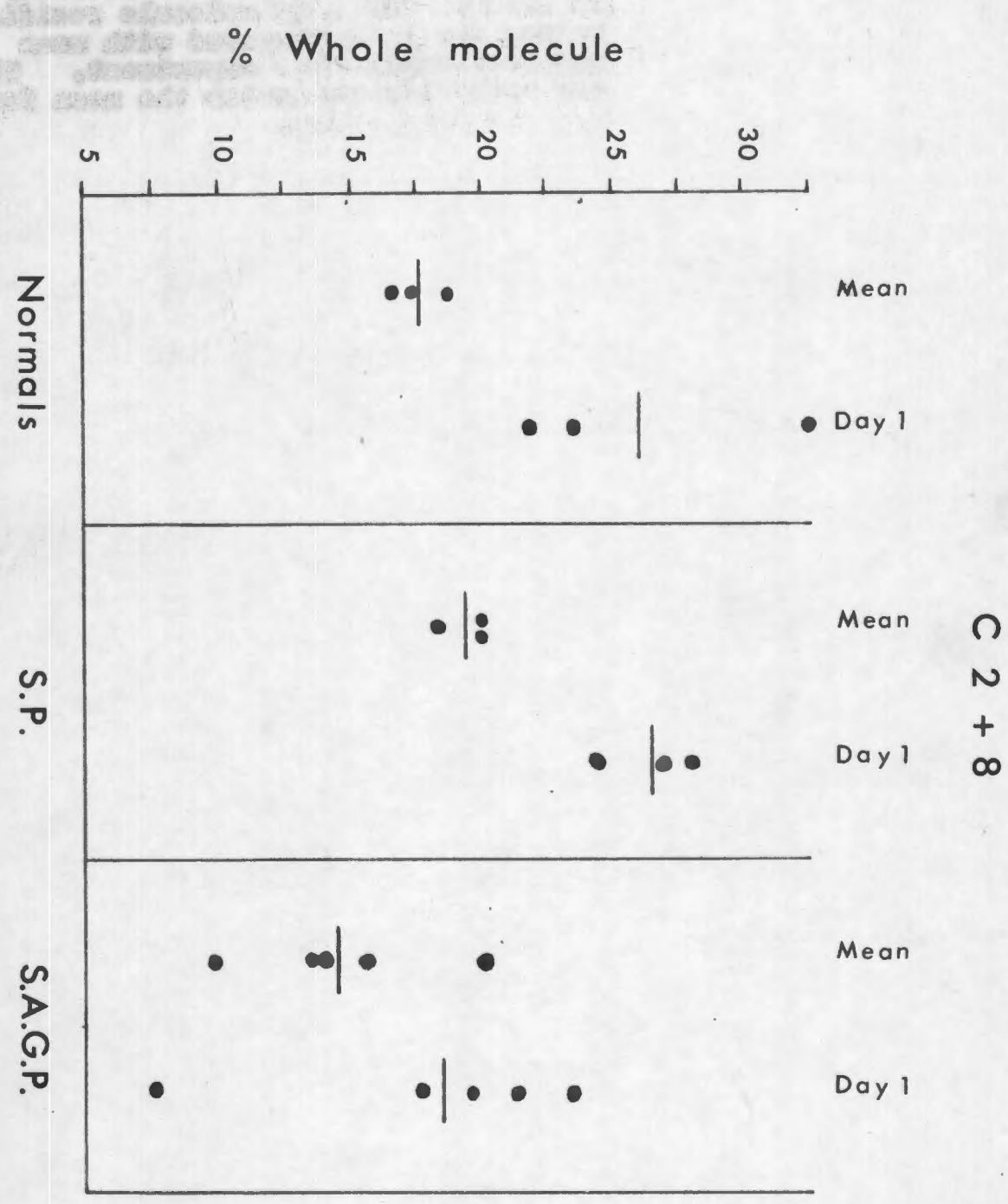


Fig. 8-7: P.T.O. for legend.



In Chapter IV, pages 215 to 219 , the rapid turnover of the glycine pool was stressed. There emerged the concept of a single "pulse" of activity in the body following the administration of labelled glycine reaching peak values well within 24 hours and of little magnitude after 48 hours. Thus isotopic uric acid will be derived, in the main, from purine compounds synthesised mainly within the first 12 to 24 hours of injecting glycine.

Following the administration of glycine-N^{15} (see Chapter IV, pages 226/228 for details) any enrichment of aspartic acid or glutamine will be derived from that $N^{15}H_3$ arising from the oxidative deamination of glycine. One would expect that the first isotopic purine moieties elaborated would have most N^{15} activity in N-7 (from glycine) whilst those made later would have an increasing proportion of the total molecule's activity residing in those nitrogen atoms derived from aspartic acid and glutamine i.e. N1+3+9. Gutman et al (168,170), Shemin et al (413) and Howell et al (196) all demonstrated quite clearly that with time, N1+3+9 comprised a progressively greater percentage of the total molecular-N^{15} activity (see Chapter IV, pages 227 and 228). Thus, the changing disposition of N^{15} activity within the uric acid-N^{15} moiety observed over days, may well reflect

what is happening over hours in the various purine precursor pools following glycine-N^{15}.

There are important analogies to be drawn from the above observations. Immediately after glycine-2-C^{14} administration, the only labelled precursor will be glycine. With time, other small molecule precursors, e.g. C-1 fragments will be increasingly labelled. The labelling of one-carbon units by glycine-2-C^{14} is a complex situation with at least 3 pathways potentially operative (Chapter IV, page 225)although Nemeth et al (319) suggest that the route via the β carbon of ALA is probably the most important one. It is feasible therefore that while the activity in the isotopic glycine pool varies in the manner described in Chapter IV, that in the one-carbon unit pool may not change in a parallel way. Isotopic activity in C-1 fragments may be relatively higher than glycine-2-C^{14} moieties after 1 hour than, for example, three hours later and this may be the explanation for the disproportionate enrichment of C2+8 in those uric acid molecules isolated from the urine in the first few days after injecting glycine-2-C^{14}.

Another explanation has already been alluded to throughout this chapter. The plateau of activity following prompt enrichment of uric acid after isotopic glycine is thought to be the composite effect of uric acid produced by the catabolism of nucleic acids of varying turnover in many

different tissues (see Chapter IV, pages 208 and 209). The liver, with its rapidly turning over purine nucleotides and nucleic acids is almost certainly a major source of early labelled uric acid.

The disproportionate enrichment of C2+8 is also only observed in this uric acid fraction. Is this merely a reflection of what is happening in the liver as opposed to other tissues? In the liver, the glycine-2-C may be a more important formyl donor or the one-carbon unit pool may turn over more rapidly than other tissues. By either mechanism, newly formed isotopic C-1 fragments derived from glycine-2-C^{14} may be of higher activity than elsewhere, thus labelling C2+8 more extensively.

Whatever explanation one accepts, two points have emerged.

(a) Disproportionate enrichment of C2+8 but not C4+5 has been noted in the first few days of giving glycine-2-C^{14} to both normals and porphyrics.

(b) No difference in the early or overall pattern of uric acid-C^{14}, C4+5 or C2+8 excretion has been observed between normals and the forms of human porphyria studied in this thesis.

II. SERUM BILIRUBIN-C^{14} STUDIES.

The pattern of C^{14} incorporation into serum bilirubin following the administration of glycine-2-C^{14} has been studied in normal and porphyric human subjects. Results, which have been presented in the previous chapter, will be discussed under the following headings:-

(a) The first fraction of early-labelled bilirubin

(b) The second fraction of early-labelled bilirubin

(c) The serum bilirubin-C^{14} pattern in S.A. genetic and symptomatic porphyrias.

A. FIRST FRACTION OF EARLY-LABELLED BILIRUBIN.

(1) Timing of peak activity.

In the 5 normal subjects studied, the early peak of activity occurred from 1½ to 12 hours after giving glycine-2-C^{14}. Whilst O.M. and C.R. both exhibited an early spike of activity after 1½ hours, in the latter, there was a double kick in activity, the second rise at about 12 hours (fig. 7-55). The early peak occurred at 3 hours (F.J.), 6 to 9 hours (P.R.) and 12 hours (D.D.) in the other normal studies (figs. 7-53, -57).

Of the three symptomatic porphyrics studied, R.N. exhibited maximal activity at 6 hours whilst as with C.R. above, P.T. had a bifid early peak with maxima at 2½ hours and 10 hours (7-59). T.M. had a bifid peak at 3 and 9 hours (fig. 7-61).

In the S.A. genetic porphyrics studied, Z.M. and M.S. exhibited peak values at 3 hours and A.Jov. at 6 hours. (Fig. 7-61,7-64).

(2) The extent of the first peak.

It is easy in bile fistula animals to quantitate the amount of activity excreted in bilirubin over a given period. However, with serum bilirubin C^{14} studies, as the serum bilirubin pools are not known, one can only roughly estimate the extent of the various fractions of early-labelled bilirubin, using such crude guides as the height of the peaks and their shape and duration. Thus only gross differences between patients are worthy of comment.

In the normal subjects studied, there was a wide variation in the size and shape of the early component of shunt bilirubin. In D.D. and C.R. not only was maximal specific activity of serum bilirubin-C^{14} high (1,787 dpm and 1,238 dpm/mg respectively) but activity was sustained for 24 to 48 hours. In D.D., the second peak was dwarfed by the first whilst in C.R., the experiment was not conducted long enough for the second peak to be fully developed. By contrast, F.R. exhibited an early peak whose maximal activity was 344 to 353 dpm/mg extending from 1½ to 15 hours and not dwarfing the second peak. One could argue that the bilirubin pool in this patient may have been large with a slow rate of turnover

accounting for the relatively small amount of activity present in early-labelled serum bilirubin. All one can say is that F.R. was of average weight (128 lbs.) and his serum bilirubin in normal limits (0.5 to 1 mg.) The other two normal subjects O.M. and E.J. had serum bilirubin-C^{14} patterns falling in between the two extremes described above.

Two of the symptomatic porphyric subjects, R.M. and P.T. had similar curves, the activity being elevated for about 12 to 18 hours with maxima of 400 to 600 dpm/mg. The second peaks although of the same height were much broader.

By contrast, T.M. exhibited an early peak of much greater magnitude than other patients studied. There was a bifid early peak with maxima at 3 hours (5,398 dpm/mg) and from 12 to 24 hours of over 6,000 dpm/mg bilirubin. High levels of activity were still present after 48 hours (3,130 dpm/mg. and the absence of the 2nd peak (buried in the first) has already been commented on in Chapter VII, page 447 (448.). Fig. 7-63 has been reproduced in fig. 8-8 to illustrate the enormous difference between the serum bilirubin pattern of T.M. and that of R.N., another symptomatic porphyric whose early peak is of a similar order of magnitude as those of other patients studied. Note how whilst peak 1 of early-labelled serum bilirubin dwarfs the newly-formed circulating haem

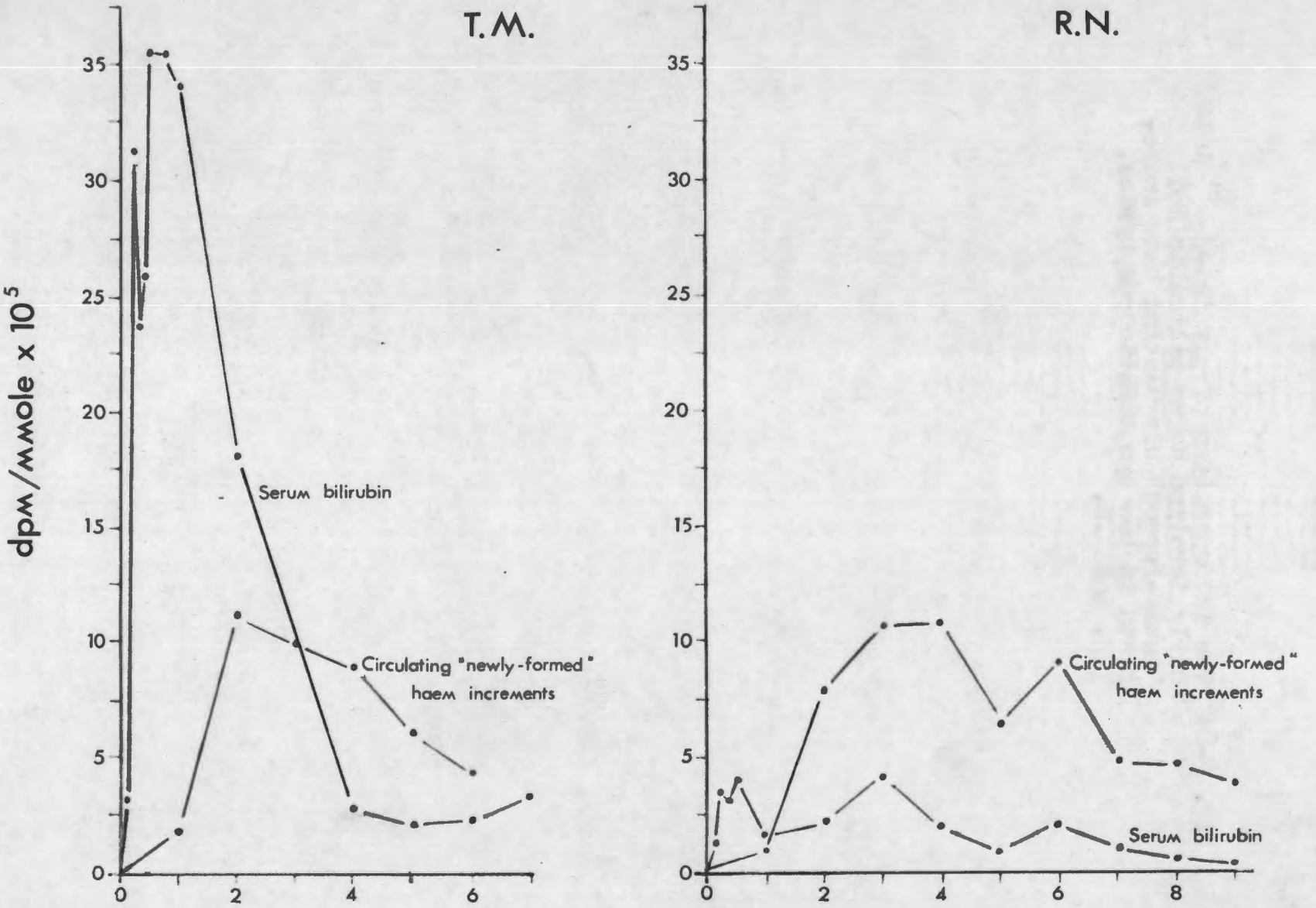


Fig. 8.8: Plot for Patient

increment curve in T.M., the situation is reversed in R.N.

In the three cases of acute S.A. genetic porphyria studied, the serum bilirubin-C^{14} patterns showed no remarkable features. Z.M. exhibited a puny first peak with a maximum of 301 dpm/mg bilirubin by 3 hours, falling to zero by 15 hours. A. Jov. and M.S. showed more activity than Z.M. but not nearly to the same degree as T.M. Their observed patterns were comparable to those of normal and symptomatic porphyrics other than T.M.

(3) Summary of results.

The essential features that have emerged from my study on serum bilirubin-C^{14} patterns in normal and porphyric subjects receiving glycine-2-C^{14} are:-

(1) That maximal activity occurs in the early component of shunt bilirubin 1½ to 12 hours after giving glycine,

(2) that in 3 cases, C.R. a normal, P.T. a symptomatic porphyric and T.M. a symptomatic porphyric, there is a distinct bifid early peak, the first rise occurring 1½ hours to 3 hours and the second 9 to 12 hours after glycine administration, and

(3) that there is a wide variation as to the extent to which glycine-2-C^{14} is incorporated into peak 1 of early-labelled bilirubin in all clinical groups most marked in T.M. , a symptomatic porphyric and least in an acute S.A. genetic porphyric, Z.M.

(4) Interpretation.

(a) Timing of maximal activity in peak 1.

The various patterns of C^{14} excretion in early-labelled stercobilin, bile bilirubin and serum bilirubin following glycine-2-C^{14} or ALA-C^{14} have already been described, discussed and interpreted in detail in Chapter III, pages 117-125.

Israels and his group (205,207,498) report maximal activity 12 to 24 hours after glycine-2-C^{14}. All the cases discussed in these papers are presented in more detail by Yamamoto, Skanderberg, Zipursky and Israels (498) where in 3 normals, there was a single early peak of serum bilirubin-C^{14} activity at about 12 hours, activity falling rapidly thereafter. In two cases of pernicious anaemia, where in addition to abnormal erythropoiesis, disorder of liver haem metabolism was inferred, the first peak was noted at 6 hours, earlier but not more prolonged, nor of higher activity. In neither of these 5 cases was the first peak dominant, nor was it dwarfed by the second peak. In one case of shunt hyperbilirubinaemia where the jaundice was almost certainly due to ineffective erythropoiesis, the first peak occurred at 6 to 12 hours.

In this thesis, in studies on 5 normal humans, the first peak varied from 1½ to 12 hours after glycine-2-C^{14} a much wider range than that recorded in the literature.

There was no consistent difference in the porphyric subjects studied.

Animal experiments are not strictly comparable to human observations but it is worth recording that in bile fistula dogs (206,207) Israels et al noted that the more ill-defined early peak occurred about 12 to 24 hours after glycine-2-C^{14}. In Sedormid porphyria (207) one dog was quoted as having maximal activity in bile bilirubin at 3 hours indicating more rapid liver haem turnover in this animal. It is rather dangerous to make such a comment on one case, especially as in humans, such a wide variation in pattern occurs from subject to subject irrespective of the underlying disease.

(b) The bifid early peak.

The bifid first peak observed in three patients, C.R., P.T. and T.M. is interesting and of potential importance. Dowdle et al (78) noted similar double early peaks in 3 of 6 subjects receiving ALA-5-C^{14} but these were not seen in the 5 cases reported by Yamamoto et al (498).

What do these findings mean? There are several possibilities:-

(i) A technical reason,

(ii) a double source of the first fraction of early-labelled bilirubin, one directly from haem precursors

and the other via non-haemoglobin haem-C^{14}, and

(iii) a reflection of varying turnover of different non-haemoglobin haem proteins in the liver or of the same haem proteins in different organs.

(i) Technical reasons.

A double early peak could represent other non-bilirubin serum fractions of high activity not separated from serum bilirubin during isolation procedures.

Two methods of isolating bilirubin from serum have been described in detail in Chapter VI, pages 311-326. One has in part been modified from the work of Ostrow, Hammaker and Schmid (331) with additional steps adapted from the methods described by Israels (208) and Yamamoto, Skanderbeg, Zipursky and Israels (498). The other is entirely derived from the latter 2 sources (208,498). Yamamoto et al (498) showed that when the bilirubin eluted from the aluminium oxide column was rechromatographed through the Kieselguhr column system of Cole and Lathe (63), specific activity remained constant, from which they inferred that the initial alumina column preparation was purified enough for counting purposes. It was this preparation that was combusted and counted in this thesis.

