STUDIES IN SYMPTOMATIC PORPHYRIA IN THE AFRICAN

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Medicine in the Faculty of Medicine, University of Cape Town.

by

BRIAN CHRISTOPHER SHANLEY, M.B. Ch.B (U.C.T.)

February 1969

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

P R E F A C E

Since the first case of acute porphyria was recorded in South Africa in 1939, it has come to be realised that this country has the highest incidence of hepatic porphyria in the world. Three types of hepatic porphyria are commonly encountered in this country. Two of these, variegate porphyria and acute intermittent porphyria, are inherited conditions. On the other hand, the third type, symptomatic porphyria (porphyria cutanea tarda sumptomatica) which occurs in the White, Cape Coloured and African population groups, does not appear to be hereditary and is generally regarded as an acquired disorder associated with chronic abuse of alcohol. This association has been stressed as the most constant feature of the clinical history in these patients and alcohol is held to be a major aetiological or aggravating factor.

Histological examination of the liver which is the site of the disorder of haem precursor metabolism in the hepatic group of porphyrias, has revealed that some degree of parenchymal liver pathology is present in all cases of the symptomatic variety. Of the pathological changes described, the most frequently encountered is siderosis and it has been suggested that the presence of abnormal amounts of iron in the liver also plays an important part in the pathogenesis of this condition.

While much valuable work has been done in characterising the porphyrin excretion pattern in symptomatic porphyria with a view to elucidation of the underlying metabolic lesion, little attention has been paid to obtaining direct evidence of the role of alcohol and hepatic siderosis in the development and course of the disorder.

The *first* aim of the present thesis, therefore, was to determine the effect of ethanol on porphyrin biosynthesis and excretion both in animals (rats) and in African patients with symptomatic porphyria and thereby to assess the part played by alcohol in this disease (Section A: Chapters 6 and 7).

Secondly, it was intended to attempt an evaluation of the relationship between hepatic siderosis and disordered porphyrin metabolism by means of an experimental model (Section A: Chapter 8).

The *third* object was to investigate possible mechanisms whereby alcohol could influence porphyrin biosynthesis (Section B: Chapters 9 - 12).

In view of the number and variety of experiments involved, further details of the rationale for individual studies will not be given here but will be found at the commencement of the relevant chapter and in the introduction to Section B of the present work.

CONTENTS

LIST OF ABBREVIATIONS

AIA	Allyl*iso*propylacetamide
AIP	Acute intermittent porphyria
ALA	δ-Aminolaevulinic acid
Alb	Albumin
ATP	Adenosine triphosphate
CoA	Coenzyme A
COPRO	Coproporphyrin
COPROgen	Coproporphyrinogen
DDC	3,5-Dicarbethoxy-1,4-dihydrocollidine
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
Glob	Globulin
GP	Glycerophosphate
GSH	Glutathione (reduced)
GTP	Guanosine triphosphate
HCB	Hexachlorobenzene
NAD, NADH	Oxidised and reduced forms of nicotinamide adenine dinucleotide
NADP, NADPH	Oxidised and reduced forms of nicotinamide adenine dinucleotide phosphate
PBG	Porphobilinogen
PCT	Porphyria cutanea tarda
PEP	Phosphoenolpyruvate
P.I.	Prothrombin index
POPOP	p-*bis*-[2-(5-Phenyloxazolyl)]-benzene
PPO	2,5-Diphenyloxazole
PROTO	Protoporphyrin
PROTOgen	Protoporphyrinogen
RNA	Ribonucleic acid
S.D.	Standard deviation
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
TCA	Tricarboxylic acid
$T_{\frac{1}{2}}$	Half-life
UDP	Uridine diphosphate
UDPG	UDPglucose
UDPGA	UDPglucuronic acid
URO	Uroporphyrin
UROgen	Uroporphyrinogen
VP	Variegate porphyria

ENZYME NOMENCLATURE ACCORDING TO THE RECOMMENDATIONS OF THE INTERNATIONAL UNION OF BIOCHEMISTRY 1964, COVERING THE MORE IMPORTANT ENZYMES MENTIONED IN THE TEXT.

TRIVIAL NAME	SYSTEMATIC NAME	NUMBER
ALA dehydratase	5-Aminolaevulinate hydro-lyase (adding 5-aminolaevulinate & cyclising)	4.2.1.24
ALA synthetase	Succinyl CoA : glycine N-succinyltransferase	2.3.1.13
Alcohol dehydrogenase	Alcohol : NAD oxidoreductase	1.1.1.1
Citrate synthetase	Citrate oxaloacetate-lyase (CoA acetylating)	4.1.3.7
Coproporphyrinogen oxidase	Precise mechanism unknown	Unclassified
Fumarase	L-Malate hydro-lyase	4.2.1.2
Fumarate reductase	Reduced NAD : (fumarate) oxidoreductase	1.6.99.?
Galactose oxidase	D-Galactose : oxygen oxidoreductase	1.1.3.9
β-Glucuronidase	β-D-Glucuronide glucuronohydrolase	3.2.1.31
Glutamate dehydrogenase	L-Glutamate : NAD oxidoreductase (deaminating)	1.4.1.2
Glutamic-oxaloacetic transaminase	L-Aspartate : 2-oxoglutarate aminotransferase	2.6.1.1
Glutamic-pyruvic transaminase	L-Alanine : 2-oxoglutarate aminotransferase	2.6.1.2
Haem synthetase (Ferrochelatase)	Protohaem ferro-lyase	4.99.1.1
Isocitrate dehydrogenase	*threo*-D_s-Isocitrate : NAD(P) oxidoreductase (decarboxylating)	1.1.1.41(42)
α-Ketoglutarate dehydrogenase	2-Oxoglutarate - lipoate oxidoreductase (acceptor-acylating)	1.2.4.2
Malate dehydrogenase	L-Malate : NAD oxidoreductase	1.1.1.37

Malic enzyme	L-Malate : NADP oxidoreductase (decarboxylating)	1.1.1.40
Methylmalonyl CoA isomerase	Methylmalonyl-CoA CoA-carbonyl mutase	5.4.99.2
$NADH_2$ oxidase	Reduced NAD : (acceptor) oxidoreductase	1.6.99.3
PBG deaminase (UROgen I synthetase)	Mechanism unknown	Unclassified
PEP carboxylase	GTP : oxaloacetate carboxy-lyase (transphosphorylating)	4.1.1.32
Pyruvate carboxylase	Pyruvate : carbon dioxide ligase (ADP)	6.4.1.1
Succinate dehydrogenase	Succinate : (acceptor) oxidoreductase	1.3.99.1
Succinyl CoA synthetase	Succinate : CoA ligase (GDP)	6.2.1.4
UDPgalactose-4-epimerase	UDPglucose 4-epimerase	5.1.3.2
UDPglucuronyltransferase	UDPglucuronate glucuronyltransferase (acceptor unspecified)	2.4.1.17
Uroporphyrinogen decarboxylase	Uroporphyrinogen-III carboxy-lyase	4.1.1.37
Uroporphyrinogen isomerase (Uroporphyrinogen III cosynthetase)	Mechanism unknown	Unclassified

CHAPTER 1

THE PORPHYRIAS.

1.1 General historical background. Although disorders of porphyrin metabolism undoubtedly occurred in earlier times, they went unrecognised until the latter half of the nineteenth century. This is nicely illustrated by the retrospective diagnosis of acute intermittent porphyria as the malady affecting King George III (1738-1820) for the greater part of his reign (Macalpine and Hunter 1966). Hitherto this unfortunate sovereign's 'madness' had traditionally been called manic-depressive psychosis. More recently Macalpine and co-workers (1968) have published the results of further extensive researches, purporting to show the transmission of porphyria from Mary Queen of Scots (1542-1587) through 13 generations and three Royal houses to two living descendents. Their thesis which has been the subject of much enthusiastic debate among authorities in the field (Dean 1968, Goldberg 1968, Rimington 1968, Eales and Dowdle 1968, Dent 1968) has by no means found general acceptance but, nevertheless, represents a fascinating exercise in medical history.

The name porphyrin was first introduced in 1871 by Hoppe-Seyler. It derives from the Greek word purphuros, meaning purple, and refers to the colour of an iron-free extract of blood previously described by Mulder in 1844 (Mulder 1844). Hoppe-Seyler (1871) found that Mulder's 'iron-free haematin' was a mixture of 2 substances; the main one he named 'haematoporphyrin'.

Several years later a substance exhibiting a spectrum resembling that of haematoporphyrin was discovered by Baumstark (1874) in the urine of a patient suffering from 'Pemphigus leprosus', as diagnosed by Schultz (1874). This was the first association of porphyrins with disease in man. The patient, a 33 year old weaver, had shown dermal photosensitivity since the age of three months and was found to have splenomegaly and icteric conjunctivae at the time of presentation. His urine was wine-red in colour. Later at autopsy the skeleton showed intense red-brown discoloration. Clearly this was the first

recorded case of congenital porphyria.

Drugs as precipitating factors in acute porphyria soon came to to the fore after the introduction in 1888 of the hypnotic, Sulphonal. An elderly woman patient of Professor B.J. Stokvis, after taking Sulphonal, excreted a dark red urine which contained a pigment resembling haematoporphyrin (Stokvis 1889). Later she died. This drug was again incriminated by Harley (1890) as causing the death of a young woman from an unusual form of nervous disturbance associated with a dark red urine.

Gradually the combination of porphyrinuria with the gastro-intestinal and neuropsychiatic manifestations of what is now known as acute intermittent porphyria (AIP) were recognised more frequently. In some cases symptoms arose spontaneously but in the majority (Gunther 1911) Sulphonal or one of the allied drugs had been administered. Three years after their introduction in 1903, barbiturates were also recognised as precipitating agents (Dobrschansky 1906).

1.2 Classification of the porphyrias. The problems involved in arriving at a satisfactory classification of the porphyrias are well illustrated by the numerous attempts that have been made over the years by authorities in this field (see Table 1.1 at end of Chapter 1). Not until the specific enzymic or other defects in the various disorders of porphyrin metabolism have been characterised will a wholly satisfactory classification be achieved. A brief outline will therefore be given of the evolution of contemporary opinion.

Not many years after the introduction of Sulphonal and related drugs, Hans Günther (1911, 1922) published the first classification comprising four types of 'haematoporphyria'. Although this is now only of historical interest, it is noteworthy that he recognised acute and chronic forms and that he first defined and named congenital (erythropoietic) porphyria. Garrod (1923) credits Günther with the first recognition that this disease was an 'inborn error of metabolism'.

Following the demonstration by Hans Fischer and others that the

naturally-occurring excreted porphyrins were structurally different from haematoporphyrin which was thought to be a chemical artefact, Waldenström (1937) introduced the term porphyria to replace Günther's haematoporphyria. From a study of 103 cases of acute porphyria he distinguished five clinical sub-types. It is now realised that these represent different manifestations of the same disease. Waldenström (1937) also renamed Günther's chronic porphyria, porphyria cutanea tarda, indicating the late onset of photosensitivity.

The first departure from the purely clinical classification was that of Schmid, Schwartz and Watson (1954). From analyses of the porphyrin and porphobilinogen (PBG) content of bone marrow and liver, they showed that the porphyrias are clearly divisible into erythropoietic and hepatic types according to the site of the major defect in porphyrin production. These two major sub-divisions found general acceptance as shown by later classifications and are still in use. Waldenström (1957), however, opposed the idea that acute porphyria involved a disturbance of hepatic function and rather held the view that the lesion was present in all haem synthesizing cells. This theory seems untenable in the light of the work of Schmid et al. (1954) and subsequent workers. In the erythropoietic group the originally described congenital variety has been joined more recently by erythropoietic protoporphyria described by Magnus et al. (1961) and characterised by overproduction of protoporphyrin in erythropoietic tissue rather than uroporphyrin I as occurs in the congenital type. Classification in this group does not appear to be a problem. The complexities lie in the subdivision of the hepatic types as evidenced by the different approaches shown in the various proposed classifications in Table 1.1. From the clinical viewpoint there is general agreement on three major subgroups of the hepatic porphyrias, viz., (a) the acute intermittent variety with no cutaneous manifestations, (b) a mixed or variegate type with both cutaneous lesions and acute attacks and (c) purely cutaneous types. On the other hand, from the genetic and biochemical aspects there is no unanimity hence the varied nomenclature introduced by different authorities in the hope of simplifying the

issue.

Waldenström's experience that acute porphyria in Sweden was entirely true to type and never mixed with cutaneous porphyria (Waldenström 1957) conflicted markedly with the findings of Dean and Barnes (1955) in the South African genetic variety (porphyria variegata, variegate porphyria, VP) that various combinations of acute and cutaneous manifestations could occur in any one family or any one individual. After comparing Swedish and South African families Dean and Barnes (1959) concluded that these two varieties were genetically distinct - each dominant and non sex-linked in character. It is of interest that variegate porphyria has recently also been reported in several families in Sweden for the first time (Waldenström 1967). Moreover, no blood relationship could be shown between these families and any families afflicted with acute intermittent porphyria.

Waldenström (1937) introduced the term 'cutanea tarda' to describe cutaneous porphyria of late onset. Subsequently he subdivided this category into 'hereditaria' and 'symptomatica' types. Most of the dissension surrounding attempts to classify the hepatic porphyrias is concerned with the cutaneous forms. Firstly, the existence of a purely cutaneous hereditary porphyria is doubted by many workers. Secondly, there is disagreement on whether the combined cutaneous and acute syndromes reported from various countries represent the same condition.

Some of the most problematical families as regards classification have been described by Watson (1960) in America; for example, he has reported the late onset of cutaneous manifestations in certain members of families where otherwise typical AIP or latent porphyria could be traced through several generations. These difficulties are reflected in classifications such as that of Goldberg and Rimington (1962) where three forms of hereditary cutaneous hepatic porphyria are distinguished as opposed to that of Eales (1963) or Tschudy (1965) where these syndromes are regarded as synonymous.

A similar approach is evident with regard to the acquired types

of porphyria. While Goldberg and Rimington (1962) recognise four subtypes most other contemporary authors prefer the overall title of symptomatic porphyria subdivisible into (a) idiosyncratic (constitutional) and (b) acquired, e.g. hexachlorobenzene-induced. As mentioned previously, no finality can be reached in regard to the controversial points in the classification of the porphyrias until the specific enzyme defects or other metabolic abnormalities have been elucidated. Therefore, for the purposes of this thesis the classification of Eales (1963)(Table 1.1) which is quite adequate has been adopted.

The patients studied in the present thesis fall into the Group 2 (iii) (a) of this classification. As will be discussed in Chapter 2, symptomatic porphyria as encountered in several racial groups in South Africa is a well recognised entity and diagnosis of the disorder is not usually problematical.

Table 1.1 Various proposed classifications of the porphyrias (partly adapted from Goldberg and Rimington 1962).

Günther (1911).

1. Haematoporphyria acuta
2. Haematoporphyria acuta toxica
3. Haematoporphyria chronica
4. Haematoporphyria congenita.

Schmid, Schwartz and Watson (1954).