In the isolation method adopted from Ostrow et al (331) even more stringent steps were taken to ensure purity of the bilirubin sample. Bilirubin isolated from serum

("hot" + carrier bilirubin) was recrystallized twice from cold methanol, then passed through an alumina column as above, then recrystallized a third time before being combusted and counted.

By whatever method bilirubin was extracted, the final preparation in chloroform exhibited a sharp single peak at 453 mu with no other peaks between 320 mu and 700 mu on the Beckman D.B. spectrophotometer.

Thus both methods are exhaustive and bilirubin obtained appears pure and free from radioactive contamination.

Another point against a technical reason for the bifid peak is the observation that in all three cases in this study and in the three cases of Dowdle et al after ALA-5-C^{14} (78) the first component of the bifid peak is of the same order of magnitude as the second. Thus in T.M., maxima were at 5,398 dpm/mg and 6,219 dpm/mg serum bilirubin. With P.T. the maxima observed were 406 dpm/mg and 394 dpm/mg whilst in the normal subject C.R., the first and second "spikes" in peak 1 were at 763 and 1238 dpm/mg respectively. As in T.M. especially, both components of the bifid peak bore no relationship to the specific activity increments of newly synthesized circulating haem-C^{14}, it is more than likely that both parts of the early peak are derived from non-haemoglobin haem-protens or their precursors.

In two of the three patients with a bifid first peak,

namely C.R. and P.T., bilirubin was isolated from the serum by the method derived from Ostrow et al (331).

(ii) Direct synthesis of early-labelled bilirubin from haem precursors.

The second possibility to be considered is that there is a double source of the early fraction of early-labelled bilirubin, the first arising directly from haem precursors and the second from non-haemoglobin haem.

In the discussion on direct synthesis of bilirubin from haem precursors in Chapter III, pages 113,114. it was concluded that to date there was no evidence in support of the direct synthesis of bilirubin without the obligatory intervention of the haem moiety as a precursor. However, recent studies of bilirubin metabolism in rats have produced provocative evidence that this may not be so.

In rats, Robinson, Owen, Flock and Schmid (364) reported the early peak of activity in bile bilirubin 1½ hours after giving glycine-2-C^{14}. Garay et al (116) went further and showed that there was a bifid early peak in shunt bilirubin isolated from the bile of rats. They injected ALA-4-C^{14} into the portal vein of isolated perfused rat liver and measured the incorporation of the C^{14} label into bilirubin and hepatic non-haemoglobin haems. They demonstrated two peaks of bilirubin-C^{14} activity in normal rats, the first at two hours and the second during

the 5th hour after ALA-4-C^{14} administration. Further, incorporation of the C^{14} label into hepatic non-haemoglobin haems was greater than into biliary bilirubin reaching a maximum during the third hour of perfusion. As this peak of haem-C^{14} activity occurred after the first rise and before the second peak in biliary bilirubin-C^{14} activity, it appeared that this second bilirubin-C^{14} peak was a reflection of liver haem catabolism whilst the first might represent a more direct pathway of synthesis from porphyrins or other pyrroles prior to the formation of haem.

In carbon tetrachloride intoxicated rats (116) there were two essential differences. Firstly, whilst the two-hour peak in biliary bilirubin C^{14} activity was conspicuous, the five hour rise was absent. Secondly, incorporation of the C^{14} label into hepatic haems was much lower than controls, possibly due to decreased haem synthesis consequent upon mitochondrial damage following the hepatotoxin. This observation provides further evidence that there is a fraction of bilirubin made in the liver whose immediate precursor may not be haem.

The possibility of a double precursor source of the first fraction of early labelled bilirubin makes it difficult to accurately time that portion relating to liver haem protein turnover. For example, in O.M., (fig.7-55) a normal subject with peak 1 at 1½ hours, there may be a

second component at e.g. 12 hours, reflecting liver haem protein catabolism, whose presence is obscured by a prominent first component arising, possibly, from haem precursors.

(iii) Varying rates of turnover of different non-haemoglobin haemproteins.

In Chapter III, pages 96-98 when the turnover of various haem proteins was reviewed, it was emphasized that a number of different haem enzymes in the liver turn over rapidly but at different rates. Thus 1/3 to ½ of liver catalase may turn over daily (70,383) whilst about 10 to 15% of cytochrome C may be newly synthesized every day (81,82). Further, rat liver may synthesize catalase 3.5 times more rapidly than rat kidney (349) and there are at least two different catalases in rat liver (349).

Thus it is quite conceivable that a bifid first peak of early-labelled serum bilirubin may be a reflection of the varying turnover not only of different non-haemoglobin haem proteins in the liver but also of the same haem-proteins in different organs or possibly even different forms of the same haemprotein in the liver.

B. THE SECOND FRACTION OF EARLY-LABELLED BILIRUBIN.

The nature and origin of the 2nd peak of early-labelled bilirubin has been reviewed in detail in Chapter III, page 124/126. By virtue of its supposed origin from ineffective erythropoiesis, this part of the serum bilirubin-C^{14} pattern was not closely observed in the present study as most of the attention was focused on that fraction (peak 1) thought to reflect liver haem protein metabolism. A few observations are worthy of comment.

One striking feature was the consistently close correlation between the haem increment curve and that of the second bilirubin-C^{14} peak. This is in accordance with the findings of Israels and his co-workers (207,498) and emphasized the relationship of the second peak with haemopoiesis.

In E.J., a patient with haemolytic anaemia, glycine-2-C^{14} was rapidly incorporated into circulating haem, maximal haem-C^{14} increments being noted on the second day. The plateau of circulating haem-C^{14} activity was reached by the third day. The shortened life span of the newly formed red cells was reflected by a fall-off of haem-C^{14} activity after the 7th day, the plateau not being maintained for the usual 80 to 100 days. These findings previously recorded in Fig. 7-54 have been reproduced again this chapter in fig. 8-9.

Fig. 8-9 also shows clearly the 3 rises in serum bilirubin-C^{14} activity over the period of study. Peak 1 is unrelated to changes in the circulating haem-C^{14} and appears unaffected by the haemolytic process. Peak 2 occurs early, on day 2 coinciding with the maximal increment in circulating haem-C^{14} activity. Itis broader than usual and reflects the overactivity of a hyperplastic marrow. A third rise in serum bilirubin-C^{14} activity coincides with the fall-off of haem-C^{14} activity due to red cell destruction, both occurring acter the 8th day.

The observation that changes in circulating haem-C^{14} activity have been associated with a predictable serum bilirubin-C^{14} pattern has provided reassuring evidence that the methods employed in isolating and counting serum bilirubin are adequate for the purposes of this thesis.

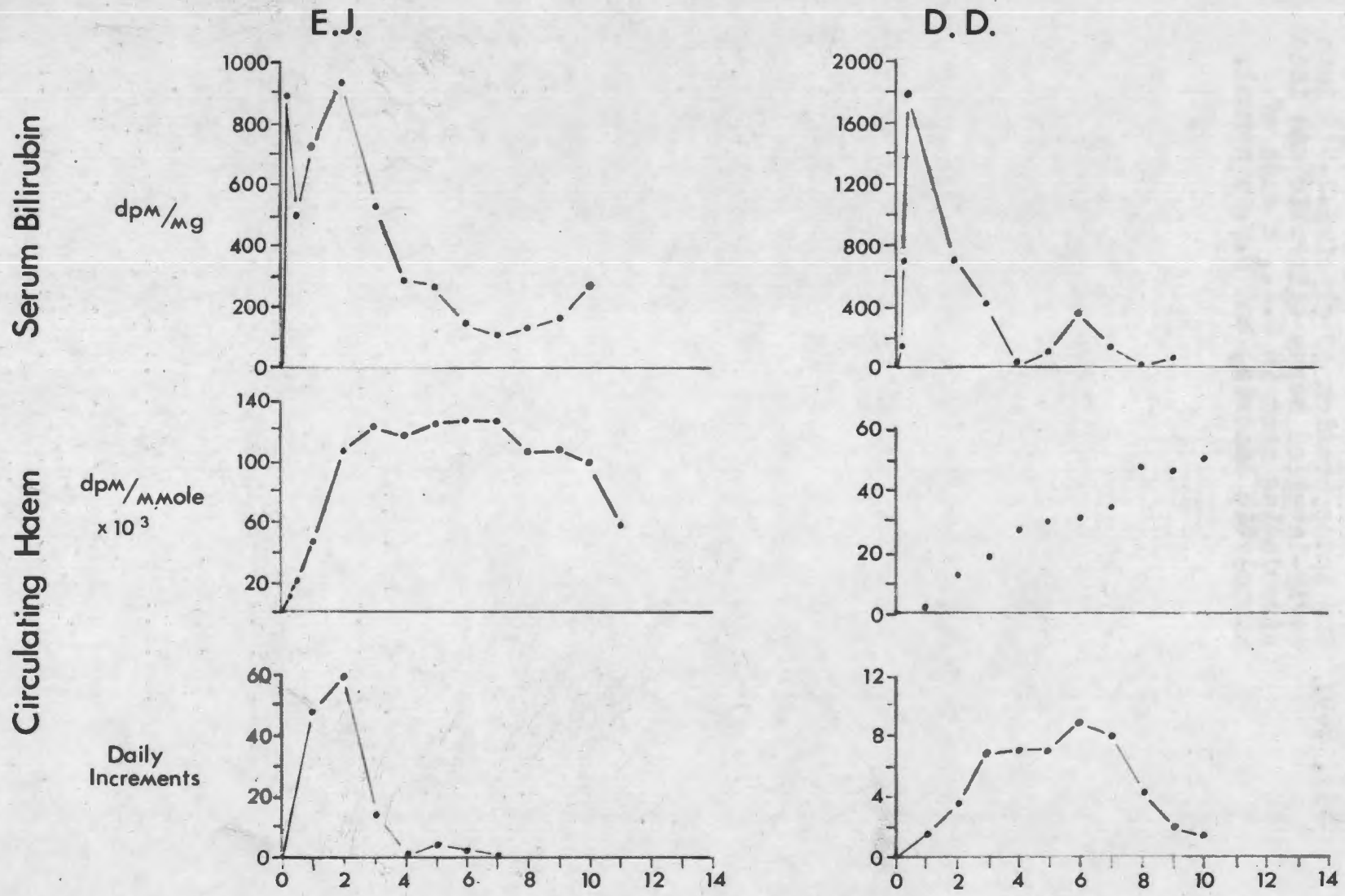


Fig. 8-9: P.T.O. for legend.

C. THE SERUM BILIRUBIN-C^{14} PATTERN IN PORPHYRIC SUBJECTS STUDIED.

The idea that motivated the present study was the supposition that any alteration in liver haemprotein metabolism would produce a detectable change in the early peak of serum bilirubin-C^{14}. Thus it was hoped that increased liver haem turnover would be reflected in a large first peak occurring earlier than normal whilst a block or difficulty in haem synthesis would delay the appearance of the C^{14} label into serum bilirubin. In this way the basic disorder of haem metabolism in the various porphyrias could be better defined.

In the symptomatic and S.A. genetic porphyrics studied, no consistent deviation from the considerably variable normal serum bilirubin-C^{14} patterns was noted. One case of symptomatic porphyria, T.M., is worthy of mention. In this subject there was clearly excessive incorporation of glycine-2-C^{14} into peak 1 of serum bilirubin-C^{14} indicating almost certainly an increased production of liver haem. While similar derangements of metabolism were most likely present in the other two symptomatic porphyrics studied, the degree of derangement was not gross enough to produce detectable changes in the first peak of serum bilirubin-C^{14} activity.

It appears that unless new techniques are developed whereby one can accurately quantitate the amount of

activity excreted as bilirubin-C^{14} (as in bile fistula animals) in the various fractions of shunt bilirubin, studies on the serum bilirubin-C^{14} pattern will not be sensitive enough to pick up the changes in liver haem and haem protein metabolism that undoubtedly occur in the hepatic forms of human porphyria.

CHAPTER IX.

CONCLUSIONS.

In summing up how much this thesis has contributed to the understanding of the fundamental biochemical lesions of human porphyria, it is necessary to consider briefly the findings of other workers in this field. Brilliant reviews on this topic have recently been presented by Tschudy (446) and Schmid (392).

A. NATURE OF THE BIOCHEMICAL LESIONS IN PORPHYRIA.

In S.A. genetic and acute intermittent porphyria, experimental evidence suggests that an expanded hepatic ALA pool is available for PBG and porphyrin synthesis (392,402). This could result from overproduction of ALA, a block in one of its alternative non-porphyrin metabolic pathways or both.

In experimental porphyria, increased mitochondrial ALA synthetase activity has been reported in the livers of DDC intoxicated guinea pigs (148) and rats (130) and in rats given AIA (445). Other enzymes involved in protoporphyrin synthesis were not similarly affected by DDC (148) suggesting that ALA synthetase was the rate limiting enzyme in hepatic porphyrin synthesis.

In porphyric rats, substances interfering with protein synthesis, e.g. actinymycin D or puromycin (267) have been shown to block induction of increased ALA synthetase activity. Granick (149) showed that exposure of avian hepatic cells in tissue culture media containing drugs stimulating porphyrin production led to fluorescence which was similarly blocked by Actinomycin D. These findings suggested that the porphyrinogenic action of these drugs was due to enzyme induction which was under genetic control.

In human porphyria, significantly increased hepatic ALA synthetase activity has been reported by Perlroth et al (340) in acute intermittent porphyria and by Dowdle (79) in the S.A. genetic and symptomatic porphyrias. As in normal liver there are very low levels of ALA synthetase, normally under almost complete repression (446), the vexing question in human porphyria is what induces increased activity thereby promoting excessive ALA and porphyrin production.

Perlroth et al (340) postulated genetically defective control of enzyme induction which would have to be at the operator gene of the operon rather than the regulator gene to fit in with the Mendelian dominant mode of inheritance in S.A. genetic and acute intermittent porphyrias.

Thus a mutation at the operator site would allow one

allele, unresponsive to repressor substance, to maintain ALA synthetase production at a high level thus explaining the biochemical findings of human porphyrin. However, this would not account for the varying porphyrian excretory patterns in the different porphyrias or the reasons for an acute attack as porphyrins or their precursors when administered to normal or porphyric humans do not cause untoward effects even though increased porphyrin excretion may be promoted (78,133,358,402).

Thus, the induction of ALA synthetase activity may be secondary to biochemical lesions elsewhere which may in addition account for the symptoms of an acute attack.

In their quest for the basic metabolic defects of the human porphyrias, research workers have explored the following possibilities:-

(1) A block in the alternative metabolism of glycine.

(2) A block in the further metabolism of succinyl coenzyme A.

(3) A block in the alternative metabolic disposition of ALA.

(4) A block beyond PBG in the biosynthetic pathway of haem.

By alternative is meant non-porphyrin pathways of metabolism.

(1) Glycine.

Overproduction of ALA could theoretically result from the increased availability of the substrates glycine and succinyl coenzyme A although in idiopathic hyperglycinaemia, attributed to impaired conversion of glycine to serine, no abnormality of pyrrole excretion has been reported (328). Richards and Scott (358) showed that after oral glycine administration, the serum concentrations of this aminoacid in acute intermittent porphyrics were no different to those in normal subjects studied, indicating no significant increase in the glycine pool in their cases. They also reported that 3 of 6 acute porphyrics had difficulty in converting glycine to serine. The three with normal serum serine patterns were the only ones receiving Vitamin B_{12}, a substance intimately related to the metabolism of C-1 fragments (358).

Decreased ability to oxidize glycine-2-C^{14} to $C^{14}O_2$ has been demonstrated in liver preparations of animals made porphyric with HCB (75), DDC (130,131) and AIA (443) whilst no defect in the oxidation of glycine-1-C^{14} (130, 131) pyruvate, acetate or glucose (443) to CO_2 was observed. Tschudy et al (443) felt that decreased catalase may have been the common factor in these in-vitro experiments especially if the major route of oxidation of the α of glycine was via glyoxalate.

Dowdle et al (76,77) showed that in-vivo, the incorporation of glycine-2-C^{14} into respiratory CO_2, urinary uric acid, circulating haem, plasma protein and stool porphyrin was no different in the S.A. genetic and symptomatic porphyrics than in the normal subjects studied.

De Matteis and Rimington (71,72,490) have postulated that a failure to generate sufficient amounts of acetyl coenzyme A (an ATP dependent reaction) may lead to deficient synthesis of aminoacetone and acetylcholine. The former defect would allow more glycine to be available for condensation with succinyl coenzyme A to form ALA and thereby porphyrins whilst lower levels of acetylcholine could explain the neurological basis for acute porphyria. However, aminoacetone formation from liver slices is normal in experimental porphyria (130,131,446) and it is excreted in normal amounts in the urine of patients with acute intermittent porphyria (444). Perlroth et al (340) showed that aminoacetone synthetase activity (and inferentially endogenous acetylcholine production) was normal in a case of acute intermittent porphyria studied.

(2) Succinyl coenzyme A.

The non-porphyrin metabolic pathways of succinyl coenzyme A have not been extensively studied in porphyria. Tschudy (443) added α ketoglutarate-5-C^{14} to normal and to AIA induced porphyric liver slices and showed that the

$C^{14}O_2$ produced was the same in both groups. As most of the $C^{14}O_2$ was thought to be derived from a functioning tricarboxylic acid cycle rather than decarboxylation of porphyrins, the authors concluded that under their experimental conditions, no block in the alternative disposition of succinyl coenzyme A occurred in porphyric liver slices.

(3) Block in the alternative pathways of ALA utilisation.

If in acute porphyria there is difficulty in the metabolism of ALA along non-porphyrin pathways, more ALA will be available for porphyrin synthesis. In addition, a negative feedback mechanism may operate whereby ALA synthetase activity is induced, more succinyl coenzyme A combining with glycine in an attempt to overcome the block.

The succinate-glycine cycle provides an alternative route for the metabolism of ALA. If in acute porphyria there is impaired conversion of the ALA-5-C to one-carbon fragments, purine synthesis may be affected to such an extent that a deficiency of purine nucleotides and nucleic acids may arise, sufficient to disturb metabolism to the extent of producing an acute attack.

Tschudy et al (443) measured the rate of conversion of ALA-4-C^{14} to $C^{14}O_2$ by normal and AIA induced porphyric rabbit liver and showed no difference between the two groups. As $C^{14}O_2$ represents oxidation of radioactive

succinate generated by the succinate glycine cycle, these authors felt that the evidence pointed to a normally active cycle in experimental rabbit porphyria.

Haem/purine relationships have been studied from every angle by a number of research workers and the subject has been dealt with at great length in Chapter V.

Briefly, in Sedormid or AIA induced porphyria in chick embryos, Talman, Labbe et al (234,437) convincingly demonstrated decreased purine synthesis and thereby uric acid formation. In experimental animals, de Matteis (74) equally convincingly showed no impairment of purine synthesis in Sedormid, AIA or HCB induced porphyria. In human porphyria low blood urate levels may occur on grounds other than impaired uric acid production (287).