1. Porphyria erythropoietica
2. Porphyria hepatica
   (a) Intermittent acute type
   (b) 'Cutanea tarda' type
   (c) Mixed type.

Watson (1960).

1. Porphyria erythropoietica (recessive)
2. Porphyria hepatica
   (a) Hereditary acute intermittent (dominant)
      (i) Manifest
      (ii) Latent
   (b) Hereditary, mixed or "variegate" group (dominant)
      (i) Cutaneous with little or no acute manifestations
      (ii) Acute intermittent without cutaneous symptoms
      (iii) Various combinations
      (iv) Latent
   (c) Hereditary cutaneous?

Waldenström (1937).

1. Porphyria congenita
2. Porphyria cutanea tarda
3. Porphyria acuta:
   (a) Latent
   (b) Abdominal form
   (c) Nervous form
   (d) Classical acute porphyria
   (e) Comatose form commencing as (b).

Waldenström (1957).

1. Congenital porphyria
2. Porphyria cutanea tarda symptomatica
3. Porphyria cutanea tarda hereditaria (protocoproporphyria)
4. Acute intermittent porphyria.

Goldberg and Rimington (1962).

1. Congenital (erythropoietic) porphyria
2. Acute intermittent porphyria
3. Cutaneous hepatic porphyria
   (a) Hereditary forms
      (i) Porphyria cutanea tarda hereditaria or Protocoproporphyria (Waldenström 1957)
      (ii) Mixed porphyria (Watson et al. 1951, Watson 1954)
      (iii) Porphyria variegata (Dean & Barnes 1959)
   (b) Acquired forms
      (i) Porphyria cutanea tarda symptomatica (Waldenström 1957)
      (ii) Bantu Porphyria (Barnes 1959)
      (iii) Turkish porphyria (Cetingil & Özen 1960)

(d) Constitutional or idiosyncratic (porphyria cutanea tarda)
  (i) Chemicals, especially alcohol
  (ii) Idiopathic
  (iii) With systemic disease.
(e) Acquired
  (i) Secondary to hepatoma
  (ii) Secondary to fungicide - Turkish epidemic.

Eales (1963).

1. Erythropoietic porphyrias
  (i) Congenital erythropoietic porphyria
  (ii) Protoporphyria erythropoietica.
2. Hepatic porphyrias
  (i) Swedish genetic porphyria : pyrroloporphyria, AIP
  (ii) S.A. genetic porphyria : protocoproporphyria, PCT hereditaria, mixed porphyria, porphyria variegata
  (iii) Symptomatic porphyria : Urocoproporphyria, PCT symptomatica, acquired porphyria hyperporphyrismus
    (a) Idiosyncratic - associated with alcoholic liver disorder, systemic disorders, drugs, chemicals.
    (b) Acquired - (i) Hexachlorobenzene-induced porphyria
      (ii) Hepatoma of the liver
      (iii) Others?
  (iv) Porphyrin-producing hepatic adenoma (Tio et al. 1957)
4. Experimentally-induced porphyrias.

Tschudy (1965).

1. Erythropoietic porphyrias
  (a) Congenital erythropoietic porphyria (recessive)
  (b) Erythropoietic protoporphyria (dominant?)
2. Hepatic porphyrias
  (a) Acute intermittent porphyria, Swedish genetic porphyria, pyrroloporphyria (dominant)
    (i) Manifest
    (ii) Latent
  (b) Porphyria variegata, mixed porphyria, S.A. genetic porphyria, PCT hereditaria and protocoproporphyria (dominant)
    (i) Cutaneous with little or no acute manifestations
    (ii) Acute intermittent without cutaneous symptoms
    (iii) Various combinations
    (iv) Latent
  (c) Symptomatic porphyria, PCT symptomatica, urocoproporphyria, constitutional porphyria
    (i) Idiosyncratic - associated with alcoholism, liver disease, systemic disease, drugs, etc.

(ii) Acquired - hexachlorobenzene-induced and hepatoma.

Conference on the porphyrias, Cape Town (1963).

1. The hepatic porphyrias
   1. A.I.P. (Pyrroloporphyria, Swedish genetic porphyria).
   2. Variegate porphyria (Protocoproporphyria and S.A. genetic porphyria)
   3. The cutaneous porphyrias (purely cutaneous)
      a. Hereditary type
      b. Possibly genetically predisposed type(s) e.g. the form associated with alcoholism (constitutional, symptomatic, PCT)
      c. Acquired type(s) e.g. Hexachlorobenzene-induced
2. The erythropoietic porphyrias
   1. Congenital erythropoietic porphyria
   2. Erythropoietic protoporphyria.

CHAPTER 2

PORPHYRIA IN SOUTH AFRICA WITH SPECIAL REFERENCE TO SYMPTOMATIC PORPHYRIA

2.1 Historical background. The story of porphyria in South Africa begins a mere 36 years after the arrival, in 1652, of Jan van Riebeeck. Gerrit Janz, a free burgher who had come to the Cape in 1685, married Adriaantjie Jacobs (Adriaantjie Adriaanse) shortly after her arrival in 1688. She was one of a number of orphans sent out by the Dutch East India Company as prospective wives for the first free burghers. This couple, according to Dean, are the original forebears of the vast majority of white porphyric subjects in this country (Dean 1963).

It is surprising, therefore, that the first record of the disease in humans only appeared in the South African medical literature in 1939 (Kooy 1939, Eales and Chait 1939). The patient was a European man aged 33 years. He was admitted to the Groote Schuur Hospital in Cape Town "presenting the rare but well known syndrome of porphyrinuria and polyneuritis" (Kooy 1939). Prior to this, congenital porphyrinuria had been described (Fourie 1936) in a herd of shorthorn cattle from a farm in Swaziland.

The next report was that of Barnes (1945), working at the South African Institute for Medical Research, Johannesburg. This was the first of many papers over the following two decades in which Dr. Barnes made notable contributions to the study of the porphyrias in South Africa, particularly in regard to porphyria among the African population. He and Dr. G. Dean, through their individual and combined researches, have been chiefly responsible for our awareness of these diseases in this country.

By 1945 Barnes had collected the details of 11 cases of porphyrinuria, including 6 Whites and 5 Africans (Bantu). With reference to the current ideas on the subject he wrote, "The excretion of porphyrin in the urine in sufficient concentration to attract attention is a relatively uncommon occurrence and is usually regarded as of little clinical importance. It is becoming clear from recent studies by

improved methods that the phenomenon is neither a mere curiosity of clinical biochemistry nor probably quite as rare as generally believed". No attempt was made in this article to classify these eleven cases but clinical differences between the African and White patients were noted. The African cases mostly did not exhibit abdominal or neurological symptoms, both of which were prominent features in the Whites. On the other hand 4 or 5 of the African patients had skin lesions, whereas only one of the Whites is recorded as having had blisters on the hands for six months. An allusion was also made to the possibility of a familial trait in several of the White patients.

Few reports of porphyria in South Africa appeared in the following six years (Linder 1947, Findlay and Barnes 1950) before the publication in 1951 of Barnes' larger series of 40 cases of porphyrinuria (Barnes 1951). This comprised 24 cases of acute porphyria, one of congenital porphyria and eight in whom "the porphyrinuria and associated skin lesions developed in later life". Seven cases were not classified. Again it was noted that there were no African patients in the acute group but the majority of cases with skin lesions belonged to this race. Among the acute cases there were 3 pairs of sisters and "some of the patients gave information that relations in earlier generations had died in circumstances which were suggestive of acute porphyria" - further pointers to genetic transmission. Unaware at that time of the differences between the Swedish and South African inherited forms of porphyria, Barnes likened his cases to those of Waldenström and Vahlquist (1944). In so doing he minimised the significance of skin lesions found in 6 of his 24 cases and which had not been noted in the Swedish patients. Commenting on the incidence of acute porphyria, Barnes noted that, after Sweden, South Africa probably had the highest incidence of the disease.

In the ensuing years interest in porphyria in this country increased enormously, culminating in the International Conference on the Porphyrias at Cape Town in 1963. Dean, who for 5 years had been investigating the families of a number of White patients presenting

with acute porphyria, reported in 1953 that the disease was inherited as a non sex-linked Mendelian dominant character (Dean 1953). In the absence of a history of an acute attack the diagnosis was made on spectroscopic examination of the urine or the presence of "the typical skin lesions" which he described in some detail. He and Barnes confirmed these findings in subsequent papers (Dean and Barnes 1955, Dean 1956) and showed that in the White South African "acute porphyria, chronic cutaneous porphyria and 'symptomless' porphyria are different manifestations of the same inherited disorder". It was suggested that the prominence of skin lesions in the South Africans as opposed to their absence in the Swedes might be due to the disparity in hours of sunshine between the two countries (Dean and Barnes 1955).

Through his monumental genealogical researches Dean was able to trace the previously mentioned Dutch immigrant couple whom he suggests were the original forebears of most of the White porphyric subjects in South Africa (Dean 1963). To account for the high incidence of porphyria in this country - highest in the world (Eales 1961) - he pointed out that one third of our present White population hold the names of 40 original settlers, an increase of 12,500 fold (Dean 1963).

Later Dean and Barnes (1958) in a screening experiment showed the overall incidence of porphyria, based on urinary and faecal porphyrin estimations, to be approximately one per cent in their test populations comprising 608 psychiatric patients and 645 nurses. On the strength of this finding they made a plea for routine screening of patients for porphyria in South Africa.

It was becoming apparent that besides the question of skin lesions, there were other differences between the Swedish and South African genetic porphyrias (Dean and Barnes 1958, Barnes 1958). Dean, therefore, undertook a comparative study, visiting Sweden and Holland where arrangements were made for him to investigate cases and families. Stool specimens were sent to Barnes in Johannesburg for quantitative porphyrin analysis and other determinations were done in Sweden. The results confirmed the absence of skin lesions in acute intermittent porphyria (Swedish) and emphasised the different patterns of porphyrin

and porphyrin precursor excretion in the two diseases (see Table 2.3). Dean and Barnes proposed the name porphyria variegata (variegate porphyria) for the South African genetic type in recognition of its varied manifestations (Dean and Barnes 1959).

Following his earlier observations on African cases of porphyria, Barnes continued to collect data and by 1955 had discovered nearly 300 non-White cases of porphyrinuria (Barnes 1955). Most of these were African and only a few were people of mixed descent, i.e. Coloureds. Other information in this paper included the frequency of skin lesions (specifically noted as absent in only 8 cases), the association with ethyl alcohol (about 50% were questioned and the majority admitted to consumption of alcohol but not all in excessive amounts), and the incidence of liver disease (34 cases had palpable hepatomegaly). Among the 300 cases in only two instances was more than one member of a family affected.

Examination of porphyrin excretion patterns showed that stool (besides urine) analysis was essential to the differentiation of porphyria variegata from 'Bantu porphyria' (Barnes 1958, 1959, Eales 1959). Barnes (1958) found increased stool copro- and protoporphyrin to be a feature of adult White cases of the South African genetic disease while much smaller increases occurred in adult African porphyric patients. Some of the children of patients in the former ethnic group, although free of clinical signs, also exhibited increased faecal excretion of these two pigments, while in the children of African porphyric subjects no such tendency was found. Urinary porphyrin excretion in the African patients was generally much greater than in the Whites (Barnes 1959).

In view of the lack of evidence for inheritance of porphyria in the African, Barnes (1959) felt that the metabolic disorder in these people might be acquired and suggested a possible mechanism. He postulated that, under stress in adulthood, liver damage due to malnutrition in infancy led to further dysfunction, one of the manifestations being this acquired type of porphyria.

Eales (1956) had reported cutaneous porphyria with porphyrinuria

in all 3 population groups in Cape Town - White, African and Cape Coloured. Later (Eales 1959, 1960) he confirmed Barnes' findings regarding the differences between South African genetic porphyria and the acquired form encountered in the African. He also demonstrated that both forms of metabolic abnormality occur in the Cape Coloured people (Eales 1963).

In 1961 Lamont et al. (Lamont et al. 1961) described a study of 100 African cases seen at King Edward VIII Hospital, Durban, and which "conformed to the clinical and biochemical pattern originally described by Barnes". They emphasised clinical, biochemical and histological evidence of associated liver disease in many of their cases and attributed an aetiological role to ethyl alcohol against a background of malnutrition.

The rarer erythropoietic porphyrias have also been reported in South Africa. In 1950 Findlay and Barnes (Findlay and Barnes 1950) described the first recorded case of congenital erythropoietic porphyria in an African. Erythropoietic protoporphyria was reported for the first time in this country in 1963 by Sweeney et al. (1963). Other unusual cases which have been described include a 5 year old White boy who had manifested cutaneous porphyria from the age of 6 months but whose porphyrin excretion pattern was that of porphyria variegata (Frootko and Parnell 1957) and several pure blooded Africans with acute porphyria (Woods and Barnes 1951, Eales 1960).

2.2. Symptomatic porphyria in South Africa.

2.2.1 Clinical features. It is not intended to give a full account of the clinical features of symptomatic porphyria. Only those points relevant to the diagnosis, pathology and pathogenesis of the condition will be dealt with. Most of the information in the following description is taken from the clinical study of Lamont et al. (1961) who described 100 African cases from the same population group as that involved in the present study.

2.2.1.1 Symptoms. Although almost every patient demonstrates porphyric cutaneous lesions, only about half of those seen at

hospital present themselves for this reason. In the remainder the presenting complaints are many and varied, usually relevant to some other disorder of a medical nature, e.g. abdominal, nervous, respiratory or cardiac disease.

Abdominal pain is encountered in about one third of cases but in most of these it amounts to no more than a vague discomfort certainly not suggestive of that found in acute intermittent porphyria or in variegate porphyria.

Symptoms referable to the nervous system are admitted by more than 50% of patients with symptomatic porphyria but in many cases objective evidence of neurological disorder is absent.

2.2.1.2 Social and Dietary history. Symptomatic porphyria is encountered in 3 racial groups in South Africa - African (Bantu), Coloured and White. While on the Witwatersrand (Barnes 1955) and in the Durban area (Lamont et al. 1961) African patients are in the majority, in Cape Town Eales (1963) found that the Cape Coloured group contributed the bulk of his cases. The lowest incidence is encountered in the White population. A family history of the disorder is not a feature of symptomatic porphyria and if present casts doubt on the diagnosis.

While no significant differences exist between the eating habits of porphyric subjects and those of control patients from the same population group, the evidence relating to the consumption of alcohol presents a different picture. In accordance with the experience of workers in other countries (Brunsting 1954, Berman and Bielický 1956, Tio and Leijnse 1958) all South African authorities are unanimous in incriminating alcohol as a probable aetiological agent. Barnes (1955) reported that almost all the patients he questioned admitted to consumption of alcohol but not all in excessive quantities. Lamont et al. (1961) found that only 2% of their cases denied drinking alcohol at all (compared to 20% of controls); 24% claimed they confined themselves to 'Kaffir beer' only; and 74% drank 'Kaffir beer' plus illicit brews such as 'Shimiyane' and/or 'gaveen'. Among Eales' series (Eales

1963) 72% had a history of heavy drinking but "the claim of abstinence in the remaining 28% was accepted with considerable reserve". His Coloured patients consumed up to 6 bottles of cheap sweet natural wines daily while the Africans drank 'Kaffir beer' .