In experimental animals and chick embryo porphyria, and less convincingly in human porphyria, treatment with purine compounds produces both a clinical and biochemical remission (109,110,111,113,234,278,279,437). It is likely that rather than compensating for deficient synthesis, added purines act by mobilizing ferrous iron from hepatic ferritin (157,290) thus stimulating liver haem synthesis (239,279), thereby inhibiting ALA synthetase by the negative feedback mechanism envisaged by Lascelles and her group (51, 244,247). Whilst ATP is a necessary co-factor for succinyl coenzyme A formation and therefore ALA synthesis (44,221, 422), Gajdos et al (112) under special experimental conditions

showed that ATP and other purine compounds inhibited porphyrin synthesis by Rhodopseudomonas spheroides.

The inhibitory effect on porphyrin synthesis by ATP led workers to study the converse situation, the effect of decreasing ATP on porphyrin formation. Orotic acid (115), ethionine (335) and AIA (74) all decrease hepatic ATP levels and are porphyrinogenic in the experimental animal. However, AIA and many barbiturates impair ATP formation without inducing experimental porphyria (74) whilst other porphyrinogenic drugs e.g. HCB do not influence hepatic ATP levels (74). Thus whilst Gajdos et al (115) and Palma-Carlos et al (335) feel that these are important avenues to follow in understanding the fundamental lesions of porphyria, de Matteis (74) is of the opinion that there is no relation between porphyria and reduced hepatic levels of ATP. As haem-containing enzymes and ATP are so intimately linked with intracellular respiration and energy trapping, it is possible that certain respiratory defects could induce ALA synthetase activity (446) and interfere with hepatic ATP levels.

Thus in all probability, the beneficial effects of purine treatment and the decreased hepatic ATP levels do not substantiate the hypothesis of Talman et al that the symptoms and biochemical features of acute porphyria are related to decreased synthesis of purines. Hitherto, no one has measured the formation of purines from common haem/

purine precursors such as glycine in human porphyria. This has been one of the major aims of this thesis where in particular, a possible block in the incorporation of the ALA-5-C has been looked for.

(4) Block beyond PBG in the formation of haem or haem-proteins

Lascelles and her co-workers (51,244,247) have indicated that in Rhodopseudomonas spheroides and other photosynthetic bacteria, ALA synthetase activity is markedly influenced by levels of haem in the medium. Thus small amounts of added haem inhibit activity and vice versa.

Rimington (362,363) and Heikel et al (187) have suggested that in some of the hepatic porphyrias, e.g. symptomatic porphyria, owing to hepatocellular damage, intracellular reducing systems may fail to operate effectively in keeping porphyrinogen intermediates in their reduced form. This could result in their oxidation and "escape" from the porphyrin biosynthetic pathway as porphyrins which would then accumulate within the liver cells and be excreted from the body in excessive quantities. Further, it is feasible that with this difficulty in making haem, induction of ALA synthetase activity would readily occur.

Schmid et al (392,383) showed a rapid fall off in liver catalase activity in rats and rabbits rendered porphyric with Sedormid. They showed that whereas glycine-2-C^{14}

was not incorporated into liver catalase in Sedormid rats, the label readily entered haem of other hepatic chromoproteins. Thus whilst Sedormid did not appear to affect haem production, it did interfere with the synthesis of haem-protein moiety of catalase. Hepatic catalase has also been shown to be deficient in DDC porphyric rat liver (130,131), in AIA porphyric rat liver (443), in human S.A. genetic porphyric liver (130) but not in the livers of acute intermittent porphyrics (392).

Tschudy et al (443), tackling the problem along different lines measured the disappearance rate of PBG in normal and AIA porphyric rat liver slices and concluded that there was no decreased utilization of PBG under these experimental conditions.

Lottefeldt et al (278,279) conclusively showed that the uptake of Fe^{59} into porphyric rat liver was greater than normal and that the incorporation of this isotope into liver haem was increased at all times in the porphyric animal (239,279). The latter was confirmed by noting increased incorporation of glycine-2-C^{14} (279), acetate-2-C^{14} (239) and succinate-2-C^{14} (237) into liver haem in AIA induced rat porphyria.

Thus, in experimental animal porphyria there is overwhelming evidence that liver haem turnover is increased even though synthesis of one of the haem-proteins, catalase is decreased. As porphyrinogenic drugs are also hepatotoxins interfering with cellular

metabolism at more than one site, one cannot extrapolate these findings to the hepatic forms of human porphyria. To date, no one has measured liver haem turnover in human porphyria and the other major aim of this thesis is to investigate this aspect of porphyrin metabolism indirectly by studying the incorporation of glycine-2-C^{14} into peak 1 of early-labelled serum bilirubin.

B. CONTRIBUTION MADE BY THESIS.

A review of research in the field of porphyria has been given in order to highlight the reasons which prompted the present study. Experimental workers have explored haem/purine relationships and liver haem turnover in animal porphyria but no-one has yet measured purine synthesis from common haem/purine precursors in human porphyria nor has anyone studied what happens to liver haem in in porphyric man. As it is difficult to obtain adequate amounts of liver tissue by conventional closed biopsy to measure liver purines and haem directly, these aspects of metabolism have been investigated indirectly by studying the incorporation of glycine-2-C^{14} into urinary uric acid and serum bilirubin in normal and porphyric human subjects.

(1) Uric acid degradation studies.

Glycine-2-C^{14} labels C5 of the purine moiety directly and C2+8 via active C-1 fragments. Nemeth et al (319) have proposed that of three possible pathways whereby the α C of glycine can label one-carbon units (443), the one via the succinate-glycine cycle is the most important.

If the findings of Talman et al (234,437) in chick embryo porphyria apply to acute human porphyria, then following glycine-2-C^{14}, significantly less activity should

reside in C2+8 of these purine moieties made in the liver.

Results of urinary uric acid degradation studies have been analysed and interpreted in Chapter VIII. In three symptomatic porphyries studied, increased incorporation of glycine-2-C^{14} into urinary uric acid, C4+5 and C2+8 was noted. This was thought to reflect difficulty in the hepatic excretion of urate in these patients with liver disease rather than an altered rate of purine synthesis. As a consequence, a relatively greater portion of the total urate excreted would be eliminated via renal channels. In normal and S.A. genetic porphyric subjects, the percentage dose incorporated into C4+5 and C2+8 over 4 or 14 days did not differ significantly but it must be re-emphasised that what has been measured is activity excreted as urinary uric acid-C^{14} and its component carbons, no correction being made for extrarenal elimination of urate along the lines suggested by Seegmiller et al (404). Further, the rate of turnover of the miscible pool was not measured in this thesis and whilst these variables may not have been large in patients without hyperuricaemia or renal disease, small differences between the clinical groups studied may not have been detected. In only one patient, C.v.H., were incorporation data rejected on the grounds of chronic renal disease and hyperuricaemia.

The above discussion has no bearing on the interpretation of C4+5: C2+8 ratios or the intramolecular disposition of

radioactivity within the uric acid molecule. By both these criteria, porphyric patients in no way differed from normal subjects studied.

There is, however, yet another important variable to consider when interpreting above data. If there were defective purine synthesis in hepatic forms of human porphyria, one would expect this to be limited to that organ where porphyrin synthesis has gone wrong, namely the liver. Uric acid derived from liver purines may well be "diluted" by that urate arising from the oxidation of normal purines made elsewhere, any defects in its elaboration thereby being masked. Evidence has been presented in Chapter VIII to the effect that a significant portion of promptly-labelled urinary uric acid may arise from the catabolism of purine compounds made in the liver and this fraction has therefore been studied separately.

In the first few days following glycine-2-C^{14} administration, relatively more activity resided in carbons 2 and 8 of the urinary urate moiety both in normals and porphyrics but no difference in either the early or overall pattern of isotope excretion was noted in the clinical groups studied. In fact, C4+5: C2+8 ratios in acute porphyria approximated normal values more closely in the first 4 days of every experiment.

Thus, while bearing in mind certain limitations as to what one can infer from available data, studies in this

thesis have shown no consistent impairment of purine synthesis in human porphyria.

From the same laboratory, Dowdle (78) has investigated the synthesis of purines from orally administered ALA-5-C^{14} in human porphyria. ALA-5-C^{14} has several important advantages over glycine-2-C^{14} as a labelled precursor. It is the only immediate source of isotopic C-1 fragments available for purine synthesis and it enters liver cells more easily than it does other tissues (401). Thus, by measuring the incorporation of ALA-5-C^{14} into C2+8 of uric acid, one is studying precisely that step thought by Talman et al (234,437) to be defective in porphyria.

Dowdle (78) could detect no impairment in the utilization of the δ carbon of ALA for purine synthesis in symptomatic and S.A. genetic porphyria thus supporting the glycine-2-C^{14} data. Thus, in these forms of human porphyria, there is now strong evidence that no block in the alternative metabolic disposition of ALA occurs and that liver purine synthesis is normal. These statements are in accordance with the observations of de Matteis et al (74) that in experimental animal porphyria, liver purine levels are unchanged.

(2) Serum bilirubin-C^{14} studies.

The first peak of early-labelled serum bilirubin-C^{14} is thought to be derived mainly from non-haemoglobin haem moieties made in the liver. In the hepatic forms of human porphyria, the disordered porphyrin metabolism should be

reflected in changes in liver haem production and turnover. Assuming normal ferrochelatase activity, one would anticipate excessive formation of liver haem in a S.A. genetic porphyria where large quantities of copro- and protoporphyrin are excreted in the bile and stool. On the other hand, if the damaged livers of symptomatic porphyrics are unable to keep porphyrinogen intermediates in their reduced form, there may be some delay in the incorporation of a labelled precursor into hepatic haem.

The early-labelled serum bilirubin-C^{14} data are extremely interesting and have evoked much discussion. Although the specific activity of the samples counted was very small, errors from statistical and technical standpoints were kept at a minimum and the results appear perfectly acceptable.

In three patients, one normal and two symptomatic porphyrics, there was a bifid first peak of activity. This has one of two likely explanations. Data from the ALA-4-C^{14} experiments of Garay et al (116) raise the possibility that the first component of the bifid peak may arise directly from haem precursors and the second from the breakdown of liver haem. On the other hand, it must be remembered that the first fraction of early-labelled serum bilirubin may reflect the catabolism of haem liberated from different rapidly-turning over haem-proteins of varying life span, and thereby, may have more than one detectable component.

A consistent finding was the wide variation in the

early-labelled serum bilirubin-C^{14} patterns of the normal subjects studied. In contrast to the observations of Israels and his co-workers (205,207,498) where maximal activity in the first peak occurred at about 12 hours after glycine-2-C^{14} administration, peak values in the present study were noted 1½ to 12 hours of giving glycine to normal controls.

With regard to symptomatic porphyrics, there was no delay in the incorporation of glycine-2-C^{14} into the first peak of early-labelled bilirubin as might have been expected from the comments of Rimington, Heikel et al (187,362,363).

On the contrary, the clearly excessive incorporation by T.M. of isotopic glycine into peak 1 shows that in this patient, there was almost certainly an over-production of liver haem. It is likely that in R.N. and P.T., the other two symptomatic porphyrics studied, similar derangements of liver haem production were present but were not of sufficient magnitude to alter the serum bilirubin-C^{14} pattern. It would seem from the observations in T.M., that in symptomatic porphyria, the damaged liver cells not only make haem at a normal rate but may in fact, overproduce this compound.

In S.A. genetic porphyria, the serum bilirubin-C^{14} patterns were all in the normal range. These findings did not corroborate the observations of Lottafeldt et al (239,279)

whose Fe^{59}, glycine-2-C^{14}, acetate-2-C^{14} and succinate 2-3-C^{14} data clearly showed increased liver haem turnover in experimental rat porphyria.

A disadvantage of serum bilirubin-C^{14} studies is that, in contrast to bile fistula animal experiments, the total bilirubin-C^{14} activity in peak 1 cannot be measured and as a consequence, many of the changes in liver metabolism that undoubtedly occur in the hepatic human porphyrias have not been detected in this thesis.

C. SUMMARY AND CONCLUSION.

It has been suggested by Talman et al (234,437) that a block in the utilization of the δ carbon of ALA in purine synthesis may be the fundamental biochemical lesion of Sedormid-induced chick embryo porphyria. The possibility of a similar defect in human porphyria has been investigated in this thesis by studying the incorporation of glycine-2-C^{14} into uric acid and its component carbons in the urine of 4 normal, 4 symptomatic porphyric and 6 South African genetic porphyric subjects.

The mean values for the percentage total dose excreted in 14 days in the urine as uric-acid were 0.172% (normals), 0.229% (symptomatic porphyrics), 0.167% (S.A. genetic porphyric in remission) and 0.186% (S.A. genetic porphyrics during an acute attack). Of this, about 60 to 75% of the activity was excreted in C4 + 5 and 15 to 20% in C2 + 8. The mean C4 + 5: C2 + 8 ratios for each clinical group were 4.09 (normals), 3.75 (symptomatic porphyrics), 3.68 (S.A. genetic porphyric in remission) and 4.51 (acute S.A. genetic porphyrics).

No significant differences between normals and porphyrics were observed with regard to the C4 + 5 : C2 + 8 ratios and the disposition of radioactivity within the uric acid molec When comparing the percentage dose of glycine-2-C^{14} excreted in the urine as uric acid, C4 + 5 and C2 + 8, it was noted that symptomatic porphyrics appeared to excrete a greater portion of the administered isotope in all the carbon atoms

of the urinary urate moiety whilst no significant difference between normals and S.A. genetic porphyric subjects could be detected. This apparent hyperincorporation of glycine-2-C^{14} into urinary uric acid by symptomatic porphyrics is possibly a reflection of reduced extrarenal elimination of urate in the bile, a consequence of the hepatocellular damage that invariably occurs in this form of porphyria. As a result, a larger fraction of the total urate excreted would leave the body in the urine.

An interesting finding was the observation that relatively more activity resided in carbons 2 and 8 of those urate moieties excreted within a few days of injecting glycine-2-C^{14}. There is experimental evidence that a significant portion of this promptly labelled urate may be derived from the oxidation of rapidly turning over ribonucleotides and ribonucleic acids elaborated in the liver and as such should reflect any abnormality of liver purine synthesis that might occur in the hepatic human porphyrias. When data for the first four days of each experiment was evaluated, no new conclusions were made.

Thus, the essential finding of this study is the failure to demonstrate any consistent impairment of hepatic purine synthesis in symptomatic and South African genetic porphyria, the latter both in an acute phase and during remission. This produces new evidence that the postulate of Talman et al (234,437) is not tenable in the two forms of human

porphyria studied.

In the hepatic forms of human porphyria, it is reasonable to assume that the derangement of porphyrin metabolism also affects liver haem production and turnover. As the first peak of early-labelled serum bilirubin-C^{14} activity following the administration of glycine-2-C^{14} is thought to arise from non-haemoglobin haem, mainly liver haem, this fraction has been studied in 5 normal subjects, 3 symptomatic porphyrics and 3 South African genetic porphyries during an acute attack.

Experiments recorded in this thesis have shown that a considerable variation both in the timing of maximal activity and in the extent of the first peak occurs both in normal and porphyric subjects. Only in one case, T.M., a symptomatic porphyric, was there clearly excessive incorporation of glycine-2-C^{14} into peak 1 raising the possibility of increased liver haem production in this form of human porphyria. This line of study is clearly not sensitive enough to pick up other than gross changes in liver haem metabolism.

LIST OF TABLES.

LIST OF FIGURES.

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ERRATUM.

The acute South African genetic porphyria referred to in the text as A.Jov. should read C.Jov. However, as this mistake was noted late in the preparation of the thesis, it has been allowed to remain.

STATISTICAL METHODS.

A. CALCULATION OF THE MEAN ($\bar{X}$)

$$\bar{X} = \frac{\Sigma x}{n}$$

Where n = number of observations and

Σx = sum of all the observations in the sample.

B. CALCULATION OF THE STANDARD ERROR (S.E.) OF THE MEAN.

The standard deviation (S.D.) of the mean was first calculated according to the formula:-

$$S.D. = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

Standard error (S.E.) was then calculated as below:-

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

Results have been expressed as $\bar{X} \pm$ S.E.

REFERENCES.

1. ABRAMS, R., & GOLDINGER, J.M., 1952. Formation of nucleic acid purines from hypoxanthine and formate in bone marrow slices. Arch. Biochem. and Biophys. 35,243-248.

2. ABRAMS, R., & BENTLEY, M., 1955. Transformation of inosinic acid to adenylic and guanylic acids in a soluble enzyme system. J. Am. Chem. Soc. 77,4179-4180.

3. ABRAMS, R., & BENTLEY, M., 1955. Biosynthesis of nucleic acid purines 11. Role of hypoxanthine and xanthine compounds. Arch. Biochem. and Biophys. 58,109-118.

4. AGNER, K., 1958. Crystalline myeloperoxidase. Acta. Chem. Scandinav. 12,89-94.

5. ALDRIDGE, W.N., & PARKER, V.H., 1960. Barbiturate and oxidative phosphorylation. Biochem. J. 76,47-56.

6. ALTMAN, K.I., CASARETT, G.W., MASTERS, R.E., NOONAN, T.R., & SALOMON, K., 1948. Haemoglobin synthesis from glycine labelled with radioactive carbon in its α-carbon atom. J. Biol. Chem. 176,319-325.

7. ANDERSON, R.L., & TOVE, S.B., 1958. Effect of copper deficiency on synthesis of haem. Nature (Lond.) 182,315.

8. ARNSTEIN, H.R.V., 1951. Biosynthesis of choline methyl groups by rat. Biochem. J. 48,27-32.

9. ARNSTEIN, H.R.V., & NEUBERGER, A., 1953. The synthesis of glycine and serine by the rat. Biochem. J. 55, 271-280.

10. ARNSTEIN, H.R.V., 1954. The metabolism of glycine. Adv. Prot. Chem., Academic Press Inc. N.Y. 9,1-81.

11. AXELROD, J., SCHMID, R., & HAMMAKER, L., 1957. A biochemical lesion in congenital, non-obstructive, non-haemolytic jaundice. Nature 180,1426-1427.

12. BAGDASARIAN, M., 1958. A new transaminase of Coryne bacterium diphtheriae. Nature (Lond.) 181,1399.

13. BALE, W.F., YUILE, C.L., De La VERGNE, L., MILLER, L.L., & WHIPPLE, G.H., 1949. Haemoglobin labeled by radioactive lysine. J. Exp. Med. 90,315-320.

14. BARAC, G., 1953. Interactions of bilirubin with plasma proteins, and the state of bilirubin in normal human plasma. Arch. International Physiol. 61,129-140.