2.2.1.3 Physical findings. Almost every patient with symptomatic porphyria exhibits easily recognisable skin lesions due to photosensitivity and fragility. Acute lesions consist of vesicles or superficial ulcers mainly in areas exposed to light, viz., the dorsa of hands and fingers, the lower legs and feet and the face (ears and forehead). Blisters may also form under the nailbeds of fingers or toes. Healing of vesicles results in scars in the aforementioned areas. All cases exhibit hyperpigmentation of the face. This is of a blotchy nature unlike the diffuse dull grey black pigmentation found in some siderotic Africans. Hypertrichosis of the forehead, evident as an extension of the hairline down toward the brows, is found in almost all female subjects and the majority of males. Pellagra is encountered concommitantly in a few patients.

While hepatomegaly is a common finding in the adult African population in general, it is nevertheless more striking in this condition. Splenomegaly is much less common. The most frequently encountered disorder of the nervous system is neuropathy including sensory disturbances, absent or exaggerated reflexes and hyperhidrosis.

2.2.2 Diagnosis. Once symptomatic porphyria is suspected on the basis of the clinical features described in Section 2.2.1, definitive diagnosis depends on the demonstration of the characteristic porphyrin excretion pattern.

At no stage in this disease is there an appreciable increase in the excretion of porphyrin precursors, viz., δ-aminolaevulinic acid (ALA) or porphobilinogen (PBG).

When the values for porphyrin excretion in patients with symptomatic porphyria (Table 2.2) are compared with those found in normal subjects (Table 2.1), it is immediately apparent that urinary porphyrin excretion, mainly uroporphyrin (URO), is greatly increased in

Table 2.1 Urine and faecal porphyrins in normal subjects (data from Eales et al. 1966)*.

Racial Group	Urine (µg/l)		Stool (µg/g dry wt.)	
	URO	COPRO	COPRO	PROTO
White	8(0-31)	47(1-130)	7(0-27)	28(0-99)
Coloured	6(0-41)	46(3-183)	7(0-36)	21(1-113)
African	6(0-35)	63(2-204)	9(0-37)	22(1-105)

* Mean values, range within brackets.

Table 2.2 Urine and faecal porphyrins in South African patients with symptomatic porphyria.*

Author	Urine (µg/100ml)		Stool (µg/g dry wt.)	
	URO	COPRO	COPRO	PROTO
Barnes (1959)	1026(75-2360)	-	47(11-91)	38(11-74)
Lamont et al. (1961)	650(68-4962)	-	50(0-578)	15(0-74)
Eales et al. (1963)	282(8-920)	56(5-348)	73(2-618)	49(4-228)

* Mean values, range within brackets.

Table 2.3 Increases of porphyrin concentration in urine and faeces that characterise the hepatic porphyrias (adapted from Sweeney 1963).

Classification	Urine				Faeces	
	ALA	PBG	URO	COPRO	COPRO	PROTO
Variegate (remission)	±	±	±	++	+++	+++
AIP	++	++ variable		±	N	±
Symptomatic	±	N	++	+	± to ++	N

N = normal values; ± = sometimes slight increase

this condition. Coproporphyrin (COPRO) excretion in the urine may also be increased but to a lesser degree.

In contrast to variegate porphyria (Table 2.3), faecal COPRO and protoporphyrin (PROTO) excretion are normal or only moderately raised in most cases of symptomatic porphyria, with COPRO usually exceeding PROTO.

Sweeney (1963) has further characterised the excreted porphyrins in these diseases. In symptomatic porphyria he reported that urinary porphyrin consists mainly of 8 and 7 carboxyl porphyrins with lesser amounts of 6, 5 and 4 carboxyl porphyrins. The 8 carboxyl compound consisted of about 70% isomer I while the other porphyrins were chiefly isomer III. Analysis of liver porphyrins showed a similar pattern. The faeces contained normal amounts of COPRO and PROTO but increased amounts of 5-8 carboxyl porphyrins.

Brilliant red fluorescence of liver biopsy specimens in ultraviolet light is also a helpful diagnostic criterion due to the usually large amounts of stored URO (Lamont et al. 1961, Uys and Eales 1963, Campbell et al. 1965). This phenomenon is not seen in variegate porphyria (Campbell et al. 1965) or AIP (Schmid et al. 1954).

2.2.3 Pathology.

2.2.3.1 Histopathology and ultrastructure of the liver. All patients with symptomatic porphyria have some degree of parenchymal liver pathology. Uys and Eales (1963) in the largest histopathological study reported to date, embracing 3 racial groups in Cape Town, classified the observed changes as (a) liver cell damage only, (b) liver cell damage accompanied by fibrosis, (c) cirrhosis and (d) siderosis.

Of these "siderosis constituted the most constant single abnormality" .... (Uys and Eales 1963). Histochemically demonstrable iron was present in all 40 cases examined. In 95% of biopsies the degree of siderosis was classified as grade-C, indicating that haemosiderin was present in liver cells and Kupffer cells and in aggregates of macrophages, chiefly in the portal tracts and fibrous bands. Perhaps the most significant finding to emerge from this study is the fact

that Africans (8 cases), Coloureds (30 cases) and Whites (4 cases) were affected to the same degree, "there being no differences in the intensity of the siderosis amongst the racial groups". Whereas siderosis occurs in the majority of the African population of South Africa (Section 2.2.4), this is not the case with the Coloured and White communities (Uys et al. 1960). Hence the association of symptomatic porphyria and excessive hepatic iron in these two ethnic groups cannot be regarded as coincidental.

Lamont et al. (1961) found histochemically demonstrable iron in liver biopsy material from 24 out of 28 (85%) African patients with symptomatic porphyria in Durban. While haemosiderin was present in the Kupffer cells, liver cells and portal phagocytes, the distribution was predominantly reticulo-endothelial. As regards intensity, these authors considered that none of their cases showed deposits of iron as heavy as is often found in non-porphyric Africans with siderosis. In Johannesburg Keeley et al. (1960) also reported a similar high incidence of siderosis in symptomatic porphyria in the African.

Uys and Eales (1963) found evidence of liver cell damage alone in 22.5% of their 42 cases. This evidence ranged from mild fatty change, through liver cell regeneration to frank necrosis of small groups of cells. In a further 50% of patients these changes were present in association with fibrosis, varying from an increase in peripheral reticulin fibres to marked septal fibrosis. Cirrhosis was diagnosed in the remaining 11 cases (27.5%). Seven of these showed the features of portal cirrhosis and one the picture of post-necrotic scarring, while in 3 these two pathologies were mixed.

Active liver cell necrosis was found in three instances but was not a feature of the cases seen by Lamont et al. (1961). Mild to moderate fatty change was found in more than half and some degree of hepatic fibrosis in all the patients in their series. As in the Cape Town material the fibrosis ranged from an increase in periportal (5 cases) and intralobular fibrous tissue (11 cases) to fully developed cirrhosis (12 cases).

Campbell et al. (1965) examined the ultrastructural appearances

Table 2.4 Results obtained in 'Liver Function Tests' in patients with symptomatic porphyria.

Investigation	Author	
	Lamont et al. (1961) % abnormal	Eales (1963) % abnormal
Serum bilirubin	(>0.8 mg%) ... 37%	(>1.2 mg%) ... 20%
Serum alkaline phosphatase	(>10 KA units) .. 54%	-
Serum albumin	Mean ... 2.81 g/100 ml	(<3.0 g%) 24%
Serum globulin	Mean ... 4.95 g/100 ml	(>3.0 g%) 62%
Thymol turbidity	-	(>4 units) ... 10%
Zinc turbidity	-	(12 + units).. 59%
Bromsulphthalein retention	-	(>5%) 23%
SGOT	(>40 Karmen units) .. 64%	(>40 Karmen units) .. 28%
Prothrombin index	(<70%) 26%	-

of the liver in 3 cases of symptomatic porphyria. They found that although mitochondria were not greatly reduced in numbers, "their cristae were scanty and short, while the customary double mitochondrial membrane was often reduced to a single membrane which was sometimes incomplete or showed small blunt protrusions". A reduction in the rough endoplasmic reticulum was also noted but smooth-walled vesicles were abundant. Iron was readily demonstrable. These workers suggested that their findings support the "generally held belief that porphyria is a mitochondrial disease ...". However, it seems highly unlikely that the pathological appearances described might be peculiar to symptomatic porphyria since both alcohol (Section 5.4.3.3) and excessive deposits of iron in the liver (Section 5.4.4) have been shown separately to be capable of causing ultrastructural changes in liver mitochondria.

2.2.3.2 Liver function. Hepatocellular dysfunction in symptomatic porphyria is reflected in varying degree by the 'liver function tests' in this condition. Table 2.4 illustrates the findings of Lamont et al. (1961) and Eales (1963) which are compatible with the picture of chronic liver disease described in Section 2.2.3.1. The most striking feature appears to be hyperglobulinaemia. Lamont et al. (1961) reported that, while serum albumin levels in their porphyric patients were not materially different from those described in Durban Africans, all globulin fractions in their patients were increased. These authors also found raised SGOT levels in the majority of their cases which they stated "can only be interpreted as evidence of active hepatocellular damage at the time of examination, because there was no clinical evidence of myocardial or skeletal muscle disease which could give rise to comparable increases".

2.2.3.3 Serum iron concentration. In keeping with the high incidence of hepatic siderosis most patients with symptomatic porphyria have hypersideraemia. Lamont et al. (1961) reported a plasma iron level of over 200 µg/100 ml in 51% of 89 cases (normal range 60-170 µg/100 ml). Eales (1963) similarly obtained results of over 180 µg/100 ml in 59% of 29 patients and Keeley et al. (1960) found raised values in 7 of 12 African patients.

Iron metabolism in symptomatic porphyria is considered in Section 5.4.4.

2.2.3.4 Diabetes mellitus and symptomatic porphyria. An association between diabetes mellitus and porphyria cutanea tarda was previously reported in America (Brunsting 1954) and Europe (Berman and Bielický 1956). More recently it has been noted that the incidence of impaired glucose tolerance and diabetes in symptomatic porphyria in South Africa is also much higher than in the general population. Of 13 porphyric patients tested by Joubert et al. (1963), 4 showed an abnormal response to a 50 g glucose tolerance test. Eales reported similar findings in 7 of 23 subjects tested. Keeley (1962) noted that, whereas the overall incidence of symptomatic porphyria in the general out-patient population of Baragwanath Hospital was 1.3%, the incidence of this disease among patients attending the diabetic clinic was 4%.

One possible cause of glucose intolerance, going on to frank diabetes, in symptomatic porphyria is pancreatic disease. Alcoholic patients are liable to develop chronic pancreatitis and Eales (1963) has reported that abnormal results were obtained in 5 out of 12 patients with symptomatic porphyria who underwent formal pancreatic function tests. Seftel et al. (1960, 1961) have stressed the association between severe siderosis and diabetes in Africans. In 4 out of 20 diabetic African subjects examined at autopsy the findings were indistinguishable from idiopathic haemochromatosis and it is suggested that siderosis of the pancreas in these cases resulted in carbohydrate intolerance. Whereas hepatic siderosis is found in virtually all cases of symptomatic porphyria (Section 2.2.3.1), the frequency of pancreatic siderosis in this disease is, however, unknown.

It has long been known that chronic liver disease is frequently associated with diabetes. The term 'hepatogenous diabetes' was coined in 1906 by Naunyn (Naunyn 1906). Recently Megyesi et al. (1967) have shown that 57% of patients with various forms of chronic liver disease have either unequivocal diabetes or impaired glucose tolerance associated with hyperinsulinaemia which is probably due to endogenous insulin resistance. In view of the frequency of chronic liver disease in

symptomatic porphyria it is quite possible that the associated diabetes found in some cases is 'hepatogenous'.

2.2.4 The pathogenesis of symptomatic porphyria. In discussing the pathogenesis of symptomatic porphyria, most authors stress the significance of 3 features mentioned in the foregoing account. These are :

1) the absence of a family history of the disorder,
2) the almost invariable history of chronic abuse of alcohol, and
3) the presence of parenchymal liver pathology of which siderosis is the most constant feature.

Heredity. As indicated previously, Dean (1953) has shown a Mendelian dominant pattern of inheritance in variegate porphyria. With regard to symptomatic porphyria, however, all authors agree that evidence of genetic influence is lacking. For this reason and on account of the associated liver pathology the disease has come to be regarded as acquired. Barnes (1955), for example, noted that of 300 case histories examined by him in only two instances was more than one member of a family affected. In a study of the children of porphyric subjects he (Barnes 1959) later observed that some of the offspring of patients suffering from variegate porphyria, although free of clinical signs, exhibited an increased faecal excretion of COPRO and PROTO, indicating latent disease. On the other hand no such tendency was found in the children of African patients with symptomatic porphyria. This latter observation is, however, of doubtful significance as evidence against a genetic factor in this disorder, since increased levels of porphyrins in the stool are not a feature of overt symptomatic porphyria.

No systematic genetic study of symptomatic porphyria appears to have been undertaken to date. Such a study would not be easy in view of the difficulty of tracing genealogies in the African population, particularly in the detribalised urban community. However, it is conceivable that a properly conducted investigation might reveal a recessive pattern of inheritance involving susceptibility to some suspected aetiological agent, e.g. alcohol.

Alcohol. As mentioned in Section 2.2.1.2, all authors are unanimous in citing alcohol as a probable causal agent in symptomatic porphyria on the basis of chronic excessive drinking by virtually all sufferers from this disease. Further supportive evidence is provided by the observation (Eales 1963) that the cutaneous lesions of patients with symptomatic porphyria have resolved during periods of abstinence with concomitant improvement in abnormal porphyrin metabolism.

Nevertheless, no controlled study of the effect of administered alcohol on porphyrin biosynthesis and excretion in this disorder appears to have been undertaken to date. One of the aims of this thesis was to provide more precise information on this aspect.

A major criticism of the postulate that alcohol is an aetiological agent in symptomatic porphyria is the fact that the incidence of this disease falls far short of the frequency of heavy drinking and alcoholism in all population groups. There are several possible explanations for this apparent anomaly.

Firstly, it is conceivable that only in individuals possessing an inherent idiosyncracy does drinking alcoholic beverages precipitate abnormal porphyrin metabolism. On the other hand, it may be argued that in unduly susceptible people the consumption of small amounts of ethanol might be expected to be sufficient to trigger porphyrinogenesis, whereas the type of porphyric patient in question is almost without exception an habitual heavy drinker.

Secondly, alcohol may play a subsidiary role to some other environmental agent which actually induces the porphyric state. Bouts of drinking might then exacerbate abnormal porphyrin metabolism while periods of abstinence might be expected to produce amelioration as observed by Eales (1963). This would appear to be the more plausible explanation. The mode whereby alcohol could exert such an effect is considered in Section 5.4.3.

Liver disease. Parenchymal liver pathology is a feature of all cases of symptomatic porphyria (Section 2.2.3.1). While no pathognomonic lesion has been described, it has been noted by various authors that siderosis is the most constant feature encountered. In order to

evaluate the significance of this finding it is necessary to review the prevalence in the population at large.

Hepatic siderosis is an extremely common condition among the African peoples of South Africa. It was first described by Strachan (1929) on the Witwatersrand and subsequent investigations revealed a high incidence in Africans in many parts of the country (Gillman et al. 1945, Higginson et al. 1953, Wainwright 1957, Gillman et al. 1958, Bothwell and Bradlow 1959, Uys et al. 1960). Reports have also come from other parts of the African continent (Edington 1954, Gelfand 1955).

Wainwright (1957) and Bothwell and Bradlow (1959) found that hepatic siderosis of varying degree was present in over 80% of adult male Africans examined at autopsy. In the former study no selection of cases was practised, while in the latter only subjects who died from acute trauma were included. Uys et al. (1960) found a somewhat lower incidence (55.9%) among 134 consecutive necropsies on African subjects in Cape Town.

By contrast the frequency of siderosis among the Cape Coloureds and Whites in the same study was only 29.1% and 30.1% respectively (Uys et al. 1960). This finding has particular significance with regard to symptomatic porphyria. As previously indicated, histochemically demonstrable iron is present in virtually all cases in this condition, irrespective of racial group. While this association could be attributed to coincidence in the African among whom siderosis is so common, an alternative explanation is necessary in the case of the Coloureds and Whites. Serious consideration must, therefore, be given to the possibility that excessive deposits of iron in the liver play a part in the pathogenesis of symptomatic porphyria. With this in mind it was intended in the present thesis to examine the effect of experimental siderosis on porphyrin metabolism. Iron metabolism in symptomatic porphyria is considered in more detail in Section 5.4.4.

CHAPTER 3

BIOSYNTHESIS OF PORPHYRINS AND HAEM

3.1 Introduction. The unravelling of the porphyrin biosynthetic chain (Fig. 3.1), a subject which has aroused world wide interest among biochemists and clinicians alike, began with the early studies in Shemin's laboratory in 1945.

Shemin and Rittenberg (1945, 1946) using ^{15}N-labelled glycine first showed that this amino acid is a specific precursor of haem protoporphyrin in both man and the rat. Subsequently *in vitro* studies demonstrated that isotopically labelled glycine is incorporated into haem by nucleated duck or chicken erythrocytes (Shemin et al. 1948, Dresel and Falk 1954) and by the non-nucleated erythrocyte of the rabbit, made anaemic by bleeding or administration of phenylhydrazine (London et al. 1950).

The next problem was the distribution of the C and N atoms of glycine in the porphyrin molecule. Protoporphyrin IX is an asymmetric tetrapyrrole consisting of two types of pyrrole units as shown in Fig. 3.2. Through various degradative steps rings A and B can be converted to methylethylmaleimide and rings C and D to haematinic acid. On degrading ^{15}N-labelled haem protoporphyrin prepared in the rabbit (Muir and Neuberger 1949), or duck, or human (Wittenberg and Shemin 1949) by the administration of glycine-^{15}N, it was found that the ^{15}N concentrations of the porphyrin molecule and the two breakdown products were equal. Thus it was established that glycine provided the N atoms present in all four pyrrole units.

The fate of the C atoms of the glycine molecule in porphyrin biosynthesis was elucidated in similar fashion. Using ^{14}C- and ^{15}N-labelled glycine and other precursors Radin et al. (1950) and Muir and Neuberger (1950) found that eight of the α C atoms of glycine were incorporated into the porphyrin molecule, i.e. twice the number of N atoms used, and four of these labelled C atoms were present in the methene C fraction (Muir and Neuberger 1950). Further stepwise degradation (Wittenberg and Shemin 1950) revealed that the other four C atoms are situated in comparable positions in the pyrrole rings, viz., at A2, B2, C2 and D2 (see Fig. 3.2). On the other hand, experiments

Fig. 3.1 Schematic representation of the haem biosynthetic pathway. Stages depicted within the rectangles occur within the mitochondria, while the remainder take place in the cell cytoplasm.

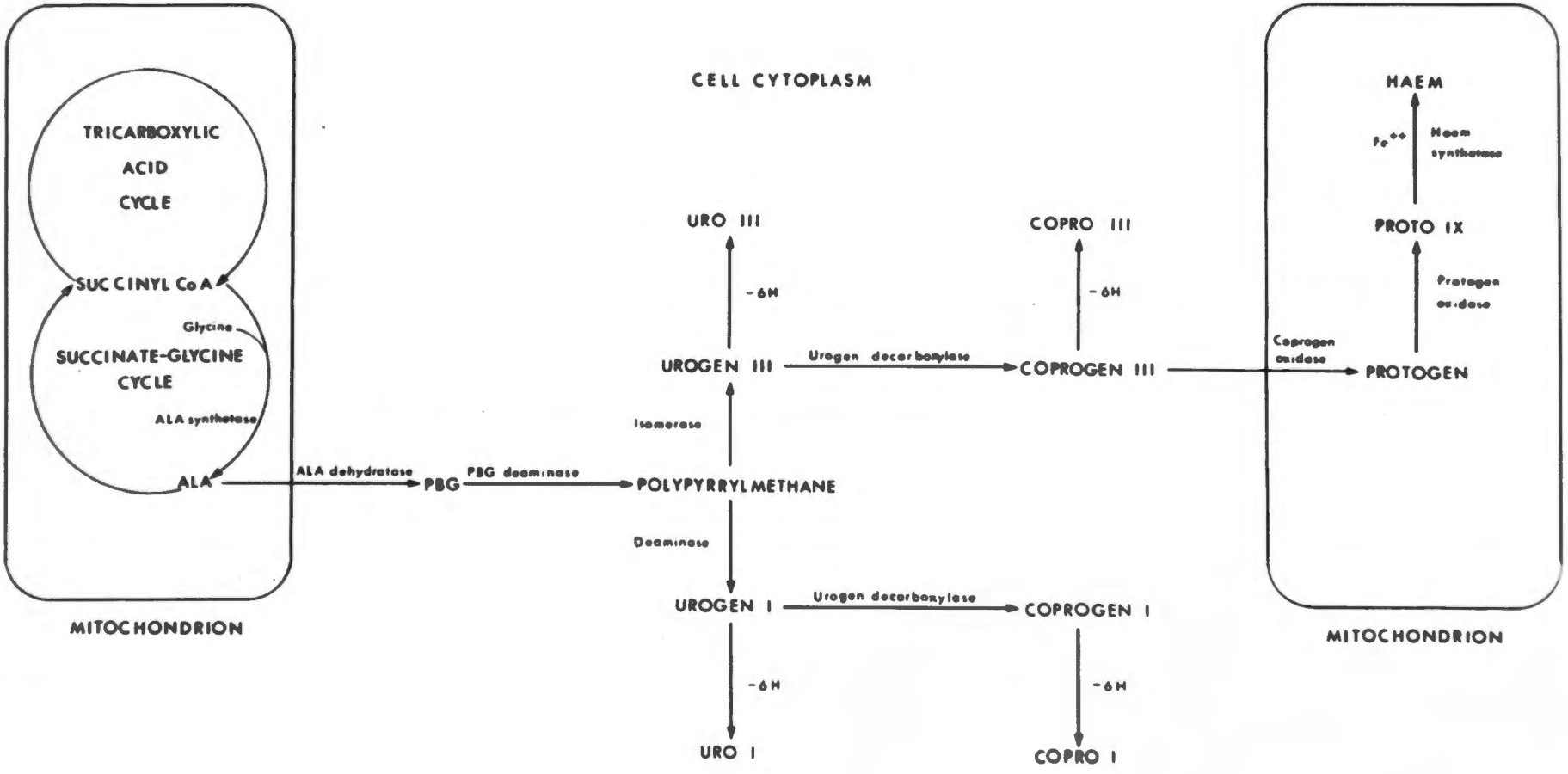
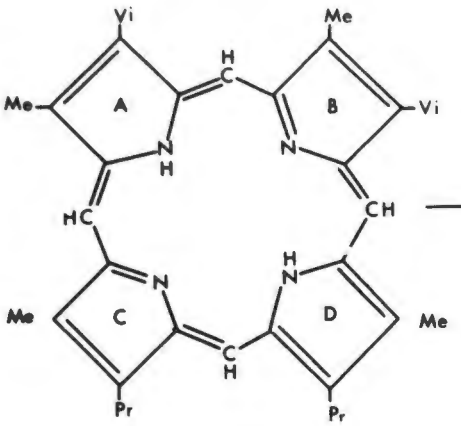
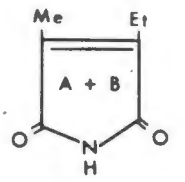

TRICARBOXYLIC
ACID
CYCLE
SUCCINYL CoA
Glycine
SUCCINATE-GLYCINE
CYCLE
ALA synthetase
ALA
MITOCHONDRION
ALA dehydratase
PBG
PBG deaminase
CELL CYTOPLASM
URO III
-6H
UROGEN III
Urogen decarboxylase
COPRO III
-6H
COPROGEN III
Isomerase
POLYPYRRYLMETHANE
Deaminase
UROGEN I
Urogen decarboxylase
COPROGEN I
-6H
URO I
-6H
COPRO I
Coprogen
oxidase
HAEM
Fe++
Haem
synthetase
PROTO IX
Protogen
oxidase
PROTOGEN
MITOCHONDRION

Fig. 3.2 Diagrammatic representation of protoporphyrin IX molecule, showing 2 types of pyrrole units. On degradation of the molecule, units A and B can be converted to methylethyl-maleimide and units C and D to haematinic acid.

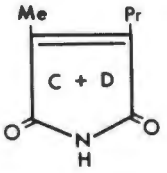
Fig. 3.3 Diagram illustrating the differences between the series I and series III isomers of UROgen and COPROgen. In each case the side chains attached to one of the pyrrole units of the I isomer have been transposed to produce the III isomer.



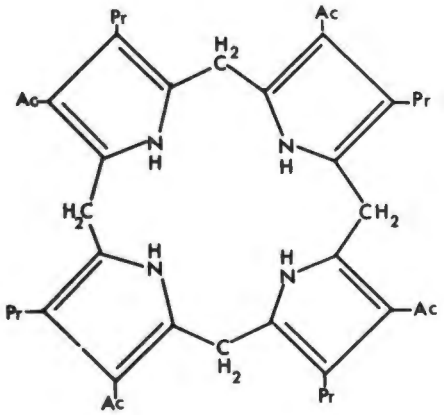
PROTOPORPHYRIN IX



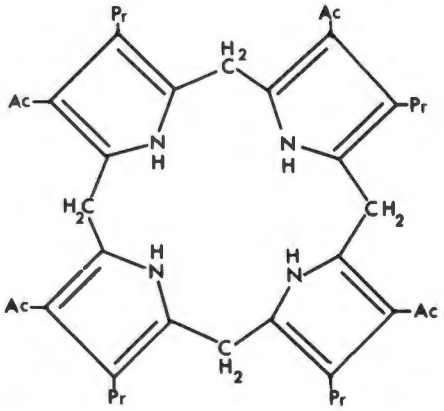
Methylethymaleimide



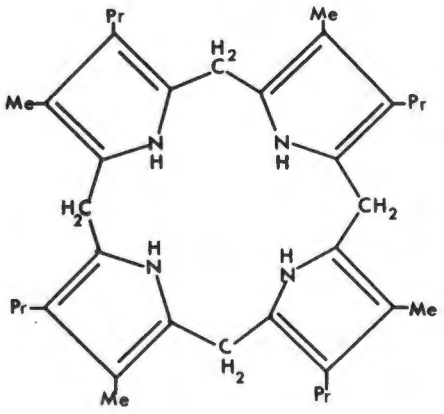
Haematinic acid



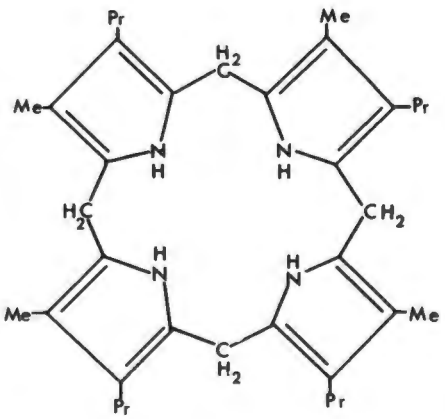
URO-gen I



URO-gen III



COPRO-gen I



COPRO-gen III

with carboxyl-labelled ^{14}C-glycine did not lead to labelling of protoporphyrin or haem (Grinstein et al. 1948, Radin et al. 1950).

The origin of the remaining 26 C atoms of protoporphyrin was likewise traced with the aid of isotopically labelled compounds. Using ^{14}C-acetate labelled either in the methyl or carboxyl position, Shemin and Wittenberg (1951) found that acetate could supply all 26 C atoms in question. Furthermore, in degradative studies of the type mentioned above, they detected equal amounts of radioactivity similarly distributed among three C atoms in each of the two residues representing the two sides of the monopyrrole molecule. Thus it seemed highly likely that the basic monopyrrole unit was formed by condensation of two molecules of the same compound with glycine (Shemin and Wittenberg 1951).

Shemin and Wittenberg (1951) reasoned that this precursor must of necessity be a three or four carbon compound, most probably an unsymmetrical four carbon molecule. In a series of further experiments using other labelled intermediates of the TCA cycle including carboxyl-labelled succinate-^{14}C, methylene-labelled succinate-^{14}C, α-ketoglutarate-5-^{14}C, α-ketoglutarate-1,2-^{14}C and citrate 1,5-^{14}C, Shemin and coworkers (Shemin and Kumin 1952, Wriston et al. 1955) identified the four carbon compound in question as a derivative of succinate, possibly a succinyl-coenzyme complex which could arise from succinate or α-ketoglutarate.

Following the earlier observation (Wittenberg and Shemin 1950) that the α C atom of glycine is used equally for both the methene bridge and the pyrrole ring and that no derivative of the α C could be substituted for glycine, it became apparent that the whole glycine molecule must participate in the initial condensation with the 'active' succinate (Shemin 1955). This led to the postulate that succinate condensed with the α C of glycine to form α-amino-β-ketoadipic acid which then underwent decarboxylation to remove the carboxyl group which does not appear in the porphyrin molecule. The resulting haem precursor would be δ-aminolaevulinic acid (ALA). It was suggested that, by means of a Knorr type condensation, two molecules of this acid could yield a pyrrole which would fit the requirements imposed by the

known distribution of the α C atom of glycine in protoporphyrin or haem (Shemin 1955) (Fig. 3.4).