15. BEEKEN, W.L., VOLWILER, W., GOLDSWORTHY, P.D., CARRY, L.E., REYNOLDS, W.E., STOGSDILL, R., & STEMLER, R.S., 1962. Studies of I^{131} albumin catabolism and distribution in normal young male adults. J. Clin. Invest. 41,1312-1333.

16. BENEDICT, J.D., FORSHAM, P.H., & STETTEN, de W., Jr., 1949. The metabolism of uric acid in the normal and gouty human studied with the aid of isotopic uric acid. J. Biol. Chem. 181,183-193.

17. BENEDICT, J.D., FORSHAM, P.H., ROCHE, M., SOLOWAY, S., & STETTEN, De W., Jr., 1950. The effect of salicylates and adrenocorticotropic hormone upon the miscible pool of uric acid in gout. J. Clin. Invest. 29,1104-1111.

18. BENEDICT, J.D., ROCHE, M., YU, T.F., BIEN, S.J., GUTMAN, A.B., & STETTEN, De W., Jr., 1952. Incorporation of glycine nitrogen into uric acid in normal and gouty man. Metabolism. 1,3-12.

19. BENEDICT, J.D., YU, T.F., BIEN, S.J., GUTMAN, A.B., & STETTEN, De W., Jr., 1953. Further study of utilization of dietary glycine nitrogen for uric acid synthesis in gout. J. Clin. Invest. 32,775-777.

20. BENNETT, L.L., Jr., & SKIPPER, H.E., 1955. In-vivo utilization of hypoxanthine and other precursors for synthesis of nucleic acid purines. Arch. Biochim. & Biophys. 54,566-569.

21. BERENDES, M., 1959. The proportion of reticulocytes in the erythrocytes of the spleen as compared with those of circulating blood, with special reference to hemolytic states. Blood. 14,558-563.

22. BERGMANN, F., & DIKSTEIN, S., 1956. Studies on uric acid and related compounds 111. Observations on the specificity of mammalian xanthine oxidase. J. Biol. Chem. 223,765-780.

23. BERLIN, N.I., TOLBERT, B.M., & LAWRENCE, J.H., 1951. Studies in (2-^{14}C) glycine metabolism in man. 1. The pulmonary excretive of $^{14}CO_2$. J. Clin. Invest. 30,73-76.

24. BERLIN, N.I., NEUBERGER, A., & SCOTT, J.J., 1956. The metabolism of δ amino-laevulic acid. 1. Normal pathways studied with aid of ^{15}N. Biochem. J. 64,80-90.

25. BERLIN, N.I., NEUBERGER, A., & SCOTT, J.J, 1956. The metabolism of δ amino-laevulic acid. 2. Normal pathways studied with the aid of ^{14}C. Biochem. J. 64,90-100.

26. BESSIS, M., BRETON-GORIUS, J., THIERY, J.P., 1961. (Fr.) Possible role of the haemoglobin accompanying the nucleus of erythroblasts in the origin of precociously eliminated stercobilin. C.R. Acad. Sci. (Paris). 252, 2300-2302.

27. BIEN, E.J., & ZUCKER, H., 1955. Uricolysis in normal and gouty individuals. Ann. Rheumat. Dis. 14,409-411.

28. BILLING, B.H., & LATHE, G.H., 1958. Bilirubin metabolism in jaundice. Am. J. Med. 24,111-121.

29. BISHOP, C., GARNER, W., & TALBOTT, J.H., 1951. Pool size, turnover rate, and rapidity of equilibration of injected isotopic uric acid in normal and pathological subjects. J. Clin. Invest. 30,879-888.

30. BISHOP, C., RAND, R., & TALBOTT, J.H., 1955. Rate of conversion of isotopic glycine to uric acid in normal and gouty human and how this is affected by vitamin B and folic acid. Metabolism. 4,174-182.

31. BLOCH, K., & RITTENBERG, D., 1945. Estimation of acetic acid formation in rat. J. Biol. Chem. 159,45-58.

32. BLOOM, B., & STETTEN, D., Jr., 1953. Pathways of glucose catabolism. J. Am. Chem. Soc. 75,5446-5447.

33. BLOOM, B., 1955. Catabolism of glucose by mammalian tissues. Proc. Soc. Exper. Biol. and Med. 88,317-318.

34. BOGORAD, L., & GRANICK, S., 1953. The enzymatic synthesis of porphyrins from porphobilinogen. Proc. Nat. Acad. Sci. Wash. 39,1176-1188.

35. BOGORAD, L., 1955. Intermediates in the biosynthesis of porphyrins from porphobilinogen. Science. 121,878-879.

36. BOGORAD, L., 1958. The enzymatic synthesis of porphyrins from porphobilinogen I Uroporphyrin I. J. Biol. Chem. 233,501-509.

37. BOGORAD, L., 1958. The enzymatic synthesis of porphyrins from porphobilinogen II. Uroporphyrin III. J. Biol. Chem. 233,510-515.

38. BOGORAD, L., 1958. The enzymatic synthesis of porphyrins from porphobilinogen III. Uroporphyrinogens as intermediates. J. Biol. Chem. 233,516-519.

39. BOGORAD, L., & MARKS, G.S., 1960. Studies on the biosynthesis of uroporphyrin III from porphobilinogen and the behaviour of uroporphyrin esters in paper chromatography. Biochim. et Biophys. Acta. 41,356-358.

40. BOOIJ, H.L., & RIMINGTON, C., 1957. Effect of pre-heating on porphyrin synthesis by red cells. Biochem. J. 65,4P.

41. BORSOOK, H., LINGREL, J.B., SCARO, J.L., & MILLETTE, R.L., 1962. Synthesis of hemoglobin in relation to the maturation of erythroid cells. Nature. 196,347-350.

42. BRECHER, G., & STOHLMANN, F., Jr., 1961. Reticulocyte size and erythropoietic stimulation. Proc. Soc. Exp. Biol. Med. 107,887-891.

43. BROWN, E.G., 1958. The relationship of the tricarboxylic acid cycle to the synthesis of δ-aminolaevulic acid in avian erythrocyte preparations. Biochem. J. 70,313-321.

44. BROWN, E.G., 1958. Evidence for the involvement of ferrous iron in the biosynthesis of δ-aminolaevulic acid by chicken erythrocyte preparations. Nature. 182,313-315.

45. BROWN, G.B., ROLL, P.M., PLENTL, A.A., & CAVALIERI, L.F., 1948. The utilization of adenine for nucleic acid synthesis and as a precursor of guanine. J. Biol. Chem. 172,469-484.

46. BUCHANAN, J.M., SONNE, J.C., & DELLUVA, A.M., 1948. Biological precursors of uric acid II. The role of lactate, glycine, and carbon dioxide as precursors of the carbon chain and nitrogen atom 7 of uric acid. J. Biol. Chem. 173,81-98.

47. BUCHANAN, J.M., & HARTMAN, S.C., 1959. Enzymic reactions in the synthesis of the purines. Advances in Enzymol. 21,199-261.

48. BUCHANAN, J.M., 1958-59. The enzymatic synthesis of purine nucleotides. Harvey Lect. 54,104-130.

49. BUCHANAN, D.L., & ROLLINS, J.M., 1961. Lack of correlation between gout and the incorporation of isotopic formate into uric acid. Yale. J. Biol. Med. 34,31-39.

50. BULLOCK, E., JOHNSON, A.W., MARKHAM, E., SHAW, K.B., 1958. A synthesis of coproporphyrin III. J. Chem. Soc. 1430, pt.2.

51. BURNHAM, B.F., & LASCELLES, J., 1963. Control of porphyrin biosynthesis through a negative-feedback mechanism. Studies with preparations of delta-aminolaevulate synthetase and delta-aminolaevulate dehydratase from Rhodopseudomonas spheroides. Biochem. J. 87,462-472.

52. BURNSTINE, R.C., & SCHMID, R., 1962. Solubility of bilirubin in aqueous solutions. Proc. Soc. Exp. Biol. Med. (N.Y.) 109,356-358.

53. BUZARD, J., BISHOP, C., & TALBOTT, J.H., 1952. Recovery in humans of intravenously injected isotopic uric acid. J. Biol. Chem. 196,179-184.

54. BUZARD, J., BISHOP, C., & TALBOTT, J.H., 1955. The fate of uric acid in the normal and gouty human. J. Chron. Dis. 2,42-49.

55. CAMERON, J.L., FILLER, R.M., IBER, F.L. ABEI, T., & RANDOLPH, J.G., 1966. Metabolism and excretion of C^{14}-labeled bilirubin in children with biliary atresia. The New Eng. J. Med. 274,231-237.

56. CAMMARATA, P.S., & COHEN, P.P., 1950. The scope of the transamination reaction in animal tissues. J. Biol. Chem. 182,439-452.

57. CANELLAKIS, E.S., TUTTLE, A.L., & COHEN, P.P., 1955. A comparative study of the end-products of uric acid oxidation by peroxidases. J. Biol. Chem. 213,397-404.

58. CAPUTTO, R., 1951. The enzymatic synthesis of adenylic acid; adenosinekinase. J. Biol. Chem. 189,801-814.

59. CARTWRIGHT, G.E., & WINTROBE, M.M., 1948. Studies on free erythrocyte protoporphyrin, plasma copper, and plasma iron in normal and in pyridoxine-deficient swine. J. Biol. Chem. 172,557-565.

60. .....See next page.

60. CASKEY, C.T., ASHTON, D.M., & WYNGAARDEN, J.B., 1964. The enzymology of feedback inhibition of glutamine phosphoribosyl-pyrophosphate amidotransferase by purine ribonucleotides. J. Biol. Chem. 239,2570-2579.

61. CHICK, H., MACRAE, T.F., MARTIN, A.J.P., & MARTIN, C.J., 1938. Water-soluble B-vitamins other than aneurin (vitamin B_1) riboflavin and nicotinic acid required by pig. Biochem. J. 32,2207-2224.

62. CHU, T.C., & CHU, E.J., 1955. Paper chromatography of iron complexes of porphyrins. J. Biol. Chem. 212,1-7.

63. COLE, P.G., & LATHE, G.H., 1953. The separation of serum pigments giving the direct and indirect van den Bergh reaction. J. Clin. Path. 6,99-104.

64. COOKSON, G.H., & RIMINGTON, C., 1953. Porphobilinogen. Chemical constitution. Nature. 171,875-876.

65. COOKSON, G.H., & RIMINGTON, C., 1954. Porphobilinogen. Biochem. J. 57,476-484.

66. CORCORAN, J.W., & SHEMIN, D., 1957. On the biosynthesis of the porphyrin-like moiety of vitamin B_{12}: the mode of utilization of δ-aminolevulinic acid. Biochim. Biophys. Acta. 25,661-662.

67. CRADDOCK, C.G., Jr., PERRY, S., & LAWRENCE, J.S., 1960. The dynamics of leukopenia and leucocytosis. Ann. Intern. Med. 52,281-294.

68. CRIDDLE, R.S., & BOCK, R.M., 1959. The physical-chemical properties of water-soluble cytochrome oxidase. Biochem. Biophys. Research Communs. 1,138-142.

69. DAVIS, A.R., NEWTON, E.B., & BENEDICT, S.R., 1922. The combined uric acid in beef blood. J. Biol. Chem. 54, 595-599.

70. de DUVE, C., 1963-64. The separation and characterization of subcellular particles. Harvey Lect. 59,49-87.

71. de MATTEIS, F., & RIMINGTON, C., 1962. The biochemical disturbance in acute intermittent and experimental porphyria. Lancet. 1,1332-1334.

72. de MATTEIS, F., 1962. An hypothesis concerning the biochemical disturbance in acute intermittent and experimental porphyria. Panminerva Med. 4,368-371.

73. de MATTEIS, F., & PRIOR, B.E., 1962. Experimental hepatic porphyria caused by feeding 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine. Comparison with sedormid porphyria. Biochem. J. 83,1-8.

74. de MATTEIS, F., SLATER, T.F., & WANG, D.Y., 1963. Liver nucleotides in experimental hepatic porphyria. Biochim. Biophys. Acta. 68,100-107.

75. DOWDLE, E.B., & EALES, L., 1963. The metabolism of glycine in experimental porphyria. S.Afr. J. Lab. Clin. Med. 9,5-11.

76. DOWDLE, E.B., SWEENEY, G.D., SAUNDERS, S.J., & EALES, L., 1963. National Conference on Nuclear Energy : Application of Isotopes and Radiation, Pretoria, Republic of South Africa. "The metabolism of [2-C^{14}] glycine by normal and porphyric human subjects".

77. DOWDLE, E.B., 1963. The metabolism of glycine-2-C^{14} in human porphyria. S.Afr. J. Lab. Clin. Med. 9,220-227.

78. DOWDLE, E.B., 1966. Metabolic fate in normals and porphyrics of orally administered ALA-5-C^{14}. — personal communication

79. DOWDLE, E.B., 1966. Personal communication.

80. DRABKIN, D.L., 1950. Cytochrome c metabolism and liver regeneration; influence of thyroid gland and thyroxine. J. Biol. Chem. 182,335-349.

81. DRABKIN, D.L., 1951. Independent biosynthesis of different hemin chromoproteins - cytochrome c in various tissues. Proc. Soc. Exper. Biol. and Med. 76,527-530.

82. DRABKIN, D.L., 1955. Independent biosynthesis of different haemin chromoproteins, with special reference to cytochrome c; The role of tissue organs. Porphyrin Biosynthesis and Metabolism (pg.) 96-127. Ed by Wolstenholme, G.E.W. and Millar, E.C.P. London: J. and A. Churchill Ltd.

83. DRESEL, E.I.B., & FALK, J.E., 1953. Conversion of δ-aminolaevulinic acid to porphobilinogen in a tissue system. Nature (Lond.) 172,1185.

84. DRESEL, E.I.B., 1955. The role of some porphyrins and porphyrin precursors in the biosynthesis of haem. Porphyrin Biosynthesis and Metabolism, pg. 72-85. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London: J. and A. Churchill Ltd.

85. DRESSEL, E.I.B., & FALK, J.E., 1956. Studies on the biosynthesis of blood pigments. 2. Haem and porphyrin formation in intact chicken erythocytes. Biochem. J. 63,72-79.

86. DRESSEL, E.I.B., & FALK, J.E., 1956. Studies on the biosynthesis of blood pigments. 3. Haem and porphyrin formation from δ-aminolaevulinic acid and from porphobilinogen in haemolysed chicken erythrocytes. Biochem. J. 63,80-87.

87. DUNN, J., & WYNGAARDEN, J.B. : Unpublished results.

88. DUTTON, G.J., & STOREY, D.E., 1954. Uridine compounds in glucuronic acid metabolism I. The formation of glucuronides in liver suspensions. Biochem. J. 57, 275-283.

89. EALES, L., 1961. The porphyrins and the porphyrias. Ann. Rev. Med. 12,251-270.

90. EALES, L., 1963. Porphyria as seen in Cape Town. A survey of 250 patients and some recent studies. S.Afr. J. Lab. Clin. Med. 9,151-162.

91. ELLIOTT, W.H., 1960. Aminoacetone formation by Staphylococcus aureus. Biochem. J. 74,478-485.

92. ELWYN, D., & SPRINSON, D.B., 1950. The role of serine and acetate in uric acid formation. J. Biol. Chem. 184, 465-474.

93. ENGELMAN, K., WATTS, R.W.E., KLINENBERG, J.R., SJOERDSMA, A., & SEEGMILLER, J.E., 1964. Clinical physiological and biochemical studies of a patient with xanthinuria and pheochromocytoma. Am. J. Med. 37,839-861.

94. ENGSTED, L., 1957. Endogenous formation of carbon monoxide in hemolytic disease. Acta. Med. Scand. No. 332 Vol 159.

95. ERIKSEN, L., 1955. Lead intoxication I. The effect of lead on the in-vitro biosynthesis of heme and free erythrocyte porphyrins. Scand. J. Clin. Lab. Invest. 7,80-85.

96. ERIKSEN, L., 1955. Relation of free erythrocyte porphyrins to haemoglobin biosynthesis. Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 185-195. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

97. EVANS, R., 1954. Short-lived red cells in normal individuals. Nature 173,129-130.

98. FALCONER, R., & GULLAND, J.M., 1939. The constitution of purine nucleotides VIII. Uric acid riboside. J. Chem. Soc. pg. 1369.

99. FALK, J.E., DRESEL, E.I.B., & RIMINGTON, C., 1953. Porphobilinogen as a porphyrin precursor, and the interconversion of porphyrins, in a tissue system. Nature. Lond. 172,292-294.

100. FALK, J.E., 1955. Haem and porphyrin formation from glycine, δ-aminolaevulic acid and porphobilinogen. Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 63-71. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

101. FLAKS, J.G., ERWIN, M.J., & BUCHANAN, J.M., 1957. Biosynthesis of purines XVI. The synthesis of adenosine-5'-phosphate and 5'-amino-4-imidazole-carboxamide ribotide by a nucleotide pyrophosphorylase. J. Biol. Chem. 228,201-213.

102. FLAKS, J.G., WARREN, L., & BUCHANAN, J.M., 1957. Biosynthesis of the purines XVII. Further studies of the inosinic transformylase system. J. Biol. Chem. 228,215-229.

103. FLAKS, J.G., ERWIN, M.J., & BUCHANAN, J.M., 1957. Biosynthesis of the purines XVIII. 5-amino-1-ribosyl-4-imidazolecarboxamide 5'-phosphate transformylase and inosinicase. J. Biol. Chem. 229,603-612.

104. FOLIN, O., 1934. The preparation of crystallized uric acid. Lab. Manuel of Biol. Chem. 5th Ed. N.Y. pg. 299.

105. FOUTS, P.J., HELMER, O.M., & LEPKOVSKY, S., 1940. Nutritional microcytic hypochromic anemia in dogs cured with crystalline factor I. Am. J. M. Sc. 199,163-166.

106. FRIEDKIN, M., 1952. Enzymatic synthesis of desoxyxanthosine by the action of xanthosine phosphorylase in mammalian tissue. J. Am. Chem. Soc. 74,112-115.

107. FROMM, H.J., 1958. On the equilibrium and mechanism of adenylosuccinic acid synthesis. Biochim. Biophys. Acta. 29,255-262.

108. FURST, S.S., ROLL, P.M., & BROWN, G.B., 1950. On the renewal of the purines of the desoxypentose and pentose nucleic acids. J. Biol. Chem. 183,251-266.

109. GAJDOS, A., GAJDOS-TÖRÖK, M., 1961. The therapeutic effect of adenosine-5-monophosphoric acid in porphyria. Lancet. 2,175-177.

110. GAJDOS, A., & GAJDOS-TÖRÖK, M., & DANIELI, G., 1963. Treatment by adenosine-5-monophosphoric acid of experimental saturnism in rabbits. Nature. Lond. 193, 183-184.

111. GAJDOS, A., & GAJDOS-TÖRÖK, M., 1962. Clinical and experimental trial of adenosine-5-monophosphoric acid in porphyria. Panminerva Med. 4,332-340.