That ALA is in fact a physiological haem precursor was subsequently shown by a number of workers. Dresel and Falk (1953) demonstrated the biosynthesis of porphobilinogen (PBG) from ALA incubated with chicken red cell haemolysates. PBG, the monopyrrole which had previously been isolated by Westall (1952) from the urine of a patient with acute porphyria, on incubation in the chicken red cell haemolysate system gave rise to a mixture of PROTO, COPRO and URO (Falk, Dresel and Rimington 1953).

In Shemin's laboratory (Shemin and Russell 1953) unlabelled ALA was shown to dilute the radioactivity of haemin synthesised by duck red cell haemolysates from glycine-^{14}C or succinate-^{14}C. This would be expected if the nonradioactive ALA were diluting ALA-^{14}C formed in the incubate from the labelled glycine or succinate. Finally, experiments with ALA-5-^{14}C (Shemin, Russell and Abramsky 1955) and ALA-1,4-^{14}C (Schiffman and Shemin 1955) gave precisely the predicted labelling pattern for haemin, i.e. ^{14}C in the 5 position behaved in the same fashion as the α C of glycine and that in the 1 and 4 positions in the same way as the carboxyl groups of succinate.

3.2 Biosynthesis of δ-aminolaevulinic acid (ALA). The initial step of the porphyrin biosynthetic pathway proper has been extensively studied in a number of different systems, including micro-organisms (Kikuchi et al. 1958, Gibson 1958, Lascelles 1959), avian erythrocytes (Brown 1958a, Laver et al. 1958, Granick 1958) and mammalian liver (Granick and Urata 1963, Tschudy et al. 1964, Marver et al. 1966). In each system the enzymatic formation of ALA by condensation of glycine and 'active' succinate, viz., succinyl-CoA, has been demonstrated (Kikuchi et al. 1958, Gibson et al. 1958, Granick and Urata 1963).

Although this enzyme, ALA synthetase, could be demonstrated in particle-free extracts of the photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* (Kikuchi et al. 1958), in avian erythrocyte preparations the activity was found to be associated with a

particulate fraction (Laver et al. 1958, Gibson et al. 1958). Sano et al. (1959) concluded that mitochondria were involved since these organelles are to be found in the avian erythrocyte and the reticulocyte but not in the mature mammalian red blood cell which is incapable of synthesising ALA. Granick and Urata (1963) in examining the induction of guinea pig liver ALA synthetase by DDC clearly demonstrated the intramitochondrial localisation of this enzyme.

Brown (1958b, c) showed that ferrous iron was involved at some stage in the synthesis of ALA in particulate preparations of chicken erythrocytes. However, it would appear that this does not apply to all ALA-synthesising systems. Burnham and Lascelles (1963) found, for example, that iron does not participate in the action of ALA synthetase prepared from *Rhodopseudomonas spheroides*.

A requirement for the coenzyme, pyridoxal phosphate, has been demonstrated in a number of different ways. Schulman and Richert (1957), studying vitamin B_6 deficient ducklings, found a decreased rate of red cell haem synthesis; and furthermore, pyridoxal-5-phosphate added *in vitro* to red cells from these birds stimulated haem formation from glycine-2-^{14}C or succinate-2-^{14}C. Induction of pyridoxine deficiency in a patient with acute intermittent porphyria likewise resulted in decreased urinary excretion of ALA and PBG. Subsequent pyridoxine loading of this patient markedly increased excretion of these precursors (Elder and Mengel 1965).

In vitro studies with microbiological (Lascelles 1957), avian erythrocyte (Gibson et al. 1958) and mammalian liver (Granick and Urata 1963) preparations have all shown that ALA or porphyrin formation is substantially increased when pyridoxal phosphate is added. Conversely, the reaction is strongly inhibited by substances such as L-penicillamine (Laver et al. 1958) which is thought to interact with pyridoxal phosphate. The mechanism whereby this cofactor promotes the biosynthesis of ALA, it is suggested, is the formation of a Schiff base between the amino group of glycine and enzyme-bound pyridoxal phosphate. This would have the effect of 'activating' the α C of glycine to condense with succinyl CoA (Granick 1958, Gibson et al. 1958).

The immediate condensation product would then be α-amino-β-ketoadipic acid (Shemin 1955) and in fact evidence is available that the diethyl ester of this acid administered to rats is metabolised to PBG (Weliky and Shemin 1957). Nevertheless, this does not constitute proof that α-amino-β-ketoadipic acid is a true intermediate in ALA biosynthesis and later Shemin et al. (1962) suggested that decarboxylation of the pyridoxal derivative of glycine and condensation with succinyl CoA may occur simultaneously.

Glycine has been shown to condense not only with succinyl CoA but with other acyl CoA compounds such as acetyl CoA and propionyl CoA (Urata and Granick 1963) to yield aminoacetone and other aminoketones. The possible significance of these compounds in porphyrin synthesis will be discussed in Section 3.3.

3.3 Biosynthesis of Porphobilinogen (PBG). PBG was first isolated from the urine of a patient with acute porphyria by Westall (1952) and soon afterwards the position of this monopyrrole as a porphyrin precursor was established (Falk et al. 1953, Bogorad and Granick 1953).

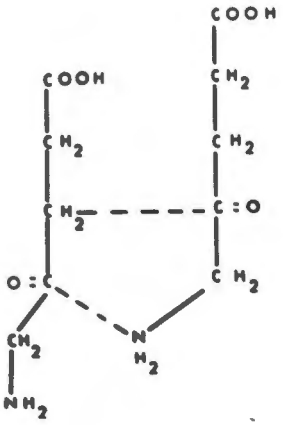
Then followed the demonstration that ALA could be enzymatically converted to PBG by chicken erythrocyte extract (Granick 1954) and by highly soluble enzyme preparations from ox liver (Gibson et al. 1955), from duck erythrocytes (Schmid and Shemin 1955) and from *Rhodopseudomonas spheroides* (Nandi et al. 1968).

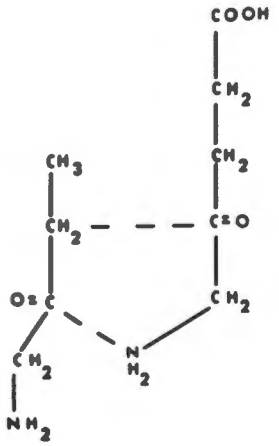
The enzyme which is known as ALA dehydratase requires reduced glutathione (GSH) for activation before it can effect the synthesis of PBG (Gibson et al. 1955, Schmid and Shemin 1955). Hence thiol inhibitors such as iodoacetamide are inhibitory as is ethylenediaminetetraacetic acid (EDTA) (Gibson et al. 1955). The latter finding pointed to the requirement for a metal and Cu was suggested by Iodice et al. (1958). These workers found 0.1% Cu in purified ALA dehydratase from beef liver, and decreased activity of this enzyme in the livers of rats or blood of ducklings fed Cu deficient diets. In a subsequent paper, however, (Wilson et al. 1959) they noted that further purification of liver ALA dehydratase could remove all copper without loss of activity.

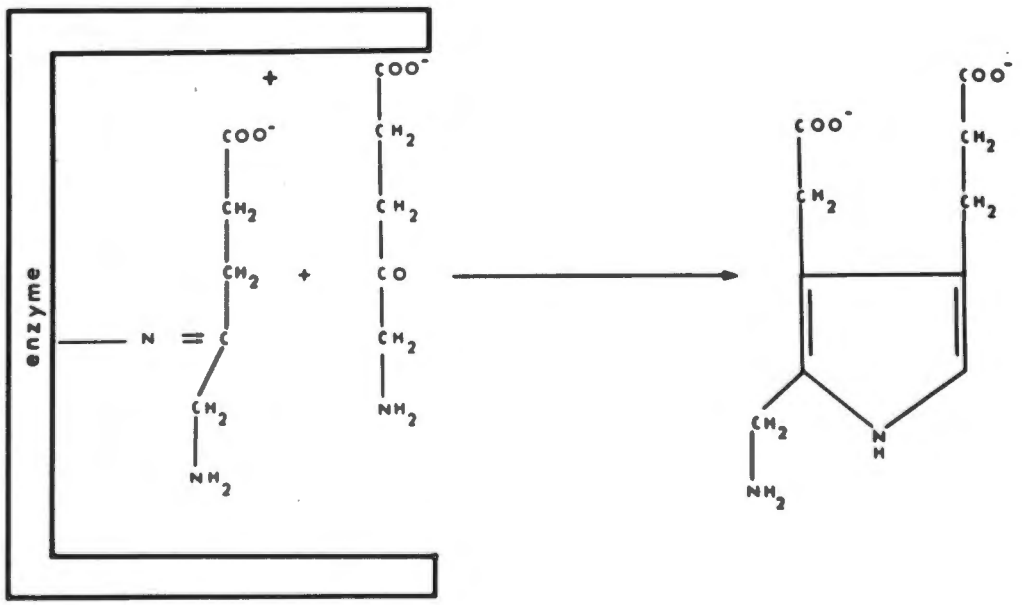
Fig. 3.4 (top left) Diagram representing Knorr condensation of two molecules of ALA to yield PBG. This involves (1) an aldol condensation followed by the elimination of water between the β-carbon of the ALA molecule on the left of the figure and the carbonyl group of the molecule on the right, and (2) the formation of a Schiff base with elimination of water between the carbonyl group of the left-hand molecule and the amino group of the right-hand molecule.

Fig. 3.6 (top right) Diagram illustrating the hypothetical condensation (by means of a Knorr type mechanism) of one molecule of 1-amino-2-butanone (left side of figure) with one molecule of ALA. The resulting pyrrole could represent the basic sub-unit of COPRO.

Fig. 3.5 (below) The mechanism of PBG synthesis from two molecules of ALA according to Nandi and Shemin (1968). The molecule on the left of the figure is held to the enzyme by (1) the formation of a Schiff base and (2) an ionic bond between a positively charged group on the enzyme (denoted $_+$) and the carboxylate group of the molecule (see Section 3.3 for further details).






enzyme

More recently Nandi et al. (1968), in characterising purified ALA dehydratase from *Rh. spheroides*, found many features of an allosteric enzyme, subject to activation by allosteric effectors, viz., the monovalent cations - K^+, Li^+, Rb^+ and NH_4^+.

On the basis of the positions of the labelled C atoms in protoporphyrin synthesised from glycine-2-^{14}C and succinyl CoA, Shemin et al. (1955) proposed the formation of a monopyrrole by condensation of two molecules of ALA via a Knorr type of mechanism (Fig. 3.4). It will be seen that the proposed structure of this monopyrrole is identical with that put forward by Cookson and Rimington (1953) for PBG. Additional support for the condensation idea came from the finding that the molar activity of PBG synthesised from ALA-5-^{14}C was exactly twice that of the latter molecule (Schmid and Shemin 1955).

The Knorr reaction (see Fig. 3.4) involves an aldol condensation followed by the elimination of H_2O between the β C of one molecule of ALA and the carbonyl group of the second molecule, plus the formation of a Schiff base with elimination of water between the carbonyl group of the first molecule and the amino group of the second.

Nandi and Shemin (1968) have recently published further evidence on the mechanism of PBG synthesis. This evidence, obtained in a series of ingenious experiments using a purified preparation of ALA dehydratase, indicates that initially one molecule of ALA forms a Schiff base with the enzyme. As a result the β-methylene C of ALA is converted to a carbanion which can then make a nucleophilic attack on the carbonyl group of a second ALA molecule. Following this aldol condensation, a molecule of water is lost as shown in Fig. 3.5 and the amino group of the second ALA molecule displaces the amino group of the enzyme by a transimination or transaldimination to close the pyrrole ring (Nandi and Shemin 1968).

It was found that, excluding ketoglutaric acid, only γ-ketoacids (ALA, N-acetyl-δ-ALA, laevulinic acid and ethyl laevulinate) formed a Schiff base with the enzyme. These γ-ketoacids varied in their ability to inhibit ALA dehydratase; thus only laevulinic acid and N-acetyl-δ-ALA

showed this property, while ethyl laevulinate did not. Nandi and Shemin (1968) interpret this difference as evidence that enzyme-substrate affinity depends, not only on ease of Schiff base formation, but also on the formation of an ionic bond between a positively charged group on the enzyme and the carboxylate ion of the substrate.

Heterologous pyrrole formation from ALA and laevulinic acid or ethyl laevulinate was demonstrated by the same workers by incubating ALA dehydratase with various mixtures of these substrates, both ^{14}C-labelled and unlabelled. Following chromatography of the incubates, the location of the radioactive labels was shown by auto-radiography and that of the pyrroles by treatment with Ehrlich's reagent.

This demonstration is particularly interesting in view of the earlier finding of Urata and Granick (1963) that normal guinea pig liver mitochondria can form several aminoketones by condensation of glycine with acyl CoA compounds. These include acetyl CoA, succinyl CoA and propionyl CoA and give rise to aminoacetone, ALA and 1-amino-2-butanone ($CH_3CH_2COCH_2NH_2$) respectively. If the latter compound (1-amino-2-butanone) which is formed in comparable amounts to aminoacetone (Urata and Granick 1963) condensed with ALA, the resulting pyrrole shown in Fig. 3.6 could represent the basic sub-unit of coproporphyrin. This raises the interesting possibility as to whether such a mechanism for coproporphyrin synthesis actually occurs *in vivo*.

On the basis of the work of Nandi and Shemin (1968) it seems highly unlikely that ALA dehydratase could catalyse the formation of such a pyrrole. As shown in Fig. 3.6 the 1-amino-2-butanone molecule would have to be held in Schiff base linkage with the enzyme during the synthesis of the pyrrole, i.e. it would have to compete with ALA for the active site on the protein. Nandi and Shemin (1968) showed that in fact non-carboxylated aminoketones (and 1-amino-2-butanone is one of these) cannot compete with ALA as far as affinity for ALA dehydratase is concerned, due to the absence of the terminal carboxylate ion. This of course does not preclude the formation of this heterologous pyrrole by some other as yet undiscovered enzyme and

hence the possibility remains to be investigated further.

3.4 Biosynthesis of uroporphyrinogen (UROgen). Westall (1952) had demonstrated that PGB on heating in dilute acid is easily converted to URO; and several authors (as mentioned in Section 3.3) had shown this monopyrrole to be an effective porphyrin precursor in biological preparations (Falk et al. 1953, Bogorad and Granick 1953). It was therefore a logical assumption that *in vivo* a similar condensation of four PBG molecules yielded URO with subsequent decarboxylations to COPRO and then PROTO.

However, it soon became apparent that URO could not replace PBG as substrate for COPRO and PROTO formation in a variety of biological systems, such as rat liver homogenate and bone marrow (Schwartz 1955) and chicken red cell haemolysates (Dresel and Falk 1956).

The true intermediates were shown to be reduced or hexahydroporphyrins - porphyrinogens. Bogorad (1955) found that PBG was converted by an enzyme from spinach leaf, PBG deaminase, initially to a colourless compound and that only under aerobic conditions was a small amount of porphyrin formed. In the following year Neve et al. (1956) confirmed that uroporphyrinogen (UROgen)(colourless) and not URO was converted to haem in a haemolysed duck red cell preparation. Subsequently, other authors have reported the same findings with rabbit reticulocyte (Mauzerall and Granick 1958) and *Chlorella* (Bogorad 1958c) preparations. The porphyrins, therefore, instead of being true intermediates in the biosynthesis of haem, represent by-products formed by irreversible oxidation of porphyrinogens (Rimington 1956).

Of the four possible uroporphyrins only the I and III isomers are found in nature. The same therefore applies to COPRO, a decarboxylation product of URO. There are fifteen possible isomeric forms of PROTO but only protoporphyrin IX, so named by Fischer and Orth (1937), and formally derivable from COPRO III, occurs naturally and complexes with Fe^{2+} to form haem (Gray 1964).

This raises one of the major unsolved problems in explaining porphyrin biosynthesis - "How is uroporphyrinogen III formed from PBG?

The step-wise head-to-tail condensation of four PBG molecules to make uroporphyrinogen I is simple to visualise, but - even on paper - steps required for the formation of the III-isomer are difficult to conceive" (Bogorad 1963).

Thus far several enzymes catalysing the condensation of PBG to porphyrinogens have been isolated from a variety of sources - plant (Bogorad 1958a, b) animal (Lockwood and Rimington 1957, Mauzerall and Granick 1958) and bacterial (Hoare and Heath 1959).

Earlier, Bogorad and Granick (1953) had discovered that extracts from *Chlorella* formed series III porphyrins from PBG but if the enzyme preparation was preheated to 55°C then only type I isomers were synthesised. The explanation of this phenomenon appears to be that two separate enzymes are involved. Bogorad (1958a, b) isolated and characterised PBG deaminase (UROgen I synthetase) from spinach leaf and UROgen isomerase (UROgen III cosynthetase) from wheat germ. Hoare and Heath (1959) also separated and partially purified these two enzymes in extracts of *Rh. spheroides*. The first enzyme which is stable at 58°C catalyses the production of UROgen I from PBG (Bogorad 1958a) while the second (which is heat labile) is inactive when incubated alone with PBG. However, when both enzymes are incubated together with PBG, UROgen III is produced (Bogorad 1958b). Bogorad (1963) suggested that "some polypyrrolic product of the action of uroporphyrinogen-I-synthetase on PBG is one of the two substrates required for uroporphyrinogen-III-cosynthetase action - PBG is the other substrate partner". Various evidence indicates that the polypyrrolic product may be a dipyrrylmethane which is isomerised by UROgen-III-cosynthetase, i.e. one of the rings is "flipped over" (Mauzerall 1964) (Fig. 3.3). This concept is supported by the isolation of an intermediate between PBG and UROgen I which has the properties of a dipyrrylmethane (Bogorad 1961).

Nevertheless, the exact mechanism of the isomerisation remains unsolved despite a great deal of further speculation which it is not pertinent to detail here.

3.5 Metabolism of uroporphyrinogen (UROgen) and biosynthesis of coproporphyrinogen (COPROgen). The biological fate of the uroporphyrinogens is either oxidation to uroporphyrin or enzymatic decarboxylation to coproporphyrinogen (Mauzerall and Granick 1958, Bogorad 1958, Hoare and Heath 1958). Normally a small fraction of the UROgen formed in the cell undergoes irreversible step-wise oxidation to URO and is thereby lost to the haem biosynthetic pathway. The oxidative process has been observed to occur either enzymatically (Bogorad 1958) or by means of photocatalytic autoxidation, sensitised by the product URO (Mauzerall and Granick 1958). The photo-oxidation is inhibited *in vitro* by antioxidants such as GSH and it is quite conceivable that in the living cell this substance, aided by the exclusion of light, plays a similar role to limit porphyrin formation. On the other hand, at acid pH photo-oxidation is assisted by ferric ions (Mauzerall and Granick 1958).

Sequential enzymatic decarboxylation of most of the uroporphyrinogens formed from PBG leads to the formation of a series of porphyrinogens with less than 8 carboxyl groups (Mauzerall and Granick 1958). The enzyme uroporphyrinogen decarboxylase has been isolated by zone electrophoresis on starch from haemolysed rabbit erythrocytes (Mauzerall and Granick 1958). Both series I and III isomers are acted upon by this enzyme but at different rates- UROgen I is decarboxylated at only one third of the rate for UROgen III. ALA, PBG and uroporphyrin cannot substitute for the UROgens as substrates (Mauzerall and Granick 1958).

In addition to rabbit erythrocytes, uroporphyrinogen decarboxylase activity has been demonstrated in the red cells of ducks (Neve et al. 1956) and in preparations of *Chlorella* (Bogorad 1958c) and of *Rh. spheroides* (Hoare and Heath 1958). Although it is generally assumed that the same sequence of events occurs in human erythrocytes and in mammalian liver cells, to date the fate of UROgen in these tissues does not appear to have been studied.

The exact mechanism of decarboxylation of UROgen (8 carboxyl groups) to COPROgen (4 carboxyl groups) is not known but there is some evidence that the process occurs step-wise. Mauzerall and Granick (1958) were able to separate intermediate compounds with 7, 6 and 5 carboxyl groups following partial enzymic decarboxylation of UROgen. Considering all these intermediates and their possible isomers there would be altogether 34 different substrates for this enzyme. Since it has not been established just how many enzymes are involved, it remains convenient to describe the collective 'enzyme' as UROgen decarboxylase (Mauzerall and Granick 1958).

3.6 Biosynthesis of protoporphyrin (PROTO). The enzymatic conversion of coproporphyrinogen III to protoporphyrin IX has been demonstrated in a number of systems, including - chicken erythrocytes (Granick and Mauzerall 1958a), *Euglena* (Granick and Mauzerall 1958b) and beef liver mitochondria (Sano and Granick 1961). The enzyme coproporphyrinogen oxidase (coproporphyrinogenase) is located intramitochondrially. Sano and Granick (1961) found over 80% of coproporphyrinogenase activity in the mitochondrial fraction of guinea pig liver. They considered that the low activities found in the fluffy layer and microsome fractions were due to contamination by damaged mitochondria.

Unlike UROgen decarboxylase this enzyme is substrate-specific for COPROgen III and does not act on the I isomer. Only two specific propionic acid groups are oxidatively decarboxylated to vinyl groups yielding protoporphyrinogen (PROTOgen). The exact mechanism(s) involved are still uncertain but the available evidence suggests that decarboxylation and oxidation by molecular oxygen of the propionic acid groups are simultaneous (Mauzerall 1964). Several intermediates such as haematoporphyrinogen and acrylic acid-substituted porphyrinogens which would be involved in step-wise reactions have been found to be unreactive (Mauzerall 1964). On the other hand, Sano and Granick (1961) reported that an intermediate '3-carboxyl compound' appears and disappears in the course of the reaction.

PROTOgen like the other porphyrinogens can undergo autoxidation. In the presence of light protophyrin IX is readily formed but in the dark this is not the case unless mitochondria are present. It is therefore suggested that an enzyme (protoporphyrinogen oxidase) operates in the cell to convert PROTOgen to PROTO IX (Sano and Granick 1961, Porra and Falk 1964).

3.7 Incorporation of Fe^{2+} into protoporphyrin - biosynthesis of haem.

The final step in haem biosynthesis is the insertion of the ferrous ion into the tetrapyrrole molecule. This could occur at the PROTOgen stage or following the oxidation of this hexahydroporphyrin to PROTO IX. The available evidence favours the latter alternative since it has been shown *in vitro* that the porphyrinogens bind metal poorly, while the porphyrins, including PROTO, in neutral aqueous solution readily complex with Fe^{2+} (Mauzerall and Granick 1958, Heikel et al. 1958).

Early studies using avian erythrocyte preparations suggested that the biosynthesis of haem from PROTO and Fe^{2+} was enzyme dependant since the activity was abolished by CN^- and significantly inhibited by preheating of the haemolysate for 30 minutes at 56°C (Goldberg et al. 1956).

More recently this iron-incorporating enzyme system (ferrochelatase, haem synthetase) has been purified from mitochondria of avian erythrocytes (Oyama et al. 1961) and mammalian liver (Porra and Jones 1963). A broad specificity toward 2-carboxyl porphyrins with corresponding haem formation was observed. There was practically no effect on 4- or 8-carboxyl porphyrins (Yoneyama et al. 1962). Addition of reducing substances - ascorbic acid, GSH and cysteine - has been shown to markedly potentiate iron-incorporating enzyme activity in rabbit bone marrow but not in liver preparations (Lockhead et al. 1963).

3.8 Shemin cycle - Succinate glycine cycle. The metabolic fate of ALA is not porphyrin synthesis alone. Shemin et al. (1955) discovered that the δ-carbon atom of this aminoketone could be utilised

as a 'one carbon fragment' in a number of situations - for the ureido group of purines, the β-carbon of serine, and the methyl group of methionine. It could also be converted to formic acid (Nemeth et al. 1957). This pattern was identical with that shown for the α-carbon of glycine. Since the δ-C of ALA is derived from the α-C of glycine, Shemin et al. (1955) proposed a 'succinate-glycine cycle' to explain these phenomena (Fig. 3.7). In terms of this cycle some of the ALA, formed by condensation of succinyl CoA and glycine, is transaminated to yield α-ketoglutaraldehyde; the latter is then decarboxylated, yielding the one-carbon fragment mentioned above and succinate which re-enters the pool.

Subsequently ALA transaminise activity was demonstrated in several different systems (Bagdasarian 1958, Neuberger and Turner 1963). Also, incubation of duck red cell haemolysates with ALA-5-^{14}C yielded ^{14}C-labelled α-ketoglutaric acid (Shemin et al. 1955); and injection of ALA-1,4-^{14}C into rats was followed by the appearance of succinic acid-1,4-^{14}C in the urine (Nemeth et al. 1957).

It seems, therefore, that this cycle does in fact operate but to what extent is another matter. Certain evidence indicates that in mammalian liver this pathway is probably not very active (Tschudy 1965) :

(a) ALA synthetase is the rate-controlling enzyme for liver haem biosynthesis (Section 4.2); if the succinate-glycine cycle were very active in liver it would be reasonable to expect the rate-controlling step to be beyond the stage of ALA synthesis; and

(b) the rate of conversion of ALA-4-^{14}C to $^{14}CO_2$ by normal rabbit liver slices is poor (Tschudy et al. 1962).

3.9 Intracellular distribution of enzymes. The intracellular distribution of enzymes involved in haem biosynthesis is illustrated in Fig. 3.1. The fact that the enzyme catalysing the initial step (ALA synthetase) and those controlling the final stages (from COPROgen to haem) are located in the mitochondria, whereas the intermediate reactions

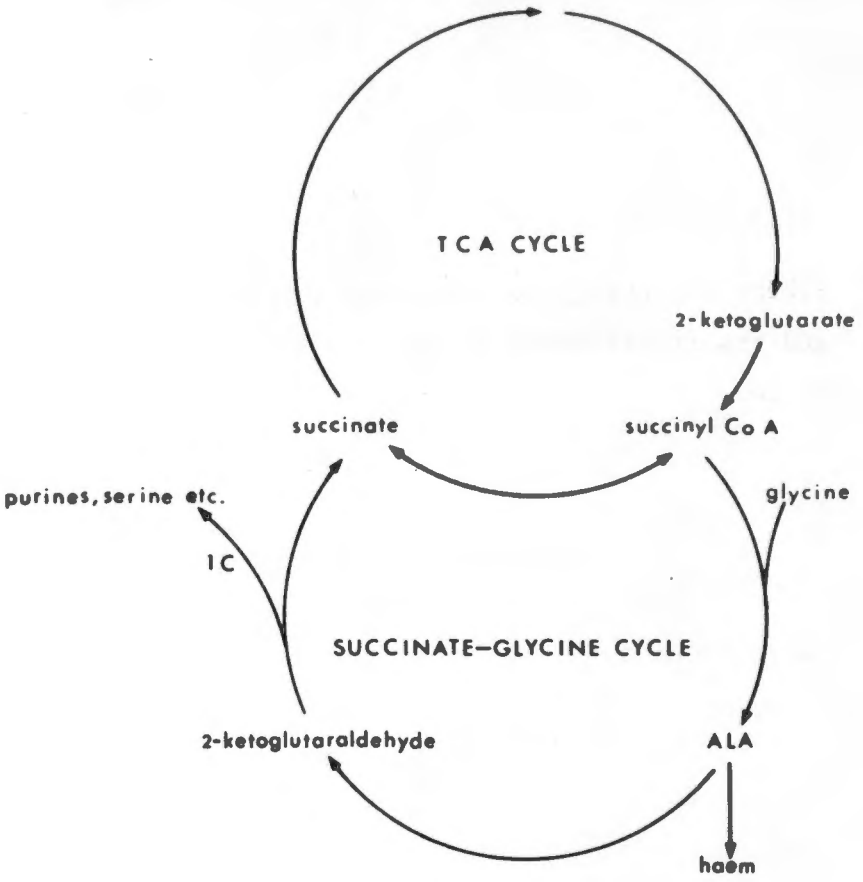
Fig. 3.7 Scheme depicting the succinate-glycine (Shemin) cycle and its relationship to the tricarboxylic acid (TCA) cycle.

Fig. 3.8 Diagram illustrating the possible sources of succinyl CoA for haem biosynthesis, viz.,

(1) oxidative (clockwise in fig.) TCA cycle activity,

(2) reversed (anti-clockwise in fig.) TCA cycle activity, and

(3) propionate via propionyl CoA and methylmalonyl CoA.


TCA CYCLE
2-ketoglutarate
succinate
succinyl Co A
purines, serine etc.
glycine
1C
SUCCINATE–GLYCINE CYCLE
2-ketoglutaraldehyde
ALA
haem

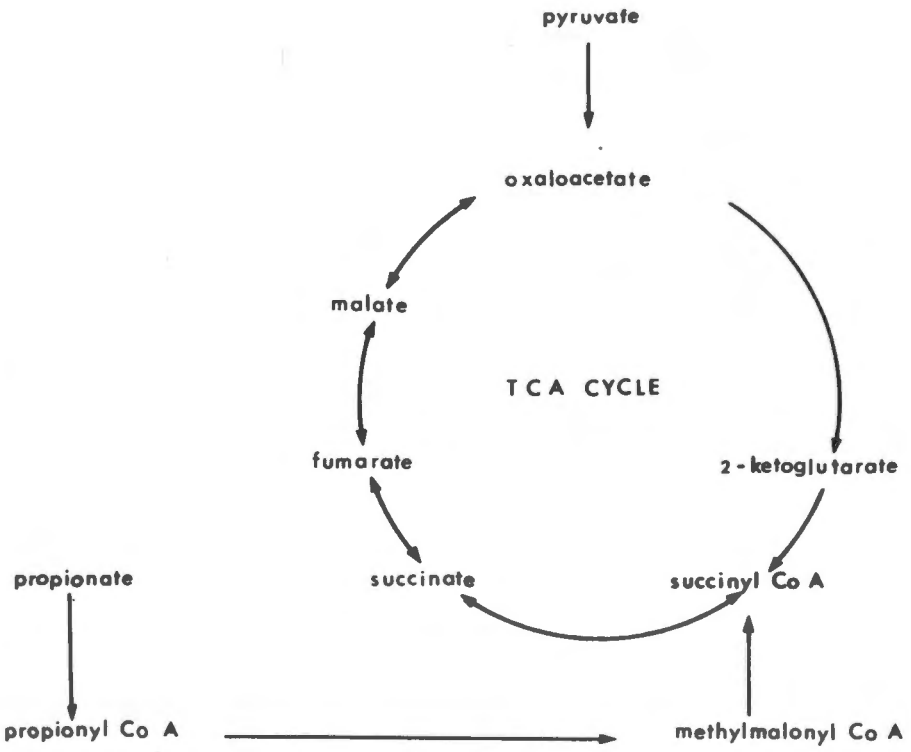

pyruvate
oxaloacetate
malate
TCA CYCLE
fumarate
2-ketoglutarate
propionate
succinate
succinyl Co A
propionyl Co A
methylmalonyl Co A

Table 3.1 Enzyme reactions producing succinyl CoA (adapted from Lascelles, 1964).