112. GAJDOS, A., & GAJDOS-TÖRÖK, M., 1963. Inhibitory effect of purine nucleosides and nucleotides on the porphyrin synthesis by Rhodopseudomonas spheroides. S.Afr. J. Lab. Clin. Med. 9,232-234.

113. GAJDOS, A., & GAJDOS-TÖRÖK, M., 1965. Effect of adenosine-5-monophosphoric acid or inosine on porphyria caused by Griseofulvin in rats. Nature. 206,297-298.

114. GAJDOS, A., & GAJDOS-TÖRÖK, M., 1965. Mechanism of the inhibitory effect of adenine nucleotides on porphyrin synthesis by Rhodopseudomonas spheroides. Nature. 207, 640-641.

115. GAJDOS, A., GAJDOS-TÖRÖK, M., PALMA-CARLOS, A., & PALMA-CARLOS, L., 1966. Porphyria induced in rats by orotic acid and the effect of adenine-5-monophosphoric acid. Nature. 211,974-975.

116. GARAY, E.A.R., OWEN, C.A., Jr., & FLOCK, E.V., 1966. Formation of bilirubin in normal, damaged and Gunn rat livers. J. Lab. Clin. Med. 67,817-829.

117. GARFINKEL, D., & LAJTHA, A., 1963. A metabolic inhomogeneity of glycine in-vivo. I. Experimental determination. J. Biol. Chem. 238,2429-2434.

118. GERBRANDY, J., van LEEUWEN, A.M., HOLLENDOORN, H.B., & de VRIES, L.A., 1960. The binding between electrolytes and serum proteins calculated from an in-vivo filtration method. Clin. Sci. 19,181-193.

119. GREEN, W., BENDICH, A., BODANSKY, O., & BROWN, G.B., 1950. Fate of uric acid in man. J. Biol. Chem. 183,21-31.

120. GIBSON, K.D., NEUBERGER, A., & SCOTT, J.J., 1954. The enzymic conversion of δ-aminolaevulic acid to porphobilinogen. Biochem. J. 58,xli.

121. GIBSON, K.D., NEUBERGER, A., & SCOTT, J.J., 1955. The purification of properties of δ-aminolaevulic acid dehydrase. Biochem. J. 61,618-629.

122. GIBSON, K.D., 1955. Some properties of δ-aminolaevulic dehydrase. Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 27-42. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

123. GIBSON, K.D., 1958. Biosynthesis of δ-aminolaevulic acid by extracts of Rhodopseudomonas spheroides. Biochim. Biophys. Acta. Amst. 28,451.

124. GIBSON, K.D., LAVER, W.G., & NEUBERGER, A., 1958. Formation of δ-aminolaevulic acid in-vitro from succinyl-coenzyme A and glycine. Biochem. J. 68,17p.

125. GIBSON, K.D., LAVER, W.G., & NEUBERGER, A., 1958. Initial stages in the biosynthesis of porphyrins 2. The formation of δ-aminolaevulic acid from glycine and succinyl coenzyme A by particles from chicken erythrocytes. Biochem. J. 70,71-81.

126. GIBSON, K.D., MATTHEW, M., NEUBERGER, A., & TAIT, G.H., 1961. Biosynthesis of porphyrins and chlorophylls. Nature. Lond. 192,204-208.

127. GIBSON, K.D., NEUBERGER, A., & TAIT, G.H., 1962. Studies on the biosynthesis of porphyrin and bacteriochlorophyll by Rhodopseudomonas spheroides I. The effect of Growth Conditions, Biochem. J. 83,539-549.

128. GIBSON, K.D., NEUBERGER, A., & TAIT, G.H., 1962. Studies on the biosynthesis of porphyrin and bacteriochlorophyll by Rhodopseudomonas spheroides 2. The effects of ethionine and threonine. Biochem J. 83,550-559.

129. GILBERTSEN, A.S., LOWRY, P.T., HAWKINSON, V., & WATSON, C.J. 1959. Studies of the dipyrryl-methene ("fusion") pigments. 1. The anabolic significance of the fecal mesobilifuscin. J. Clin. Invest. 38,1166-1174.

130. GINSBURG, A.D., & DOWDLE, E.B., 1963. Biochemical changes in dicarbethoxy-dihydrocollidine-induced porphyria in the rat. S.Afr. Lab. Clin. Med. 9,206-211.

131. GINSBURG, A.D., 1964. Biochemical studies in drug induced porphyrias in the rat. Thesis, U.C.T.

132. GJØRUP, S., POULSEN, H., & PRAETORIUS, E., 1955. Uric acid concentration in serum determined by enzymatic spectrophotometry. Scandinav. J. Clin. Invest. 2, 201-203.

133. GOLDBERG, A., PATON, W.D.M., & THOMPSON, J.W., 1954. Pharmacology of the porphyrins and porphobilinogen. Brit. J. Pharm. 9,91-94.

134. GOLDBERG, A., ASHENBRUCKER, H., CARTWRIGHT, G.E., & WINTROBE, M.M., 1956. Studies on the biosynthesis of heme in-vitro by avian erythrocytes. Blood. 11,821-833.

135. GOLDBERG, A., 1959. The enzymatic formation of haem by the incorporation of iron into protoporphyrin : Importance of ascorbic acid, ergothioneine and glutathione. Brit. J. Haemat. 5,150-157.

136. GOLDBERG, A., 1962. Acute intermittent porphyria (Aetiology & pathogenesis). Panminerva Med. 4,328-332.

137. GOLDTHWAIT, D.A., GREENBERG, G.R., & PEABODY, R.A., 1955. The involvement of 5-phosphoribosylamine in the biosynthesis of glycinamide ribotide. Biochim. et Biophys. Acta. 18,148-149.

138. GOLDTHWAIT, D.A., PEABODY, R.A., & GREENBERG, G.R., 1956. On the mechanism of synthesis of glycinamide ribotide and its formyl derivative. J. Biol. Chem. 221,569-577.

139. GOLDTHWAIT, D.A., 1956. 5-phosphoribosylamine, a precursor of glycinamide ribotide. J. Biol. Chem. 222, 1051-1068.

140. GOLDTHWAIT, D.A., 1957. Mechanisms of synthesis of purine nucleotides in heart muscle extracts. J. Clin. Invest. 36,1572-1578.

141. GRANICK, S., 1954. Enzymatic conversion of δ-aminolevulinic acid to porphobilinogen. Science. 120,1105-1106.

142. GRANICK, S., & MAUZERALL, D., 1958. Enzymes of porphyrin synthesis in red blood cells. pg. 115-121.

143. GRANICK, S., & MAUZERALL, D., 1958. Enzymic formation of protoporphyrin from coproporphyrinogen III. Fed. Proc. 17,233.

144. GRANICK, S., 1958. Porphyrin biosynthesis in erythrocytes. I. Formation of δ-aminolevulinic acid in erythrocytes. J. Biol. Chem. 232,1101-1117.

145. GRANICK, S., & MAUZERALL, D., 1958. Porphyrin biosynthesis in erythrocytes. II. Enzymes converting δ-aminolevulinic acid to coproporphyrinogen. J. Biol. Chem. 232,1119-1140.

146. GRANICK, S., & MAUZERALL, D., 1961. The metabolism of heme and chlorophyll. Metabolic Pathways Vol. 2, 2nd. Edition. Ed. by David M. Greenberg. Academic Press New York and London. pg. 525-616.

147. GRANICK, S., 1962. Porphyrin biosynthesis, porphyria diseases, and induced enzyme synthesis in chemical porphyria. Trans. N.Y. Acad. Sci. 25,53-65.

148. GRANICK, S., & URATA, G., 1963. Increase in activity of δ-aminolevulinic acid synthetase in liver mitochondria induced by feeding of 3,5-dicarbethoxy-1,4-dihydrocollidine. J. Biol. Chem. 238,821-827.

149. GRANICK, S., 1963. Induction of the synthesis of δ-aminolevulinic acid synthetase in liver parenchyma cells in culture by chemicals that induce acute porphyria. J. Biol. Chem. 238, p. 2247-2249.

150. GRAY, C.H., & KEKWICK, R.A., 1948. Bilirubin-serum protein complexes and the van den Bergh reaction. Nature. Lond. 161,274.

151. GRAY, C.H., NEUBERGER, A., & SNEATH, P.H.A., 1950. Studies in congenital porphyria. 2. Incorporation of ^{15}N in the stercobilin in the normal and in the porphyric. Biochem. J. 47,87-92.

152. GRAY, C.H., 1952. Isotope studies in porphyria. Brit. Med. Bull. 8,229-233.

153. GRAY, C.H., 1953. The bile pigments. Methuen. London.

154. GRAY, C.H., & SCOTT, J.J., 1959. The effect of haemorrhage on the incorporation of (α-^{14}C) glycine into stercobilin. Biochem. J. 71,38-42.

155. GRAY, C.H., & NICHOLSON, D.C., 1963. Pathways of bile pigment metabolism. Ann. N.Y. Acad. Sciences. 3, 281-289.

156. GRAYZEL, A.I., LIDDLE, L., & SEEGMILLER, J.E., 1961. Diagnostic significance of hyperuricemia in arthritis. New. Eng. J. Med. 265,763-768.

157. GREEN, S., & MAZUR, A., 1957. Relation of uric acid metabolism to release of iron from hepatic ferritin. J. Biol. Chem. 227,653-668.

158. GREENBERG, G.R., & JAENICKE, L., 1957. On the activation of the one-carbon unit for the biosynthesis of purine nucleotides. pgs. 204-232. Ciba Foundation Symposium on the chemistry and biology of purines. Ed. by Wolstenholme, G.E.W., and O'Connor, C.M. J. and A. Churchill Ltd., London.

159. GREGORY, C.H., 1963. Studies of conjugated bilirubin. III. Pigment 1, a complex of conjugated and free bilirubin. J. Lab. and Clin. Med. 61,917-925.

160. GRIFFITHS, M., 1952. Oxidation of uric acid catalyzed by copper and by the cytochrome-cytochrome oxidase system. J. Biol. Chem. 197,399-407.

161. GRINSTEIN, M., KAMEN, M.D., & MOORE, C.V., 1948. Observation on the utilization of glycine in the biosynthesis of haemoglobin. J. Biol. Chem. 174,767-768.

162. GRINSTEIN, M., BANNERMAN, R.M., VAVRA, J.D., & MOOR, C.V., 1960. Haemoglobin metabolism in thalassemia. In-vivo studies. Am. J. Med. 29,18-32.

163. GRODSKY, G.M., & CARBONE, J.V., 1957. The synthesis of bilirubin glucuronide by tissue homogenates. J. Biol. Chem. 226,449-458.

164. GUTMAN, A.B., & YU, T.F., 1957. Renal function in gout with commentary on the renal regulation of urate excretion, and the role of the kidney in the pathogensis of gout. Am. J. Med. 23,600-622.

165. GUTMAN, A.B., YÜ, T.F., BLACK, H., YALOW, R.S., & BERSON, S.A., 1958. Incorporation of glycine 1-C^{14}, glycine 2-C^{14} and glycine-N^{15} into uric acid in normal and gouty subjects. Am. J. Med. 25,917-932.

166. GUTMAN, A.B., YÜ, T.F., & BERGER, L., 1959. Tubular secretion of urate in man. J. Clin. Invest. 38,1778-1781.

167. GUTMAN, A.B., & YÜ, T.F., 1961. A three-component system for regulation of renal excretion of uric acid in man. Tr. Ass. Amer. Physicians. 74,353-365.

168. GUTMAN, A.B., YÜ, T.F., ADLER, M., & JAVITT, N.B., 1962. Intramolecular distribution of uric acid-N^{15} after administration of glycine-N^{15} and ammonium N^{15} chloride to gouty and non-gouty subjects. J. Clin. Invest. 41, 623-636.

169. GUTMAN, A.B., & YÜ, T.F., 1963. On the nature of the inborn metabolic error(s) of primary gout. Tr. Ass. Amer. Physicians. 76,141-151.

170. GUTMAN, A.B., & YÜ, T.F., 1963. An abnormality of glutamine metabolism in primary gout. Am. J. Med. 35, 820-831.

171. GUTMAN, A.B., & TSIAI-FAN YÜ, 1965. Uric acid metabolism in normal man and in primary gout. The New Eng. J. Med. 273,252-260; 313-321.

172. HAMILTON, L.D., 1955. Nucleic acid metabolism in chronic leukaemia. J. Clin. Invest. 34,938.

173. HANDLER, J.S., 1960. The role of lactic acid in the reduced excretion of uric acid in toxemia of pregnancy. J. Clin. Invest. 39,1526-1532.

174. HARPER, H.A., 1965. Review of Physiological Chemistry. Lange Medical Publications.

175. HARTMAN, S.C., LEVENBERG, B., & BUCHANAN, J.M., 1956. Biosynthesis of the purines XI. Structure, enzymatic synthesis and metabolism of glycinamide ribotide and (α-N formyl) - glycinamide ribotide. J. Biol. Chem. 221,1057-1070.

176. HARTMAN, S.C., & BUCHANAN, J.M., 1958. Biosynthesis of the purines. XXI. 5-phosphoribosylpyrophosphate amidotransferase. J. Biol. Chem. 233,451-455.

177. HARTMAN, S.C., & BUCHANAN, J.M., 1958. Biosynthesis of the purines. XXII. 2-amino-N-ribosylacetamide-5'-phosphate kinosynthase. J. Biol. Chem. 233,456-461.

178. HARTMAN, S.C., & BUCHANAN, J.M., 1959. Biosynthesis of purines. XXVI. The identification of the formyl donors of the transformylation reactions. J. Biol. Chem. 234, 1812-1816.

179. HARTMAN, S.C., 1963. Phosphoribosyl pyrophosphate amidotransferase : purification and general catalytic properties. J. Biol. Chem. 238,3024-3035.

180. HATEFI, Y., OSBORN, M.J., KAY, L.D., & HUENNEKENS, F.M., 1957. Hydroxymethyl tetrahydrofolic dehydrogenase. J. Biol. Chem. 227,637-647.

181. HATFIELD, D., & FORREST, H.S., 1962. Biosynthesis of 3-ribosyluric acid (uric acid riboside). Biochim. et Biophys. Acta. 62,185-187.

182. HATFIELD, D., & WYNGAARDEN, J.B., 1964. 3 ribosyl purines II. Studies on (3-ribosyl xanthine) - 5'-phosphate and on ribonucleotide derivatives of certain uracil analogues. J. Biol. Chem. 239,2587-2592.

183. HAWKE, P.B., OSER, B.L., & SUMMERSON, W.H., 1954. Practical Physiological Chemistry 13th Ed. New York: Blakiston. pg. 911.

184. HAWKINS, W.B., & JOHNSON, A.C., 1939. Bile pigment and hemoglobin inter-relation in anemic dogs. Am. J. Physiol. 126,326-336.

185. HEALEY, A.A., 1964. The effect of fasting and ketosis on uric acid excretion. Arthritis and Rheumatism. 7, 313.

186. HEATH, H., & HOARE, D.S., 1959. The biosynthesis of porphyrins from porphobilinogen by Rhodopseudomonas spheroides. Biochem. J. 72,14-22.

187. HEIKEL, T., LOCKWOOD, W.H., & RIMINGTON, C., 1958. Formation of non-enzymic haem. Nature, London. 182,313.

188. HEINRICH, M.R., & WILSON, D.W., 1950. The biosynthesis of nucleic acid components studied with C^{14} 1. Purines and pyrimidines in the rat. J. Biol. Chem. 186,447-460.

189. HELWIG, H.L., & GREENBERG, D.M., 1952. Studies on metabolism of iron-containing proteins with radioactive carbon and iron. J. Biol. Chem. 198,703-712.

190. HEPPEL, L.A., & HILMOE, R.J., 1951. Purification and properties of 5-nucleotidase. J. Biol. Chem. 188,665-676.

191. HEPPEL, L.A., & RABINOWITZ, J.C., 1958. Enzymology of nucleic acids, purines, and pyrimidines. Ann. Rev. Biochem. 27,613-642.

192. HIATT, H.H., 1958. Studies of ribose metabolism VI. Pathways of ribose synthesis in man. J. Clin. Invest. 37,1461-1464.

193. HIGGINS, J.T., ASHTON, D.M., SPEAS, M., & WYNGAARDEN, J.B., 1961. An evaluation of relative roles of substrate diversion and feedback inhibition in the control of purine synthesis. Clin. Res. 9,181a.

194. HOARE, D.S., & HEATH, H., 1959. The biosynthesis of porphyrins from porphobilinogen by Rhodopseudomonas spheroides 2. The partial purification and some properties of porphobilinogen deaminase and uroporphyrinogen decarboxylase. Biochem. J. 73,679-690.

195. HOWELL, R.R., & WYNGAARDEN, J.B., 1959. Uricolysis by haematin proteins. Clin. Res. 7,257-258.

196. HOWELL, R.R., SPEAS, M., & WYNGAARDEN, J.B., 1961. A quantitative study of recycling of isotope from glycine-1-C^{A}, α-N^{15} into various subunits of the uric acid molecule in a normal subject. J. Clin. Invest. 40, 2076-2082.

197. HOWELL, R.R., ASHTON, D.M., & WYNGAARDEN, J.B., 1962. Glucose-6-phosphatase deficiency glycogen storage disease. Studies on the inter-relationships of carbohydrate, lipid and purine abnormalities. Pediatrics, Springfield. 29, 533-565.

198. HOARE, D.S., & HEATH, H., 1958. Intermediates in the biosynthesis of porphyrins from porphobilinogen by Rhodopseudomonas spheroides. Nature, London. 181, 1592-1593.

199. HUENNEKENS, F.M., NURK, E., & GABRIO, B.W., 1956. Erythrocyte metabolism. 1. Purine nucleoside phosphorylase. J. Biol. Chem. 221,971-981.

200. HURWITZ, J., ANDERS, M., GOLD, M., & SMITH, I., 1965. The enzymatic methylation of ribonucleic acid and deoxyribonucleic acid. VII. The methylation of ribosomal ribonucleic acid. J. Biol. Chem. 240,1256-1266.

201. IBRAHIM, G., & WATSON, C.J., 1964. Early appearance of a bile bilirubin of high specific activity after administration of C^{14}- δ-aminolevulinic acid to a dog with bile fistula. Gastro-enterology. 46,299.

202. IBRAHIM, G., SCHWARTZ, S., & WATSON, C.J., The contribution of non-haemoglobin hemes to the early labelling of bile bilirubin. - In preparation.

203. IBRAHIM, G., SCHWARTZ, S., & WATSON, C.J. Conversion of C^{14} protoporphyrin to liver hemes and bilirubin in bile fistula dogs. In preparation.

204. IODICE, A.A., RICHERT, D.A., & SCHULMAN, M.P., 1958. Copper content of purified δ-aminolevulinic acid dehydrase. Fed. Proc. 17,248.

205. ISRAELS, L.G., YAMAMOTO, T., SKANDERBEG, J., & ZIPURSKY, A., 1963. Evidence for two components. Science. 139, 1054-1055.

206. ISRAELS, L.G., SKANDERBEG, J., GNYDA, H., ZINGG, W., & ZIPURSKY, A., 1963. A study of the early-labelled fraction of bile pigment. The effect of altering erythropoiesis on the incorporation of (2-^{14}C) glycine into haem and bilirubin. Brit. J. Haemat. 9,50-62.

207. ISRAELS, L.G., YAMAMOTO, T., SKANDERBEG, J., & ZIPURSKY, A., 1963. Alternate pathways of bilirubin production. Proc. 9th Congress Europ. Soc. Haematology, Lisbon. (S. Karger, Basel / New York) pgs. 891-897.

208. ISRAELS, L.G., 1964. Method of estimating specific activity of plasma bilirubin. Personal communication.

209. JAENICKE, L., 1956. Conversion of β-carbon of serine to N^{10}-formyltetrahydrofolic acid. Fed. Proc. 15,281.

210. JANDL, J.H., INMAN, J.K., SIMMONS, R.L., & ALLEN, D.W., 1959. The transfer of iron from serum iron-binding protein to human reticulocytes. J. Clin. Invest. 38,161-185.

211. JONES, O.P., 1959. Electron microscope studies of fetal erythropoiesis. Proceedings of the 7th Congress of the European Society of Hematology, London. Part II, pgs. 79-81.

212. JORGENSEN, S., & POULSEN, H.E., 1955. Enzyme determination of hypoxanthine and xanthine in human plasma and urine. Acta. pharmacol. et toxicol. 11,223.

213. JORGENSEN,S., & NIELSEN, A.T., 1956. Uric acid in human blood corpuscles and plasma. Scandinav. J. Clin. and Lab. Investigation. 8,108-112.

214. KAGAWA, Y., MINAKAMI, S., & YONEYAMA, Y., 1959. Prepared iron-proto chelating enzyme from avian erythrocytes in soluble form. J. Biochem. (Tokyo). 46,771.

215. KALCKAR, H.M., 1947. Differential spectrophotometry of purine compounds by means of specific enzymes III. Studies of the enzymes of purine metabolism. J. Biol. Chem. 167,461-475.

216. KALCKAR, H.M., 1947. The enzymatic synthesis of purine ribosides. J. Biol. Chem. 167,477-486.

217. KARLSSON, J.L., & BARKER, H.A., 1949. Biosynthesis of uric acid labelled with radioactive carbon. J. Biol. Chem. 177,597-599.

218. KAZIRO, K., KIKUCHI, G., & HANAOKA, C., 1955. Verdohemoglobin, an immediate precursor of biliverdin in the model reaction of hemoglobin in-vitro. J. Biochem. 42, 423.

219. KENCH, J.E., GARDIKAS, C., & WILKINSON, J.F., 1950. Bile pigment formation in-vitro from haematin and other haem derivatives. The Biochem. J. 47,129-134.

220. KIKUCHI, G., SHEMIN, D., & BACHMANN, B., 1958. The enzymic synthesis of δ-aminolevulinic acid. Biochim. Biophys. Acta. 28,219-220.

221. KIKUCHI, G., KUMAR, A., TALMAGE, P., & SHEMIN, D., 1958. The enzymatic synthesis of δ-aminolevulinic acid. J. Biol. Chem. 233,1214-1219.

222. KIKUCHI, G., KUMAR, A., & SHEMIN, D., 1959. Mechanism of enzymatic synthesis of δ-aminolevulinic acid. Fed. Proc. 18,259.

223. KLATSKIN, G., & BUNGARDS, L., 1956. Bilirubin-protein linkages in serum and their relatioship to the van den Bergh reaction. J. Clin. Invest. 35,537-551.

224. KORN, E.D., & BUCHANAN, J.M., 1955. Biosynthesis of the purines VI. Purification of liver nucleoside synthesis from 4-amino-5-imidazole carboxamide, adenine and 2,6-diamino purine. J. Biol. Chem. 212,183-191.

225. KORNBERG, A., TABOR, H., & SEBRELL, W.H., 1945. Blood regeneration in pyridoxine-deficient rats. Am. J. Physiol. 143,434-439.

226. KORNBERG, A., & PRICER, W.E., Jr., 1951. Enzymatic phosphorylation of adenosine and 2,6-diaminopurine riboside. J. Biol. Chem. 193,481-495.

227. KORNBERG, A., LIEBERMAN, I., & SIMMS, E.S., 1955. Enzymatic synthesis and properties of 5-phosphoribosyl pyrophosphate. J. Biol. Chem. 215,389.

228. KOWALSKI, E., DANCEWICZ, A., SZOT, Z., & LIPINSKI, B., & ROSEIK, O., 1959. Studies on ∫-aminolaevulic acid transamination. Acta. Biochim. Polon. 6,257-266.

229. KOWALSKI, E., DANCEWICZ, A., & SZOT, Z., 1960. ∫-aminolevulinic acid transaminase activity in tissues. Chem. Abstr. 54,4812g.

230. KRAKOFF, I.H., & BALIS, M.E., 1963. Mechanisms of uric acid production in chronic leukaemia. J. Clin. Invest. 42,947-948.

231. KRAMER, S., 1963. Iron metabolism in the porphyrias. S.Afr. J. Lab. Clin. Med. 9,283-287.

232. KRUEGER, R.C., MELNICK, I., & KLEIN, J.R., 1956. Formation of heme by broken-cell preparations of duck erythrocytes. Arch. Biochem. Biophys. 64,302-310.

233. KUN, E., DECHARY, J.M., & PITOT, H.C., 1954. The oxidation of glycolic acid by a liver enzyme. J. Biol. Chem. 210,269.

234. LABBE, R.F., TALMAN, E.L., & ALDRICH, R.A. 1954. Porphyrin metabolism II. Uric acid excretion in experimental porphyria. Biochim. Biophys. Acta. 15,590-591.

235. LABBE, R.F., TALMAN, E.L., & ALDRICH, R.A., 1955. Purine metabolism in porphyria. Fed. Proc. 14,241.

236. LABBE, R.F., & NISHIDA, C., 1957. A new method of Hemin isolation. Biochim. et Biophys. 26,437.

237. LABBE, R.F., ZASKE, M.R., & ALDRICH, R.A., 1959. Bilirubin inhibition of heme biosynthesis. Science. 129, 1741-1742.

238. LABBE, R.F., & HUBBARD, N., 1960. Preparation and properties of the iron-protoporphyrin chelating enzyme. Biochim. Biophys. Acta. 41,185-191.

239. LABBE, R.F., HANAWA, Y., & LOTTSFELDT, F.I., 1961. Heme and fatty acid biosynthesis in experimental porphyria. Arch. Biochem. 92,373-374.

240. LABBE, R.F., 1962. Purines in the treatment of porphyria. Lancet. 282,435-436.

241. LAGERKVIST, U., 1958. Biosynthesis of guanosine 5'-phosphate. I. Xanthosine 5'-phosphate as an intermediate. J. Biol. Chem. 233,138-142.

242. LAJTHA, L.G., & VANE, J.R., 1958. Dependence of bone marrow cells on the liver for purine supply. Nature, London. 182,191-192.

243. LASCELLES, J., 1955. The formation of porphyrins by photosynthetic bacteria. Porphyrin Biosynthesis and Metabolism, Ciba. pgs. 265-284.

244. LASCELLES, J., 1956. The synthesis of porphyrins and bacteriochlorophyll by cell suspensions of Rhodopseudomonas spheroides. Biochem. J. 62,78-93.

245. LASCELLES, J., 1957. Synthesis of porphyrins by cell suspensions of tetrahymena vorax : Effect of members of the vitamin B group. Biochem. J. 66,65-72.

246. LASCELLES, J., 1959. Adaptation to form bacteriochlorophyll in Rhodopseudomonas spheroides : Changes in activity of enzymes concerned in pyrrole synthesis. Biochem. J. 72,508-518.

247. LASCELLES, J., 1961. Synthesis of tetrapyrroles by micro-organisms. Physiol. Rev. 41,417-441.

248. LASTER, L., & MULLER, A.F., 1953. Uric acid production in a case of myeloid metaplasia associated with gouty arthritis studied with N^{15}-labeled glycine. Amer. J. Med. 15,857-861.

249. LATHE, G.H., & WALKER, M., 1958. The synthesis of bili-

...../cont. next page.

rubin glucuronide in animal and human liver. Biochem. J. 70,705-712.

250. LAVER, W.G., & NEUBERGER, A., 1957. The formation of ∫-aminolaevulinic acid by chicken erythrocytes in-vitro. Biochem. J. 67,22p-23p.

251. LAVER, W.G., NEUBERGER, A., & UDENFRIEND, S., 1958. Initial stages in the biosynthesis of porphyrins I. The formation of ∫-aminolaevulic acid by particles obtained from chicken erythrocytes. Biochem. J. 70,4-14.

252. LEMBERG, R., LEGGE, J.W., & LOCKWOOD, W.H., 1939. Coupled oxidation of ascorbic acid and haemoglobin. Biochem. J. 33,754-758.

253. LEMBERG, R., LEGGE, J.W., & LOCKWOOD, W.H., 1941. Coupled oxidation of ascorbic acid and haemoglobin; formation and properties of choleglobin. Biochem. J. 35,328-338.

254. LEMBERG, R., LEGGE, J.W., & LOCKWOOD, W.H. 1941. Coupled oxidation of ascorbic acid and haemoglobin;quantitative studies on choleglobin formation. Estimation of haemoglobin and ascorbic acid oxidations. Biochem J. 35,339-352.

255. LEMBERG, R., LOCKWOOD, W.H., & LEGGE, J.W., 1941. Coupled oxidation of ascorbic acid and haemoglobin; studies on formation of bile pigments form choleglobin and verdo-haemochromagen and on their isolation from erythrocytes. Biochem. J. 35,363-379.

256. LEMBERG, R., & LEGGE, J.W., 1949. Haematin compounds and bile pigments. Interscience, New York.

257. LENNOX, W.G., 1925. A study of the retention of uric acid during fasting. J. Biol. Chem. 66,521-572.

258. LESTER, R., OSTROW, J.D., & SCHMID, R., 1961. Enterohepatic circulation of bilirubin. Nature, London. 192,372.

259. LESTER, R., & SCHMID, R., 1962. The mechanism of intestinal absorption of bilirubin. J. Clin. Invest. 41,1379.

260. LESTER, R., 1963. Intestinal absorption of bilirubin. Ann. N.Y. Acad. Sci. 111,290-294.

261. LESTER, R., & SCHMID, R., 1963. Intestinal absorption of bile pigments I. The enterohepatic circulation of bilirubin in the rat. J. Clin. Invest. 42,736-746.

262. LESTER, R., 1964. Bilirubin metabolism. The New Eng. J. Med. 270,779-786.

263. LEVENBERG, B., & BUCHANAN, J.M., 1957. Biosynthesis of the purines XII. Structure, enzymatic synthesis, and metabolism of 5-iminoimidazole ribotide. J. Biol. Chem. 224,1005-1018.

264. LEVENBERG, B., & BUCHANAN, J.M., 1957. Biosynthesis of the purines XVIII. Structure, enzymatic synthesis and metabolism of (α-N-formyl)-glycinamidine ribotide. J. Biol. Chem. 224,1019-1027.

265. LEVINTHAL, C., KEYMAN, A., & HIGA, A., 1962. Messenger RNA turnover and protein synthesis in B. subtilis inhibited by actinomycin D. Proc. Nat. Acad. Sci. (U.S.A.) 48, 1631-1638.

266. LIDDLE, L., SEEGMILLER, J.E., & LASTER, L., 1959. The enzymatic spectrophotometric method for determination of uric acid. J. Lab. and Clin. Med. 54,903-913.

267. LIEBERMAN, I., 1956. Enzymatic synthesis of adenosine 5'-phosphate from inosine-5-phosphate. J. Biol. Chem. 223,327-339.

268. LOCHHEAD, A.C., & GOLDBERG, A., 1959. The enzymic formation of haem by human and rat tissues. Biochem. J. 73, 39p-40p.

269. LOCKWOOD, W.H., & RIMINGTON, C., 1957. Purification of an enzyme converting porphobilinogen to uroporphyrin. Biochem. J. 67,8p.

270. LOCKWOOD, W.H., & BENSON, A., 1960. The enzymic condensation of porphobilinogen to porphyrins. Biochem. J. 75,372-381.

271. LOEB, J.N., HOWELL, R.R., & TOMKINS, G.M., 1965. Turnover of ribosomal RNA in rat liver. Science. 149,1093-1095.

272. LONDON, I.M., SHEMIN, D., WEST, R., & RITTENBERG, D., 1949. Heme synthesis and red blood cell dynamics in normal humans and in subjects with polycythemia vera, sickle cell anemia. J. Biol. Chem. 179,463-484.

273. LONDON, I.M., WEST, R., SHEMIN, D., & RITTENBERG, D., 1950. On the origin of bile pigment in normal man. J. Biol. Chem. 184,351.

274. LONDON, I.M., & WEST, R., 1950. Formation of bile pigment in pernicious anemia. J. Biol. Chem. 184, 359-364.

275. LONDON, I.M., WEST, R., SHEMIN, D., & RITTENBERG, D., 1950. Porphyrin formation and hemoglobin metabolism in congenital porphyria. J. Biol. Chem. 184,365-371.

276. LONDON, I.M., 1950. The conversion of hematin to bile pigment. J. Biol. Chem. 184,373-376.

277. LONDON, I.M., YAMASAKI, M., & SABELLA, G., 1951. Conversion of protoporphyrin to bile pigment. Fed. Proc. 10,217.

278. LOTTSFELDT, F.I., LABBE, R.F., 1961. Iron metabolism in experimental porphyria. Clin. Res. 9,93.

279. LOTTSFELDT, F.I., LABBE, R.F., & ALDRICH, R.A., 1961. The effect of inosine on heme synthesis in experimental porphyria. J.A.M.A. 178,928-929.

280. LOWY, B.A., WILLIAMS, M.K., & LONDON, I.M., 1962. Enzymatic deficiencies of purine nucleotide synthesis in the human erythrocyte. J. Biol. Chem. 237,1622-1625.

281. LUDWIG, G.D., 1963. Uric acid metabolism in acute intermittent porphyria. J. Clin. Invest. 42,953.

282. LUKENS, L.N., & BUCHANAN, J.B., 1957. Further intermediates in the biosynthesis of inosinic acid de novo. J. Am. Chem. Soc. 79,1511-1513.

283. LUKENS, L.N., & HERRINGTON, K.A., 1957. Enzymic formation of 6-mercaptopurine ribotide. Biochim. Biophys. Acta. 24,432-433.

284. MAGASANIK, B., MOYED, H.S., & GEHRING, L.B., 1957. Enzymes essential for the biosynthesis of nucleic acid guanine : inosine 5'-phosphate dehydrogenase of aerobacter aerogenes. J. Biol. Chem. 226,339-350.

285. MAGEE, P.N., & FARBER, E., 1962. Toxic liver injury and carcinogenesis : methylation of rat-liver nucleic acids by dimethyl-nitrosamine in-vivo. Biochem. J. 83,114-124.

286. MARTIN, N.H., 1949. Preparation and properties of serum and plasma proteins. XXI. Interactions with bilirubin. J. Amer. Chem. Soc. 71,1230.

287. MARVER, H., TSCHUDY, D., PERLROTH, M., & COLLINS, A., 1965. The regulation of heme biosynthesis. Clin. Res. 13,278.

288. MAUZERALL, D., 1964. Normal porphyrin metabolism. J. Pediat. 64,5-16.

289. MAUZERALL, D., & GRANICK, S., 1958. Porphyrin biosynthesis in erythrocytes III. Uroporphyrinogen and its decarboxylase. J. Biol. Chem. 232,1141-1162.

290. MAZUR, A., GREEN, S., SAHA, A., & CARLETON, A., 1958. Mechanism of release of ferritin iron in-vivo by xanthine oxidase. J. Clin. Invest. 37,1809-1817.

291. MEISTER, A., 1956. Metabolism of glutamine. Physiol. Rev. 36,103-127.

292. MELLINKOFF, S.M., HALPERN, R.M., FRANKLAND, D.N., & GREIPEL, M., 1959. Abnormal urinary aminoacid patterns in acute intermittent porphyria. J. Lab. Clin. Med. 53,358-363.

293. MELNICK, I., & BUCHANAN, J.M., 1957. Biosynthesis of the purines. XIV. Conversion of (∠-N-formyl)-glycinamide ribotide to (∠-N-formyl)-glycinamidine ribotide; purification and requirements of the enzyme system. J. Biol. Chem. 225,157-162.

294. MERCHANTE, A., WAJCHENBERG, B.L., & SCHWARTZ, S., 1957. Conversion of porphobilinogen to porphyrin by liver homogenates of rats with experimental hepatic porphyria. Proc. Soc. Exper. Biol. Med. N.Y. 95,221-224.

295. MICHAEL, S.T., 1944. The relation of uric acid excretion to blood lactic acid in man. Amer. J. Physiol. 141,71-74.

296. MICHAELSSON, M., 1961. Bilirubin determination in serum and urine. Studies on diazo methods and a new copper-azo pigment method. Scand. J. Clin. Lab. Invest. 13, (suppl.56),1-80.

297. MILLER, R.W., & BUCHANAN, J.M., 1962. Biosynthesis of purines. XXVII. N-(5-amino-1-ribosyl-4-imidazolylcarbonyl) -L-aspartic acid 5'-phosphate kinosynthetase. J. Biol. Chem. 237,485-490.

298. MILLER, R.W., & BUCHANAN, J.M., 1962. Biosynthesis of purines. XXVIII. Mechanism of action of adenylo-succinase. J. Biol. Chem. 237,491-496.

299. MILLS, G.C., & RANDALL, H.P., 1958. Hemoglobin catabolism II. The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. J. Biol. Chem. 232,589-598.

300. MILNE, M.D., 1966. Urate excretion. Proc. Royal Soc. Med. 59,308-310.

301. MINAKAMI, S., YONEYAMA, Y., & YOSHIKAWA, H., 1958. On the biosynthesis of heme and hemeproteins in liver cells. Biochim. Biophys. Acta. 28,447-449.

302. MIYAKOSHI, T., & KIKUCHI, G., 1963. Studies on experimental porphyria. I. Increased synthesis of δ-aminolevulinic acid in allylisopropylacetamide-induced porphyric rat. Tohoku J. Exp. Med. 79, 199-209.

303. MOAT, A.G., WILKINS, C.N., Jr., & FRIEDMAN, H., 1956. Biotin in purine biosynthesis. Fed. Proc. 15,605.

304. MORGAN, E.J., 1926. The distribution of xanthine oxidase. I. Biochem. J. 20,1282-1291.

305. MORRISON, M., ESTERBROOK, R.W., & STOTZ, E., 1954. Multiple prosthetic groups in cytochrome c. J. Amer. Chem. Soc. 76,6409-6410.