Enzyme system	Reaction catalyzed	Cofactors
1) α-Ketoglutarate oxidase	α-Ketoglutarate → succinyl CoA + CO_2 + $NADH_2$	Thiamine pyrophosphate; lipoic acid, NAD, CoA
2) Succinyl CoA synthetase	Succinate + nucleotide triphosphate + CoA ⇌ succinyl CoA + Nucleotide diphosphate + Pi	GTP (Animals); ATP (bacteria, plants)
3) Acetoacetyl CoA transferase	Succinate + acetoacetyl CoA ⇌ succinyl CoA + acetoacetate	
4) Methylmalonyl CoA isomerase	Methylmalonyl CoA ⇌ succinyl CoA	B_{12} coenzyme

are governed by cytoplasmic enzymes, may be of particular significance in the control of tetrapyrrole formation in the cell (Section 4.2.7).

3.10 Origin of the primary precursors : glycine and succinyl CoA.

3.10.1 Glycine. The source of most of this amino acid in animals is probably the diet. Glycine can also be formed from serine by the action of hydroxymethyltransferase (Lascelles 1964).

3.10.2 Succinyl CoA. The immediate sources from which succinyl CoA can be synthesised in the intact organism are shown in Table 3.1. The relative contributions of each of these sources is not known with any certainty but the major one in animal tissue is probably oxidative decarboxylation of α-ketoglutarate (Lascelles 1964). This compound is an intermediate of the tricarboxylic acid (TCA) cycle and hence it follows that succinyl CoA for haem biosynthesis may normally arise from oxidative TCA cycle activity (Figs. 3.8, 9.1).

In this connection it has been shown that the labelling patterns in protoporphyrin and haem following incubation of red cell haemolysates with α-ketoglutarate-5-^{14}C, α-ketoglutarate-1,2-^{14}C, citrate-1,5-^{14}C, acetate-1-^{14}C and acetate-2-^{14}C were in complete agreement with the patterns predicted on the basis of succinyl CoA synthesis via oxidative TCA cycle activity (Shemin and Wittenberg 1951, Wriston et al. 1955).

Succinate was established as an immediate precursor of succinyl CoA (succinyl CoA synthetase reaction, Table 3.1) by Shemin and Kumin (1952), using carboxyl-labelled succinate-^{14}C which cannot give rise to ^{14}C-labelled succinyl CoA via an oxidative turn of the TCA cycle. This raises the question of what source(s) would normally supply succinate for direct conversion to succinyl CoA and thence to haem. Labbe and co-workers (Section 4.2.2) have produced evidence that conversion of pyruvate to succinyl CoA via a reductive dicarboxylic acid pathway (Figs. 3.1, 9.1) involving malate, fumarate and succinate occurs both in normal and porphyric mammalian liver. These aspects are considered in more detail in Section 4.2.2 and Chapter 9.

Transfer of CoA from acetoacetyl CoA to succinate (Reaction 3, Table 3.1) does not occur in liver since the relevant enzyme is not found in this organ (Harper 1967).

The fourth possible immediate source of succinyl CoA is methylmalonyl CoA (Table 3.1, Figs. 3.8, 9.1) which undergoes isomerisation under the influence of methylmalonyl CoA isomerase. This reaction provides a mechanism whereby propionate can be converted to TCA cycle intermediates and thereby act as a carbon source for gluconeogenesis. In ruminants, where propionate is a major source of glucose, this is obviously an important pathway. However, there is at present no information on the relative importance of methylmalonyl CoA (and therefore of propionate) as a source of succinyl CoA for haem synthesis in animals and man.

CHAPTER 4

CONTROL OF HAEM BIOSYNTHESIS.

4.1 Introduction. Knowledge of the normal biosynthetic pathway for haem (Fig. 3.1) is essential to an understanding of the disorders of porphyrin metabolism. However, the ultimate elucidation of the biochemical lesions in the porphyrias depends rather on an appreciation of the control mechanisms governing the rate of haem biosynthesis. The study of these control mechanisms is inseparable from the study of the actions of porphyrinogenic substances. Hence this chapter is as much concerned with mechanisms of porphyrinogenesis as with normal control of haem biosynthesis.

4.2 Control mechanisms. Normally in the intact organism haem formation is well regulated. Only minute amounts of haem precursors accumulate in the tissues or find their way into the excreta. The most feasible site for metabolic control in the haem biosynthetic pathway (Fig. 3.1) which involves mainly irreversible enzyme-catalyzed reactions is a branch point, whence an intermediate may follow two or more paths. Such a point would be the formation and disposal of ALA and in fact a number of authors have demonstrated that control is exerted at the stage of ALA synthesis. ALA synthetase has been shown to be the rate-limiting enzyme for haem biosynthesis in a number of systems including the bacterium, *Rhodopseudomonas spheroides* (Lascelles 1960, Burnham and Lascelles 1963), guinea pig liver cells (Granick 1963, Granick and Urata 1963), chick embryo liver cells (Granick 1966), rabbit reticulocytes (London et al. 1964), and chick erythroid precursor cells (Levere and Granick 1965, 1967).

4.2.1 End product inhibition and repression of ALA synthetase. Based mainly on bacterial studies, Jacob, Monod and Wollman (cit. in Lwoff 1962) proposed two general mechanisms for control of rate-limiting enzymes in a biosynthetic sequence, viz., end product inhibition and end product repression. In the former case the end product allosterically inhibits the activity of the rate-limiting enzyme, while

in the latter, inhibition of enzyme synthesis is the mode of control.

The proposed repressor mechanism is shown in Fig. 4.1. Transcription of the genetic code contained in the DNA of the structural gene into messenger RNA and thence into a polypeptide chain is under the influence of the adjacent operator gene. When this operator gene is blocked no messenger RNA and consequently no protein can be formed. The repressor is depicted as a protein synthesised under the direction of a regulator gene and capable of blocking the action of the operator gene. In the case of end product repression the effective repressor is the combination of end product (co-repressor), usually a small molecule, and the repressor protein. Similarly, in the case of induction, the inducing molecule combines with the repressor protein, thereby rendering it inactive and allowing derepression to ensue.

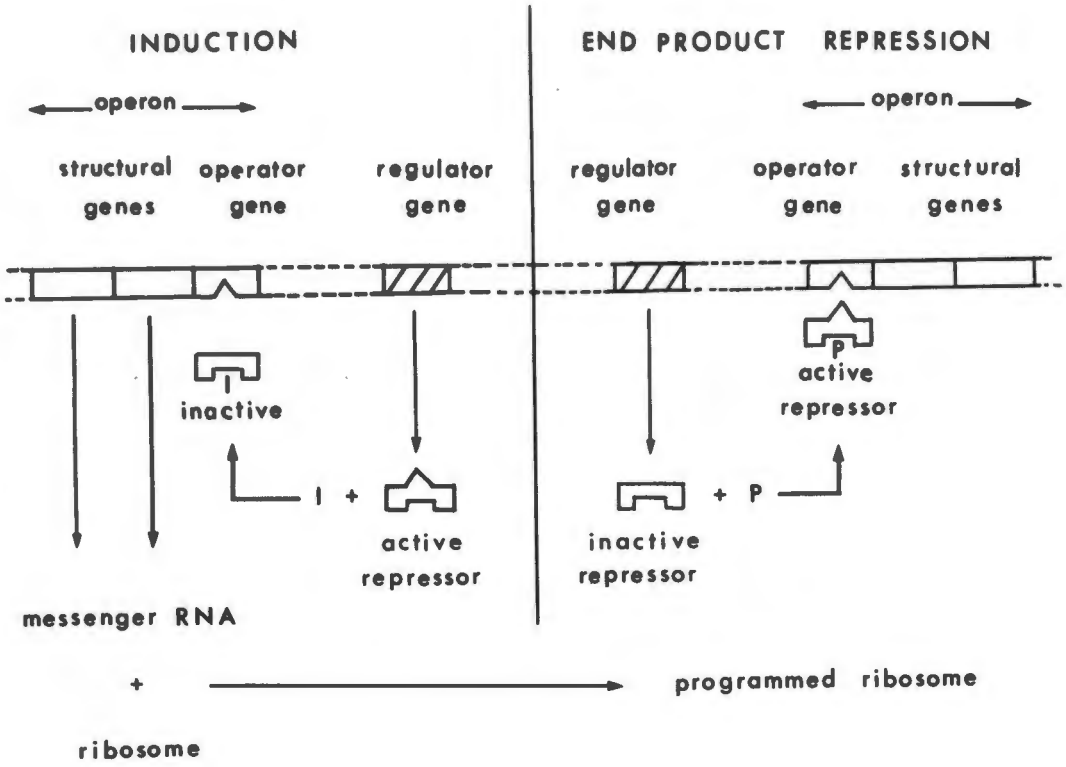
In the control of tetrapyrrole biosynthesis in the photosynthetic bacterium, *Rh. spheroides*, Burnham and Lascelles (Lascelles 1960, Burnham and Lascelles 1963) found that both of the above-mentioned mechanisms operated. Haem allosterically inhibited ALA synthetase activity and repressed formation of ALA synthetase.

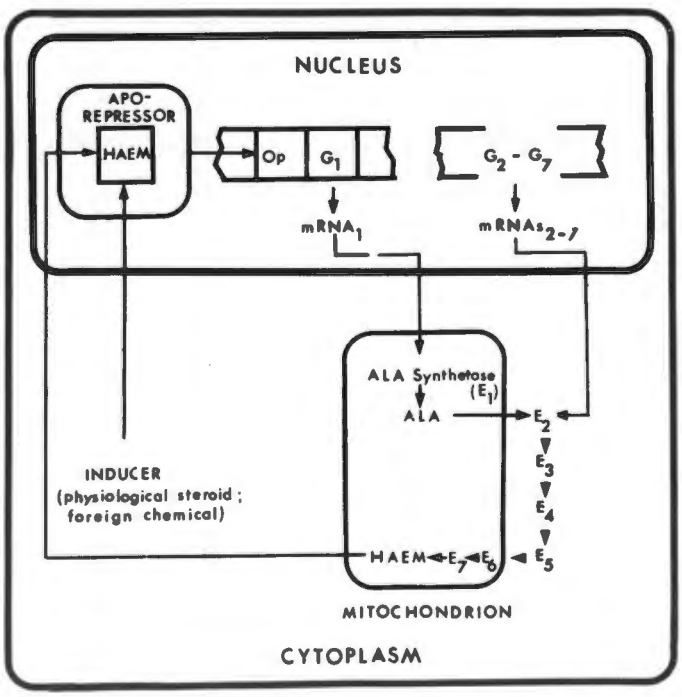
End product inhibition of ALA synthetase in rabbit reticulocytes was demonstrated by London et al. (1964). In the early differentiating erythroblast of the chick, Levere and Granick (1967) found that haem formation was limited only by a lack of ALA synthetase which they proposed was under the control of a repressor mechanism.

Granick and Urata (1963) demonstrated that the porphyrinogenic action of 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) lay in its effect on hepatic ALA synthetase. In guinea pigs, acutely poisoned with DDC, liver ALA synthetase activity was increased more than 40-fold. This increase was found to be due to induction of enzyme synthesis rather than to activation of preformed protein (Granick 1963). Similar results were obtained with DDC and other drugs (Section 5.2) in experiments on chick embryo liver. Haem was noted to inhibit the induction process but no end product inhibition of ALA synthetase was

Fig. 4.1 Schematic representation of end product repression and induction (derepression). I is the small molecule inducer and P the small molecule end product. The systems are depicted as they would function in the presence of I and P. Opposite effects would occur in their absence. The affinity of repressors for operator genes is indicated by their complementary shapes (adapted from Hoagland 1966).

Fig. 4.2 Diagram illustrating a proposed mode of induction of liver ALA synthetase by inducing substances which compete with haem for a site on the aporepressor molecule. (Op. = operator gene, G = structural gene, E_{1-7} = enzymes of haem biosynthetic pathway) (adapted from Granick 1966, Kappas and Granick 1968).


INDUCTION
END PRODUCT REPRESSION
operon
operon
structural genes
operator gene
regulator gene
regulator gene
operator gene
structural genes
inactive
I
I +
active repressor
P
active repressor
+ P
inactive repressor
messenger RNA
+
ribosome
programmed ribosome


NUCLEUS
APO-REPRESSOR
HAEM
Op
G_1
G_2 - G_7
$mRNA_1$
$mRNAs_{2-7}$
ALA Synthetase (E_1)
ALA
E_2
E_3
E_4
E_5
E_6
E_7
HAEM
INDUCER
(physiological steroid ; foreign chemical)
MITOCHONDRION
CYTOPLASM

found (Granick 1966).

These findings led Granick (1966) to postulate that the repressor mechanism for ALA synthetase consists of a protein aporepressor and a corepressor which is haem. Inducer substances compete with haem for an active site on the aporepressor molecule. The combination of aporepressor and inducer fails to repress the operator gene for ALA synthetase. More of this enzyme is therefore formed, leading to accelerated porphyrin biosynthesis (Fig. 4.2).

Among the chemicals and drugs initially shown to be capable of inducing excessive porphyrin synthesis in chick embryo liver cells (Section 5.2) were a number of steroid hormones (Granick 1966). In further studies (Granick and Kappas 1967, Kappas and Granick 1968) the potent inducers among them were found to be of the 5β-H type including aetiocholanolone, pregnanediol, 11-ketopregnanolone and pregnanetriol. The inducing potential of the free steroid was found to equal or exceed that of the most active porphyrinogenic drugs or chemicals originally tested. On the other hand, the glucuronide derivatives of even potent inducers were devoid of activity (Granick and Kappas 1967). The pattern of induction by these natural steroids as regards time of onset, course of development and prevention by inhibitors of nucleic acid and protein synthesis was found to be very similar to that of foreign drugs and chemicals (Kappas and Granick 1968).