306. MUIR, H.M., & NEUBERGER, A., 1949. The biogenesis of porphyrins. The distribution of ^{15}N in the ring system. Biochem. J. 45,163-170.

307. MUIR, H.M., & NEUBERGER, A., 1950. Biogenesis of porphyrins 2. The origin of the methyne carbon atoms. Biochem. J. 47,97-104.

308. MULLER, A.F., & BAUER, W., 1953. Uric acid production in normal and gouty subjects, determined by N^{15} labeled glycine. Proc. Soc. Exper. Biol. and Med. 82,47-50.

309. MURPHY, R., & SHIPMAN, H., 1963. Hyperuricemia during total fasting : renal factors. Arch. Intern. Med. (Chicago). 112,954-959.

310. MURRAY, R.K., CONNELL, G.E., & PERT, J.H., 1961. The role of haptoglobin in the clearance and distribution of extra corpuscular hemoglobin. Blood. 17,45-53.

311. NAJJAR, V.A., & CHILDS, B., 1953. The crystallization and properties of serum bilirubin. J. Biol. Chem. 204, 359-366.

312. NAKADA, H.I. & WEINHOUSE, S., 1953. Studies of glycine oxidation in rat tissues. Arch. Biochem. 42,257-270.

313. NAKADA, H.I., FRIEDMANN, B., & WEINHOUSE, S., 1955. Pathways of glycine catabolism in rat liver. J. Biol. Chem. 216,583-592.

314. NAKADA, H.I., & SUND, L.P., 1958. Glyoxylic acid oxidation by rat liver. J. Biol. Chem. 233,8-13.

315. NAKAJIMA, H., TAKEMURA, T., NAKAJIMA, O., & YAMAOKA, K., 1963. Studies on heme alpha-methenyl oxygenase. I. The enzymatic conversion of pyridine-hemichromogen and hemoglobin-haptoglobin into a possible precursor. J. Biol. Chem. 238,3784-3796.

316. NAKAJIMA, H., 1963. Studies on heme alpha-methenyl oxygenase II. The isolation and characterization of the final reaction product, a possible precursor of biliverdin. J. Biol. Chem. 238,3797-3801.

317. NARROD, S.A., & JAKOBY, W.B., 1958. Enzymatic conversion of ethanolamine to glycolaldehyde. Fed. Proc. 17,281.

318. NATHAN, D.G., & GARDNER, F.H., 1962. The relationship of increased erythropoiesis to globin synthesis in man. J. Clin. Invest. 41,1389.

319. NEMETH, A.M., RUSSELL, C.S., & SHEMIN, D., 1957. The succinate-glycine cycle. II. Metabolism of δ-aminolevulinic acid. J. Biol. Chem. 229,415-422.

320. NEUBERGER, A., & SCOTT, J.J., 1953. Aminolaevulinic acid and porphyrin biosynthesis. Nature, London. 172,1093-1094.

321. NEUBERGER, A., SCOTT, J.J., & GRAY, C.H., 1954. The metabolism of δaminolaevulic acid in acute porphyria. Biochem. J. 58,xli.

322. NEUBERGER, A., & TURNER, J.M., 1963. γ, δ-dioxovalerate aminotransferase activity in Rhodopseudomonas spheroides. Biochim Biophys. Acta. 67,342-345.

323. NEVE, R.A., LABBE, R.F., & ALDRICH, R.A., 1956. Reduced uroporphyrin III in the biosynthesis of heme. J. Am. Chem. Soc. 78,691-692.

324. NICHOLS, J., MILLER, A.T.,Jr., & HIATT, E.P., 1951. Influence of muscular exercise on uric acid excretion in man. J. Appl. Physiol. 3,501-507.

325. NIERLICH, D.P., & MAGASANIK, B., 1965. Regulation of purine ribonucleotide synthesis by end product inhibition. J. Biol. Chem. 240,358-365.

326. NISHIDA, G., & LABBE, R.F., 1959. Heme biosynthesis; on the incorporation of iron into protoporphyrin. Biochim. Biophys. Acta. 31,519-524.

327. NUGENT, C.A., & TYLER, F.H., 1959. The renal excretion of uric acid in patients with gout and in non-gouty subjects. J. Clin. Invest. 38,1890-1898.

328. NYHAN, W.L., & CHILDS, B., 1964. Hyperglycinemia V. The miscible pool and turnover rate of glycine and the formation of serine. J. Clin. Invest. 43,2402-2409.

329. OSBORN, M.J., & HUENNEKENS, F.M., 1957. Participation of anhydroleucovorin in the hydroxymethyl tetrahydrofolic dehydrogenase system. Biochim. et Biophys. Acta. 26, 646-647.

330. OSBORN, M.J., & TALBERT, P.T., 1957. Hydroxymethyl tetrahydrofolic dehydrogenase. Fed. Proc. 16,230.

331. OSTROW, J.D., HAMMAKER, L., & SCHMID, R., 1961. The preparation of crystalline bilirubin-C^{14}. J. Clin. Invest. 40,1442-1452.

332. OSTROW, J.D., JANDL, J.H., & SCHMID, R., 1962. The formation of bilirubin from hemoglobin in-vivo. J. Clin. Invest. 41,1628-1637.

333. OSTROW, J.D., & SCHMID, R., 1963. The protein binding of C^{14} bilirubin in human and murine serum. J. Clin. Invest. 42,1286-1299.

334. PADOVA, J., & BENDERSKY, G., 1962. Hyperuricemia in diabetic ketoacidosis. New Eng. J. Med. 267,530-534.

335. PALMA-CARLOS, A., PALMA-CARLOS, L., GAJDOS-TÖRÖK, M., & GAJDOS, A., 1966. Experimental porphyria induced in rats by ethionine. Nature. 211,977-978.

336. PAPPENHEIMER, A.M., 1947. Diphtheria toxin : reinvestigation of effect of iron on toxin and porphyrin production. J. Biol. Chem. 167,251-259.

337. PASS, I.J., SCHWARTZ, S., & WATSON, C.J., 1945. The conversion of hematin to bilirubin following intravenous administration in human subjects. J. Clin. Invest. 24, 283-291.

338. PATWARDHAN, M.V., 1957. Role of ferrous iron in enzymatic transamination. Nature, London. 181,187.

339. PAUL, K.G., 1950. The conversion of ferriporphyrin hemins to their corresponding porphyrins by means of pyruvic acid. Acta. Chem. Scand. 4,1221.

340. PERLROTH, M.G., TSCHUDY, D.P., MARVER, H.S., COSTAN, W.B., ZEIGEL, R.F., RECHCIGL, M., & COLLINS, A., 1966. Acute intermittent porphyria. Am. J. Med. 41,149-162.

341. PETERS, J.M., & GREENBERG, D.M., 1958. Citrovorum factor cyclodehydrase. J. Am. Chem. Soc. 80,2719-2722.

342. POLLYCOVE, M., TOLBERT, B.M., LAWRENCE, J.H., & HARMAN, D., 1957. Uric acid metabolism : the oxidation of uric acid in normal subjects and patients with gout, polycythemia and leukemia. Clin. Res. Proc. 5,38-39.

343. PORRA, R.J., & FALK, J.E., 1961. Protein-bound porphyrins associated with protoporphyrin biosynthesis. Biochem. Biophys. Res. Commun. 5,179-184.

344. PORRA, R.J., & JONES, O.T., 1963. Studies on ferrochelatase. I. Assay and properties of ferrochelatase from a pig-liver mitochondrial extract. Biochem. J. 87,181-185.

345. PORRA, R.J., & JONES, O.T., 1963. Studies on ferrochelatase 2. An investigation of the role of ferrochelatase in the biosynthesis of various haem prosthetic groups. Biochem. J. 87,186-192.

346. PORRA, R.J., & FALK, J.E., 1964. The enzymic conversion of coproporphyrinogen III into protoporphyrin IX. Biochem. J. 90,69-75.

347. PRAETORIUS, E., & KIRK, J.E., 1950. Hyperuricemia : with evidence for tubular elimination of uric acid. J. Lab. and Clin. Med. 35,865-868.

348. PREISS, J., & HANDLER, P., 1957. Enzymatic synthesis of nicotinamide mononucleotide. J. Biol. Chem. 225,759-770.

349. PRICE, V.E., STERLING, W.R., TARANTOLA, V.A., HARTLEY, R.W., Jr., & RECHCIGL, M., Jr., 1962. The kinetics of catalase synthesis and destruction in-vivo. J. Biol. Chem. 237, 3468-3475.

350. RABINOWITZ, J.C., & PRICER, W.E.Jr., 1956. Formimino tetrahydrofolic acid and methenetetrahydrofolic acid as intermediates in the formation of N^5 formyl tetrahydrofolic acid. J. Am. Chem. Soc. 78,5702-5704.

351. RABINOWITZ, J.C., & PRICER, W.E., Jr., 1957. An enzymatic method for the determination of formic acid. J. Biol. Chem. 229,321-328.

352. RADIN, N.S., RITTENBERG, D., & SHEMIN, D., 1950. The role of glycine in the biosynthesis of heme. J. Biol. Chem. 184,745-753.

353. RAJAGOPALAN, K.V., & SARMA, P.S., 1958. Effect of sulphanilamide on formation of sterols in saccharomyces cerevisiae and neurosporacrassa. Biochem. J. 69,53-56.

354. RASETTI, L., PETTINATI, L., & RUBINO, G.F., 1962. Effect of glycine administration in aquired porphyria cutanea tarda. Panminerva Med. 4,390-391.

355. RATNER, S., NOCITO, V., & GREEN, D.E., 1944. Glycine oxidase. J. Biol. Chem. 152,119-133.

356. RECTOR, F.C., Jr., & ORLOFF, J., 1959. The effect of the administration of sodium bicarbonate and ammonium chloride on the excretion and production of ammonia : the absence of alterations in the activity of renal ammonia-producing enzymes in the dog. J. Clin. Invest. 38,366-372.

357. REMY, C.N., REMY, W.T., & BUCHANAN, J.M., 1955. Biosynthesis of the purines. VIII. Enzymatic synthesis and utilization of α-5-phosphoribosyl-pyrophosphate. J. Biol. Chem. 217,885-895.

358. RICHARDS, F.F., & SCOTT, J.J., 1961. Glycine metabolism in acute porphyria. Clin. Sci. 20,387-400.

359. RIMINGTON, C., 1954. Porphyrin metabolism. Chem. Soc. Ann. Rep. 51,311.

360. RIMINGTON, & BOOIJ, H.L., 1957. Porphyrin biosynthesis in human red cells. Biochem. J. 65,3p.

361. RIMINGTON, C., 1959. Biosynthesis of haemoglobin. Brit. Med. Bull. 15,19-26.

362. RIMINGTON, C., 1962. Suggestions concerning the biochemical defects in the different porphyrin diseases. Panminerva Med. 4,307-311.

363. RIMINGTON, C., 1963. Types of porphyria : some thoughts about biochemical mechanisms involved. Ann. N.Y. Acad. Sci. 104,666-675.

364. ROBINSON, S.H., OWEN, C.A., FLOCK, E.V., & SCHMID, R., 1965. Direct hepatic synthesis of bilirubin : source of early labelling bile pigment (abstract). Proceedings of the American Association for the Study of Liver Disease. Gastroenterology. 48,496.

365. ROLL, P.M., BROWN, G.B., Di CARLO, F.J., & SCHUTZ, A.S., 1949. The metabolism of yeast nucleic acid in the rat. J. Biol. Chem. 180,333-340.

366. ROSSI-FANELLI, A., & ANTONINI, E., 1956. Heterogeneity of human myoglobin. Arch. Biochem. Biophys. 65,587-590.

367. RUBINO, G.F., 1962. The role of lead in porphyrin metabolism. Panminerva Med. 4,[illegible]-344.

368. RUBINO, G.F., RASETTI, L., GIARRUSSO, P., 1962. Effect of glycine administration on δaminolaevulic acid and porphobilinogen excretion in lead poisoning. Panminerva Med. 4,388-389.

369. SAFFREN, M., & SCARANO, E., 1953. Mechanism of the incorporation of adenine into adenosine monophosphate. Nature, London. 172,949-951.

370. SAKAMI, W., 1949. The conversion of glycine into serine in the intact rat. J. Biol. Chem. 178,519-520.

371. SALOMON, K., RICHMOND, J.E., & ALTMAN, K.I., 1952. Tetrapyrrole precursors of protoporphyrin IX. I. Uroporphyrin III. J. Biol. Chem. 196,463-468.

372. SANADI, D.R., & LITTLEFIELD, J.W., 1951. Studies on α-ketoglutaric oxidase I. Formation of "Active" succinate. J. Biol. Chem. 193,683-689.

373. SANDBERG, A.A., LEE, G.R., CARTWRIGHT, G.E., & WINTROBE, M.M., 1955. Purine nucleoside phosphorylase activity of blood. I. Erythrocytes. J. Clin. Invest. 34,1823-1829.

374. SANO, S., INOUE, S., TANABE, Y., SUMIYA, C., & KOIKE, S., 1959. Significance of mitochondria for porphyrin and heme biosynthesis. Science. 129,275-276.

375. SANO, S., & GRANICK, S., 1961. Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J. Biol. Chem. 236,1173-1180.

376. SARKER, N., & COMB, D.G., 1965. Methylation of transfer-like-RNA associated with ribosomes. Fed. Proc. 24,292.

377. SAUNDERS, S.J., 1963. Iron metabolism in symptomatic porphyria. A preliminary communication. S.Afr. J. Lab. Clin. Med. 9,277-282.

378. SCHACHTER, D., 1963. The formation and excretion of bilirubin. Med. Clin. N.America. 47,621-628.

379. SCHENKER, S., DAWBER, N.H., & SCHMID, R., 1964. Bilirubin metabolism in the fetus. J. Clin. Invest. 43,32-39.

380. SCHIFFMAN, E., & SHEMIN, D., 1957. Further studies on the utilization of ∫-aminolevulinic acid for porphyrin synthesis. J. Biol. Chem. 225,623-628.

381. SCHMID, R., & SCHWARTZ, S., 1952. Experimental porphyria III. Hepatic type produced by sedormid. Proc. Soc. Exper. Biol. and Med. N.Y. 81,685-689.

382. SCHMID, R., FIGEN, J.F., & SCHWARTZ, S., 1955. Experimental porphyria. IV. Studies of liver catalases and other heme enzymes in sedormid porphyria. J. Biol. Chem. 217, 263-274.

383. SCHMID, R., & SCHWARTZ, S., 1955. Studies of some liver heme proteins and porphyrins in experimental sedormid porphyria. Porphyrin Biosynthesis and Metabolism, pg. 196-205. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

384. SCHMID, R., & SHEMIN, D., 1955. The enzymatic formation of porphobilinogen from ∫-aminolevulinic acid and its conversion to protoporphyrin. J. Am. Chem. Soc. 77, 506-507.

385. SCHMID, R., HAMMAKER, L., & AXELROD, J., 1957. The enzymatic formation of bilirubin glucuronide. Arch. Biochem. and Biophys. 70,285-288.

386. SCHMID, R., AXELROD, J., HAMMAKER, L., & SWARM, R.L., 1958. Congenital jaundice in rats, due to a defect in glucuronide formation. J. Clin. Invest. 37,1123-1130.

387. SCHMID, R., 1958. Jaundice and bilirubin metabolism. A.M.A. Arch. Int. Med. 101,669-674.

388. SCHMID, R., 1960. "Hyperbilirubinemia" in "The Metabolic Basis of Inherited Disease", pg. 226-270. Ed. by Stanbury, J.B., Wyngaarden, J.B., and Fredrikson, D.S., New York, McGraw Hill, 1960.

389. SCHMID, R., 1960. "The Porphyrias" in "The metabolic Basis of Inherited Disease", pg. 939. Ed. by Stanbury, J.B., Wyngaardon, J.B., and Fredrickson, D.S., New York, McGraw-Hill, 1960.

390. SCHMID, R., 1963. Studies of congenital non-hemolytic jaundice with C ^{14}-bilirubin. Ann. N.Y. Acad. Sci. 111, 451-460.

391. SCHMID, R., & HAMMAKER, L., 1963. Metabolism and disposition of C ^{14}-bilirubin in congenital non-haemolytic jaundice. J. Clin. Invest. 42, 1720-1734.

392. SCHMID, R., 1966. "The Porphyrias" in "The metabolic Basis of Inherited Disease", pg. 813-870. Ed. by Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., New York, McGraw-Hill, 1966.

393. SCHMID, R., 1966. "Hyperbilirubinemia" in "The Metabolic Basis of Inherited Disease", pg. 871-902. Ed. by Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., New York, McGraw-Hill, 1966.

394. SCHMIDT, G., 1955. Nucleases and enzymes attacking nucleic acid components, in "The Nucleic Acids", Ed. by E. Chargaff and J.N. Davidson, Vol. 1, Academic, New York.

395. SCHULMAN, M.P., & RICHERT, D., 1956. Utilization of glycine, succinate and δ-aminolevulinic acid for heme synthesis. Fed. Proc. 15, 349-350.

396. SCHULMAN, M.P., & RICHERT, D.A., 1957. Heme synthesis in Vit. B_6 and pantothenic acid deficiencies. J. Biol. Chem. 226, 181-189.

397. SCHWARTZ, H.C., CARTWRIGHT, G.E., & WINTROBE, M.M., 1957. Studies on the synthesis of heme from protoporphyrin. Clin. Research. Proc. 5, 29.

398. SCHWARTZ, H.C., HILL, R.L., CARTWRIGHT, G.E., & WINTROBE, M.M., 1959. Enzymic synthesis of heme from iron and protoporphyrin. Fed. Proc. 18, 545.

399. SCHWARTZ, S., 1955. Porphyrins and porphyrin precursors in human and experimental porphyria. Fed. Proc. 14, 717-722.

400. SCHWARTZ, S., IBRAHIM, G., & WATSON, C.J., 1964. The contribution of non-hemoglobin hemes to the early labeling of bile bilirubin. J. Lab. and Clin. Med. 64, 1003.

401. SCHWARTZ, S., & CARDINAL, R., 1965. Incorporation of ALA-C^{14} into dog tissue hemes. Fed. Proc. 24, pg. 485.

402. SCOTT, J.J., 1955. The metabolism of δ-aminolaevulic acid. Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 43-62. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churcill Ltd.

403. SEEGMILLER, J.E., LASTER, L., & STETTEN, D., Jr., 1955. Incorporation of 4-amino-5-imidazolecarboxamide-4-C^{13} into uric acid in the normal human. J. Biol. Chem. 216,653-662.

404. SEEGMILLER, J.E., GRAYZEL, A.I., LASTER, L., & LIDDLE, L., 1961. Uric acid production in gout. J. Clin. Invest. 40,1304-1314.

405. SEEGMILLER, J.E., LASTER, L., & HOWELL, R.R., 1963. Biochemistry of uric acid and its relation to gout. New Eng. J. Med. 268,712-716,764-773,821-827.

406. SEEGMILLER, J.E., 1966. The study of human intermediary purine metabolism and its regulation. Proc. Roy. Soc. Med. 59,292-302.

407. SEGAL, S., & WYNGAARDEN, J.B., 1955. Plasma glutamine and oxypurine content in patients with gout. Proc. Soc. Exper. Biol. and Med. 88,342-345.

408. STANDARDS COMMITTEE OF COLLEGE OF AMERICAN PATHOLOGISTS, 1963. Recommendations for uniform bilirubin standard. Am. J. Clin. Path. 19,90-91.

409. SELLINGER, O.Z., & VERSTER, F.D., 1962. Glutamine synthetase of rat cerebral cortex : intracellular distribution and structural latency. J. Biol. Chem. 237, 2836-2844.

410. SHAPIRO, J.R., KLINENBERG, J.R., PECK, W., GOLDFINGER, S.E., & SEEGMILLER, J.E., 1964. Hyperuricemia associated with obesity and intensified by caloric restriction. Arthritis and Rheumatism. 7,343.

411. SHEMIN, D., & RITTENBERG, D., 1946. The biological utilization of glycine for the synthesis of the protoporphyrin of hemoglobin. J. Biol. Chem. 166,621-625.

412. SHEMIN, D., & RITTENBERG, D., 1946. The life span of the human red blood cell. J. Biol. Chem. 166,627-636.

413. SHEMIN, D., & RITTENBERG, D., 1947. On the utilization of glycine for uric acid synthesis in man. J. Biol. Chem. 162,875-876.

414. SHEMIN, D., LONDON, I.M., & RITTENBERG, D., 1948. The in-vitro synthesis of heme from glycine by the nucleated red blood cell. J. Biol. Chem. 173,799-800.

415. SHEMIN, D., & WITTENBERG, J., 1951. The mechanism of porphyrin formation. The role of the tricarboxylic acid cycle. J. Biol. Chem. 192,315-334.

416. SHEMIN, D., & KUMIN, S., 1952. The mechanism of porphyrin formation. The formation of a succinyl intermediate from succinate. J. Biol. Chem. 198,827-837.

417. SHEMIN, D., & RUSSELL, C.S., 1953. δ-aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. J. Amer. Chem. Soc. 75,4873.

418. SHEMIN, D., ABRAMSKY, T., & RUSSELL, C.S., 1954. The synthesis of protoporphyrin from δ-aminolevulinic acid in a cell-free extract. J. Amer. Chem. Soc. 76, 1204-1205.

419. SHEMIN, D., 1955. The biosynthesis of porphyrins. Harvey Lect. 50,258-284.

420. SHEMIN, D., 1955. "The succinate-glycine cycle : the role of δ-aminolevulinic acid in porphyrin synthesis". Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 4-26. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

421. SHEMIN, D., RUSSEL, C.S., & ABRAMSKY, T., 1955. The succinate-glycine cycle I. The mechanism of pyrrole synthesis. J. Biol. Chem. 215,613-626.

422. SHEMIN, D., KIKUCHI, G., & BACHMANN, B.J., 1958. Enzymatic synthesis of δ-aminolevulinic acid. Fed. Proc. 17,310.

423. SJÖSTRAND, T., 1951. Formation of carbon monoxide in connexion with haemoglobin catabolism. Nature. 168, 1118-1119.

424. SMITH, E.C.B., & MILLS, G.T., 1954. Uridine nucleotide compounds of the liver. Biochim. Biophys. Acta. 13, 386-400.

425. SNYDER, A.L., & SCHMID, R., 1965. The conversion of hematin to bile pigment in the rat. J. Lab. Clin. Med. 65,817-824.

426. SONNE, J.C., BUCHANAN, J.M., & DELUVA, A.M., 1948. Biological precursors of uric acid I. The role of lactate, acetate and formate in synthesis of ureido groups of uric acid. J. Biol. Chem. 173,69-79.

427. SORENSEN, L.B., 1959. Degradation of uric acid in man. Metabolism. 8,687-703.

428. SORENSEN, L.B., 1963. Current concepts of gout and its treatment. Med. Clin. N.Amer. 47,169-189.

429. SPECK, J.F., 1949. The synthesis of glutamine in pigeon liver dispersions. J. Biol. Chem. 179,1387-1403.

430. SPILMAN, S.L., 1954. Uric acid synthesis in the non-gouty and gouty human. Fed. Proc. 13,302.

431. STAEHELIN, T., WETTSTEIN, F.O., & NOLL, H., 1963. Breakdown of rat-liver ergosomes in-vivo after actinomycin inhibition of messenger RNA synthesis. Science. 140, 180-183.

432. STETTEN, De W., Jr., 1956. Current status of purine biosynthesis : an interim report. Ann. Rheumat. Dis. 15,404-405.

433. STROMINGER, J.L., KALCKAR, H.M., AXELROD, J., & MAXWELL, E.S., 1954. Enzymatic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. J. Amer. Chem. Soc. 26,6411.

434. TAKEDA, Y., & REEVE, E.B., 1963. Studies of the metabolism and distribution of albumin with antologous $1^{/3/}$-albumin in healthy men. J. Lab. and Clin. Med. 61,183-202.

435. TALMAN, E.L., CASE, J.D., NEVE, R.A., LABBE, R.F., ALDRICH, R.A., 1955. Porphyrin metabolism I. Experimental porphyria in chick embryos. J. Biol. Chem. 212,663-675.

436. TALMAN, E.L., LABBE, R.F., & ALDRICH, R.A., 1957. Porphyrin metabolism. IV. Molecular structure of acetamide derivatives affecting porphyrin metabolism. Arch. Biochem. Biophys. 66,289-300.

437. TALMAN, E.L., LABBE, R.F., ALDRICH, R.A., & SEARS, D., 1959. Porphyrin metabolism. V. The metabolism of purines in experimental porphyria. Arch. Biochem. Biophys. 80, 446-457.

438. TANAKA, T., & KNOX, W.E., 1959. The nature and mechanism of the tryptophan pyrrolase (peroxidase-oxidase) reaction of Pseudomonas and of rat liver. J. Biol. Chem. 234,1162-1170.

439. TANFORD, C., & LOVRIEN, R.E., 1959. Abstr. Amer. Chem. Soc. 14c.

440. TAXAY, E.P., 1961. Serum-uric-acid in porphyria. Lancet. 280,936.

441. TESAR, C., & RITTENBERG, D., 1947. The metabolism of L-histidine. J. Biol. Chem. 170,35-53.

442. THEORELL, H., BEZNAK, M., BONNICHSEN, R., PAUL, K.O., & AKESON, A., 1951. On the distribution of injected radioactive iron in guinea pigs and its rate of appearance in some hemoproteins and ferritins. Acta. Chem. Scandinav. 5,445.

443. TSCHUDY, D.P., ROSE, J., HELLMAN, E., COLLINS, A., & RECHCIGL, M., Jr., 1962. Biochemical studies of experimental porphyria. Metabolism. 11,1287-1301.

444. TSCHUDY, D.P., WELLAND, F.H., COLLINS, A., & HUNTER, G., 1963. Aminoacetone in acute intermittent porphyria. Lancet. 2,660-662.

445. TSCHUDY, D.P., WELLAND, F.H., COLLINS, A., & HUNTER, G., Jr., 1964. The effect of carbohydrate feeding on the induction of δ-aminolevulinic acid synthetase. Metabolism. 13,396-406.

446. TSCHUDY, D.P., 1965. Biochemical lesions in porphyria. J.A.M.A. 191,718-730.

447. URATA, G., & GRANICK, S., 1961. Aminoacetone formation and decomposition in liver. Biochem. Biophys. Res. Communication. 4,96-100.

448. URATA, G., & GRANICK, S., 1961. Aminoacetone formation and decomposition in liver. Biochem. Biophys. Res. Communication. 4,96-100.

449. VAN SLYKE, D.D., PLAZIN, J., & WEISIGER, J.R., 1951. Reagents for the Van Slyke-Folch wet carbon combustion. J. Biol. Chem. 191,299-304.

450. VANNOTTI, A., 1955. Experimental studies of porphyrin metabolism in cytochrome c synthesis. Ciba Foundation Symposium : Porphyrin Biosynthesis and Metabolism, pg. 128-142. Ed. by Wolstenholme, G.E.W. and Millar, E.C.P. London : J. and A. Churchill Ltd.

451. VILLA, L., ROBECCHI, A., & BALLABIO, C.B., 1958. Physiopathology, clinical manifestations, and treatment of gout. Part I Physiopathology-pathogenesis. Ann. Rheumat. Dis. 17,9-15.

452. VOGEL, A.I., 1956. A text book of practical organic chemistry including qualitative organic analysis. 3rd. Ed. Published by Longmans, Green and Co. Ltd.

453. VON EULER, L.H., RUBIN, R.J., & HANDSCHUMACHER, R.E., 1963. Fatty livers induced by orotic acid II. Changes in nucleotide metabolism. J. Biol. Chem. 238,2464-2469.

454. WAELSCH, H., 1951. Glutamic acid and cerebral function. Advances in Protein Chem. 6,299-341.

455. WARBURG, O., & GEWITZ, H.S., 1951. Cytohämin aus Herzmuskel. Z. Physiol. Chem. 288,1-4.

456. WARREN, L., & BUCHANAN, J.M., 1957. Biosynthesis of the purines. XIX. 2-amino-N-ribosylacetamide-5'-phosphate (glycinamide ribotide) transformylase. J. Biol. Chem. 229,613-626.

457. WARREN, L., FLAKS, J.G., & BUCHANAN, J.M., 1957. Biosynthesis of the purines. XX. Integration of enzymatic transformylation reactions. J. Biol. Chem. 229,627-640.

458. WATSON, C.J., 1938. The pyrrol pigments, with particular reference to normal and pathological haemoglobin metabolism, in Handbook of Hematology, Ed. by Hal Downey, Hamilton, London, 1938.

459. WATSON, C.J., 1965. The continuing challenge of hemoglobin and bile pigment metabolism. Ann. Int. Med. 63,931-944.

460. WATSON, D., 1962. The transport of bile pigments : the binding of sodium bilirubinate to plasma protein. Clin. Sci. 22,435-445.

461. WATSON-JAMES, G., III, ABBOT, L.D., Jr., NORBERG, B., BIRKELAND, S., & EVANS, E.I., 1953. Metabolic fate of N^{15}-labelled erythrocytes transferred into normal man. J. Clin. Invest. 32,578.

462. WATSON-JAMES, G., III, & ABBOTT, L.D., Jr., 1961. Stercobilin N^{15} excretion in refractory anaemia. Trans. Amer. Clin. Climat. Ass. 73,116.

463. WATTS, R.W.E., & CRAWHALL, J.C., 1959. The first glycine metabolic pool in man. Biochem. J. 73,277-286.

464. WATTS, R.W.E., 1966. Uric acid production with particular reference to the role of xanthine oxidase and its inhibition. Proc. of the Roy. Soc. Med. 59,287-292.

465. WAY, L.J., & PARKS, R.E., 1958. Enzymatic synthesis of 5'-phosphate nucleotide of purine analogues. J. Biol. Chem. 231,467-480.

466. WEBER, A.P., SCHALM, L., & WITMANS, J., 1963. Bilirubin monoglucuronide (pigment I) : a complex. Acta. Med. Scand. 173,19-24.

467. WEECH, A.A., VANN, D., & GRILLO, R.A., 1941. The clearance of bilirubin from the plasma. A measure of the excreting power of the liver. J. Clin. Invest. 20,323-332.

468. WEINHOUSE, S., & FRIEDMANN, B., 1951. Metabolism of labeled 2-carbon acids in the intact rat. J. Biol. Chem. 191,707-717.

469. WEINHOUSE, S., & FRIEDMANN, B., 1952. Study of precursors of formate in the intact rat. J. Biol. Chem. 197, 733-740.

470. WEINHOUSE, S., 1955. A symposium on amino acid metabolism. The Synthesis Degradation of Glycine : Amino Acid Metabolism. Ed. by McElroy, W.D., and Glass, H.B., Baltimore. Johns Hopkins Press, 1955. pg. 637-657.

471. WEISSBACH, A., HURWITZ, D., & SPRINSON, D.B., 1950. The synthesis of the methyl groups and ethanolamine moiety of choline from serine and glycine in the rat. J. Am. Chem. Soc. 72,3316-3317.

472. WESTALL, R.G., 1952. Isolation of porphobilinogen from the urine of a patient with acute porphyria. Nature, London. 170,614-616.

473. WESTERFELD, W.W., STOTZ, E., & BERG, R.L., 1943. The coupled oxidation-reduction of alcohol and pyruvate in-vivo. J. Biol. Chem. 149,237-243.

474. WHIPPLE, G.H., ROBSCHEIT-ROBBINS, F.S., 1926. Muscle hemoglobin as a source of bile pigment. Amer. J. Physiol. 78,675-682.

475. WILSON, D., BEYER, A., BISHOP, C., & TALBOTT, J.H., 1954. Urinary uric acid excretion after the ingestion of isotopic yeast nucleic acid in the normal and gouty human. J. Biol. Chem. 209,227-232.

476. WINNICK, T., MORING-CLARSSON, I., & GREENBERG, D.M., 1948. Distribution of radioactive carbon among certain amino acids of liver homogenate protein, following uptake experiments with labeled glycine. J. Biol. Chem. 175, 127-132.

477. WINTROBE, M.M., 1949-50. Factors and mechanisms in the production of red corpuscles. Harvey Lectures. 45, 87-126.

478. WINTROBE, M.M., 1956. Clinical Hematology. Lea and Febiger, Philadelphia, 1956. Fourth Edition.

479. WISE, C.D., & DRABKIN, D.L., 1964. Degradation of hemoglobin and hemin to biliverdin by a new cell-free enzyme system obtained from the hemophagous organ of dog placenta. Fed. Proc. 23,223.

480. WISE, C.D., & DRABKIN, D.L., 1965. Enzymatic degradation of hemoglobin and hemin to biliverdin and carbon monoxide. Fed. Proc. 24,222.

481. WITTENBERG, J., & SHEMIN, D., 1949. The utilization of glycine for the biosynthesis of both types of pyrroles in protoporphyrin. J. Biol. Chem. 178,47-51.

482. WITTENBERG, J., & SHEMIN, D., 1950. The location in protoporphyrin of the carbon atoms derived from the α-carbon atom of glycine. J. Biol. Chem. 185,103-116.

483. WRISTON, J.C., Jr., LACK, L., & SHEMIN, D., 1953. Citric acid cycle and porphyrin synthesis. Federation. Proc. 12,294.

484. WRISTON, J.C., Jr., LACK, L., & SHEMIN, D., 1955. The mechanism of porphyrin formation. Further evidence on the relationship of the citric acid cycle and porphyrin formation. J. Biol. Chem. 215,603-611.

485. WU, H., & RITTENBERG, D., 1949. Metabolism of L-aspartic acid. J. Biol. Chem. 179,847-856.

486. WYNGAARDEN, J.B., & STETTEN, De W., Jr., 1953. Uricolysis in normal man. J. Biol. Chem. 203,9-21.

487. WYNGAARDEN, J.B., 1955. The effect of phenylbutazone on uric acid metabolism in two normal subjects. J. Clin. Invest. 34,256-262.

488. WYNGAARDEN, J.B., 1957. Intermediary purine metabolism and the metabolic defects of gout. Metabolism. 6, 244-268.

489. WYNGAARDEN, J.B., BLAIR, A., & SCHUCK, J., 1957. On the mechanism of the "shunt" pathway of uric acid synthesis. Clin. Research Proc. 5,108.

490. WYNGAARDEN, J.B., 1957. Over-production of uric acid as the cause of hyperuricemia in primary gout. J. Clin. Invest. 36,1508-1515.

491. WYNGAARDEN, J.B., SILBERMAN, H.R., & SADLER, J.H., 1958. Feedback mechanisms influencing purine ribotide synthesis. Ann. New York, Acad. Sc. 75,45-60.

492. WYNGAARDEN, J.B., BLAIR, A.B., & HILLEY, L., 1958. On the mechanism of overproduction of uric acid in patients with primary gout. J. Clin. Invest. 37,579-590.

493. WYNGAARDEN, J.B., SEEGMILLER, J.E., LASTER, L., & BLAIR, A.B., 1959. Utilization of hypoxanthine, adenine and 4-amino-5 imidazole carboxamide for uric acid synthesis in man. Metabolism. 8,455-464.

494. WYNGAARDEN, J.B., & ASHTON, D.M., 1959. The regulation of activity of phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides : a potential feedback control of purine biosynthesis. J. Biol. Chem. 234,1492-1496.

495. WYNGAARDEN, J.B., & GREENLAND, R.A., 1963. The inhibition of succinoadenylate kinosynthetase of Escherichia coli by adenosine and guanosine 5'-monophosphates. J. Biol. Chem. 238,1054-1057.

496. WYNGAARDEN, J.B., 1966. "Gout" in "The Metabolic Basis of Inherited Disease", 2nd. Edition. Ed. by Stanbury, Wyngaarden and Fredrickson. Published by McGraw-Hill. Chapter 31. pg. 667-728.

497. YAMAGUCHI, M., NAKAJIMA, H., & YAMAOKA, K., 1961. Enzymic degradation of heme proteins into bile pigments. VI. Proc. Japan Acad. 32,584-587.

498. YAMAMOTO, T., SKANDERBEG, J., ZIPURSKY, A., & ISRAELS, L.G., 1965. The early Appearing Bilirubin : Evidence for two components. J. Clin. Invest. 44,31-41.

499. YÜ, T.F., & GUTMAN, A.B., 1953. Ultrafilterability of plasma urate in man. Proc. Soc. Exper. Biol. and Med. 84,21-24.

500. YÜ, T.F., & GUTMAN, A.B., 1955. Paradoxical retention of uric acid by uricosuric drugs in low dosage. Proc. Soc. Exper. Biol. N.Y. 90,542-547.

501. YÜ, T.F., WEISSMANN, B., SHARNEY, L., KUPFER, S., & GUTMAN, A.B., 1956. On the biosynthesis of uric acid from glycine-N^{15} in primary and secondary polycythemia. Amer. J. Med. 21,901-917.

502. YÜ, T.F., SIROTA, J.H., BERGER, L., HALPERN, M., & GUTMAN, A.B., 1957. Effect of sodium lactate infusion on urate clearance. Proc. Soc. Exp. Biol. N.Y. 96,809-813.

503. YÜ, T.F., & GUTMAN, A.B., 1959. Study of the paradoxical effects of salicylate in low, intermediate and high dosage on the renal mechanisms for excretion of urate in man. J. Clin. Invest. 38,1298-1315.

504. YÜ, T.F., BERGER, L., & GUTMAN, A.B., 1961. Suppression of tubular secretion of urate by pyrazinamide in the dog. Proc. Soc. Exp. Biol. Med. 107,905-908.