This was the first demonstration of porphyrinogenesis in liver cells by a group of physiological compounds. Accordingly, it seemed logical to propose that these steroids play a role in the normal control of haem biosynthesis (Granick and Kappas 1967). In terms of this hypothesis free steroid, in the physiological situation, competes with haem for a site on the aporepressor protein and thereby causes derepression (Fig. 4.2); at any given time the concentration of free steroid in the cell is governed, among other factors, by the efficiency of glucuronide formation.

Granick's hypothesis is an attractive one, particularly as regards its applications to hepatic porphyria (Sections 5.3, 5.4). However, it

must be remembered that it is essentially based on evidence obtained from non-mammalian sources. Until more direct evidence on control mechanisms in mammalian liver is available, it cannot be assumed that this theory is equally applicable to humans.

4.2.2 Terminal oxidation and porphyrin synthesis. Labbe (1967) postulated that the rate of haem biosynthesis in the liver cell is controlled by the redox state of the mitochondria and that the fundamental lesion in porphyria, both experimental and human, is defective terminal oxidation with a resultant increase in the intramitochondrial $NADH_2/NAD$ ratio.

This theory is based partly on the observation that many of the chemicals and drugs which are capable of inducing experimental porphyria in animals, or exacerbating human porphyria, are inhibitors of $NADH_2$ oxidase in its purified form or in submitochondrial particles and intact mitochondria (Labbe 1962, Cowger and Labbe 1965), and partly on experiments in animals treated with two of these porphyrinogenic compounds, viz., allyl*iso*propylacetamide (AIA) and DDC (Section 5.2.1). Labbe and co-workers found that in these animals certain metabolic effects preceded the induction of ALA synthetase by these compounds. Increased succinyl CoA formation was shown to be due to an inducible form of mitochondrial succinyl CoA synthetase (Labbe et al. 1965). Further, evidence was obtained which suggested that succinate, the substrate for this enzyme, was generated by $NADH_2$-dependent fumarate reductase activity (Kurumada and Labbe 1966).

In terms of the hypothesis, factors which lead to an increase in the intramitochondrial $NADH_2/NAD$ ratio stimulate fumarate reductase activity, leading to increased synthesis of succinate which in turn induces non-constitutive succinyl CoA synthetase. This succinyl CoA, generated in a separate intramitochondrial 'compartment' from constitutive succinyl CoA of the TCA cycle, then acts as a derepressor of ALA synthetase synthesis by substrate induction.

According to Labbe's views (Labbe 1967), the carbon source of

succinyl CoA for accelerated liver haem biosynthesis is pyruvate and the pathway concerned is a reductive one. Initially, pyruvate is carboxylated to oxaloacetate or malate. These dicarboxylic acids are then converted to succinyl CoA via a reductive pathway (reversed TCA cycle activity) involving malate, fumarate and succinate. This concept is supported by the demonstration that those ^{14}C-labelled precursors, e.g. succinate-1-^{14}C, fumarate-1-^{14}C and malate-1-^{14}C, which can label succinyl CoA (and therefore haem) only by reverse reactions of the TCA cycle gave rise to greater radioactivity of haem on incubation with porphyric than with normal liver homogenate. With citrate-6-^{14}C, however, no difference was evident between normal and porphyric liver homogenate in this respect (Haining and Labbe 1967). Pyruvate was also "demonstrated by enzyme assay to stimulate δ-aminolaevulinate formation in porphyric liver preparations" (Labbe 1967). On the other hand, oxidative TCA cycle activity was found to be unaffected in experimental porphyria (Labbe et al. 1965).

Labbe's proposals are both interesting and novel, interrelating as they do seemingly coincidental metabolic changes. However, more direct evidence that inhibition of mitochondrial terminal oxidation and porphyrinogenesis are linked as cause and effect is still lacking. It seems reasonable to expect that the more powerful porphyrinogenic agents would be the most potent inhibitors of mitochondrial terminal oxidation, yet this does not appear to be the case. AIA, an extremely potent inducer of ALA synthetase and porphyrin synthesis (Granick 1966) is a relatively poor inhibitor of terminal oxidation compared with Amytal (5-ethyl-5-*iso*amylbarbituric acid) (Mustafa, Labbe et al. 1968) which is a less efficient porphyrinogenic agent (Granick 1966). Also, as in the case of Granick's hypothesis, there is virtually no information on the applicability of the proposal to human porphyria.

4.2.3 Drug detoxification. Porphyrinogenic drugs such as barbiturates stimulate the synthesis of hepatic detoxifying enzymes (Williams 1959). These include cytochromes and haem-requiring mixed function

oxidases. Granick (1966) postulated that increased requirements for haem in an effort to detoxify these drugs may be the stimulus for porphyrinogenesis.

An argument against this hypothesis is the finding that induction of these detoxifying enzymes is not quantitatively related to porphyrinogenesis (Marver 1966).

4.2.4 Relative availability of acetyl CoA and succinyl CoA. As indicated in Chapter 3, glycine is able to condense with several acyl CoA compounds to form aminoketones. Normally aminoacetone is formed in the liver in greater amounts than ALA and on this basis De Matteis and Rimington (1962) proposed that the relative availability of acetyl CoA and succinyl CoA for condensation with glycine determines the relative amounts of these two aminoketones formed. Decreased availability of acetyl CoA would divert glycine for increased synthesis of ALA and consequently also of PBG and porphyrins.

These authors suggested that the basic lesion in the hereditary porphyrias may be a genetic abnormality of the acetylating system resulting in deficient production of acetyl CoA. This deficiency would be aggravated by drugs such as barbiturates which are known to interfere with acetylation reactions (Johnson and Quastel 1953).

Experimental and clinical evidence, however, does not support the hypothesis. In DDC-induced porphyria Granick and Urata (1963) found that increased ALA synthetase activity was accompanied by slightly enhanced rather than diminished aminoacetone synthesis; and studies in porphyric patients excreting excessive amounts of ALA have revealed normal urinary aminoacetone levels (Tschudy et al. 1963, Druyan and Haeger-Aronsen 1964).

4.2.5 Alternative pathways of ALA metabolism. The succinate-glycine cycle of Shemin (Section 3.8) provides an alternative pathway to the haem biosynthetic sequence for the metabolism of ALA; and possibly there are yet other routes for the disposal of this aminoketone. It is conceivable that variations in the demands of these

alternative pathways, e.g. purine metabolism, might exert a controlling effect on porphyrin and haem biosynthesis through regulation of the quantities of available ALA.

Although there is some evidence of defective purine synthesis in experimental porphyria (Labbe et al. 1954, Talman et al. 1959), clinical studies do not bear this out. No defect in incorporation of ^{14}C into respiratory CO_2, haemoglobin haem and urinary uric acid could be shown in patients with acute intermittent porphyria, porphyria variegata and symptomatic porphyria following intravenous administration of glycine-2-^{14}C (Dowdle 1963) or an oral dose of ALA-5-^{14}C (Dowdle et al. 1968).

4.2.6 The redox state of the cell cytoplasm. As discussed in Chapter 3, the porphyrinogens and not the porphyrins are the true intermediates in haem biosynthesis. Porphyrinogens are highly unstable compounds and readily undergo autoxidation to porphyrins. Reducing conditions are consequently necessary to prevent this irreversible oxidation. It has been suggested (Heikel et al. 1958) that in cutaneous porphyria the excessive porphyrins in the liver may originate from porphyrinogens which have 'escaped' from the haem biosynthetic pathway. Goldberg and Rimington (1962) have further proposed that in the diseased liver the cytoplasmic redox state, as reflected by NAD/$NADH_2$ and NADP/$NADPH_2$ ratios, may be sufficiently altered to promote excessive oxidation of porphyrinogens.

This situation is of course quite different from that envisaged by Labbe (Section 4.2.2) who holds that porphyrin synthesis is promoted by conditions (within the mitochondria) favouring reduction.

It is also difficult to reconcile the concept of excessive, irreversible oxidation of porphyrinogens with the fact that alcohol which lowers hepatic NAD/$NADH_2$ ratios, or conversely raises hepatic $NADH_2$/NAD ratios (Smith and Newman 1959, Isselbacher and Krane 1961), and thus favours reduction, is regarded as an aetiological agent in cutaneous (symptomatic) porphyria.

4.2.7 Intracellular compartmentation. A relative or absolute block in the haem biosynthetic pathway at a point distal to ALA formation could lead to induction of ALA synthetase through lack of feedback or end product repression by haem.

On the basis of the compartmentation of enzymes involved (Fig. 3.1), Sano and Granick (1961) suggested that porphyrin biosynthesis might be partly controlled by the permeability of the mitochondrial membrane to ALA. A similar potential control point could be the entry of COPROgen into the mitochondria or alternatively the conversion of this haem precursor to PROTOgen by coproporphyrinogen oxidase, a membrane-bound mitochondrial enzyme. Zail and Joubert (1968) suggested that the basic lesion in symptomatic porphyria might be in this area.

Evidence against this concept is the finding that hepatic haem synthesis in porphyria, including symptomatic porphyria, appeared normal as judged by the 'early-labelled' bilirubin fraction following oral ALA-5-^{14}C (Dowdle et al. 1968).

4.2.8 ATP and porphyrin biosynthesis. Following on the finding that deranged purine metabolism might be a significant factor in experimental porphyria (Talman et al. 1955), Gajdos and Gajdos-Török (1961) postulated that a deficiency of phosphorylated derivatives of adenosine in porphyria might be responsible for the clinical signs. These authors claim to have obtained therapeutic benefit in acute porphyria by administration of adenosine-5-monophosphate, and decreased porphyrin formation in experimental porphyria by administration of this nucleotide (Gajdos and Gajdos-Török 1961).

Subsequently, ATP was found to be a much more effective inhibitor of porphyrin formation "probably by the enhancement of the formation of a physiological inhibitor" (Gajdos 1966). It was proposed that decreased hepatic ATP might be the stimulus for porphyrinogenesis and in fact administration to rats of 6-mercaptopurine, orotic acid and ethionine which decreases biosynthesis of ATP was found to be associated with increased liver porphyrins.

This does not prove, however, that decreased hepatic ATP levels and increased porphyrin synthesis are directly linked as cause and effect. Labbe (1967) pointed out the intimate relationship between electron transport and oxidative phosphorylation and suggested that decreased synthesis of ATP in the liver may result from inhibition of $NADH_2$ oxidation, and that, conversely, chronic ATP deficiency could produce in effect an inhibition of electron flow, simulating inhibition of $NADH_2$ oxidation. Furthermore, it has been demonstrated that experimental porphyria can be induced without an accompanying decrease in the concentration of hepatic ATP and that certain compounds can cause a reduction in hepatic ATP levels without inducing porphyria (De Matteis et al. 1963).

4.2.9 The 'glucose effect'. Tschudy and co-workers demonstrated that diet has a profound effect on porphyrin biosynthesis and excretion, both in experimental and human porphyria. Administration of carbohydrate or protein (but not fat) was found in rats to inhibit the induction of hepatic ALA synthetase by ALA (Tschudy et al. 1964) and to effect symptomatic and biochemical improvement in patients with AIP (Welland et al. 1964) and variegate porphyria (Perlroth et al. 1968). Conversely, starvation has been shown to facilitate the induction of experimental porphyria (Tschudy et al. 1964) and has been reported as the precipitating factor in a case of acute porphyria (Knudsen et al. 1967).

Marver, Collins, Tschudy and Rechcigl (1966) have suggested that these phenomena, often referred to as the 'glucose effect', "are best explained by the hypothesis that glucose inhibits the genetically mediated induction of δ-aminolaevulinic acid synthetase", possibly by interference with the induced synthesis of certain RNA's.

In this connection, Hickman et al. (1968) who previously showed that RNA isolated from porphyric rat liver could stimulate porphyrin synthesis in chick embryo liver cells (Hickman, Saunders, Dowdle and Eales 1967), have published evidence that the 'glucose effect' is in fact mediated via RNA.

CHAPTER 5

THE BIOCHEMICAL LESIONS IN THE PORPHYRIAS WITH PARTICULAR REFERENCE TO SYMPTOMATIC PORPHYRIA.

5.1 Introduction. Haem biosynthesis in the intact organism, as discussed in Section 4.2, is finely regulated with minimal 'wastage' of porphyrin precursors and porphyrins in the excreta (Lascelles 1964). Under pathological conditions, on the other hand, failure of these highly organised control mechanisms leads to accumulation in the tissues and excretion of relatively large amounts of haem precursors. In man and other animals these diseases, the porphyrias, have been extensively studied but there are still today large gaps in our knowledge of the basic biochemical lesions.

5.2 Experimental porphyria.

5.2.1 Historical background. Following his report on the association of porphyrinuria with the taking of the hypnotic drug, Sulphonal (Section 1.1), Stokvis (1895) was the first to induce an experimental porphyria by administering this drug to rabbits and dogs.

Since that time the list of drugs and chemicals which have been shown to have porphyrinogenic properties has expanded tremendously. Most of the interest in this subject has developed since the demonstration by Schmid and Schwartz (1952) of an hepatic form of porphyria in rabbits by administration of the hypnotic, Sedormid (allyl*iso*propylacetylcarbamide). This prompted Goldberg and Rimington (Goldberg 1953, Goldberg and Rimington 1955) to investigate the activity of a related but non-hypnotic compound, allyl*iso*propylacetamide (AIA) which they found produced the same biochemical picture in rabbits, rats and fowls. Without affecting haem synthesis in the bone marrow in these animals, Sedormid, AIA and related drugs rapidly lead to hepatic accumulation of PBG, URO, COPRO and PROTO. Together with ALA these haem precursors appear in excessive quantities in the urine, while COPRO and PROTO are predominantly excreted in the bile and faeces (Schmid 1963). Study of these experimental porphyrias and of the toxic porphyrias in general has contributed much in recent years to present day knowledge and

theory regarding control of haem biosynthesis and the pathogenesis of the human disorders of porphyrin metabolism.

Four general classes of porphyrinogenic compounds are now recognised (Granick 1966), viz.,

a. the barbiturate group (including Sedormid, AIA and barbiturates),

b. the collidines (e.g. DDC),

c. the sex steroids (e.g. progesterone, testosterone) and

d. a miscellaneous group (e.g. hexachlorobenzene).

These various groups are obviously not closely related chemically and hence it is difficult to correlate structure and porphyrinogenic properties. Various authors have attempted to identify active groups within a particular class of compounds (Goldberg and Rimington 1962, Granick 1966); recently Granick (1968) has suggested a basic configuration for induction possessed by nearly all inducing compounds. This consists of "1 : a carbonyl group, free or as a sterically hindered ester or amide and, 2 : a relatively hydrophobic aliphatic, or cyclic non-planar 6-17 carbon ring" (Granick 1968).

5.2.2 Induction of ALA synthetase. Initially it was not clear whether the excessive haem precursor excretion in the experimental porphyrias was due to 'over-production' or to 'under-utilisation' as a result of a metabolic block. Evidence in favour of the latter concept was the finding that different excretion patterns were produced by different porphyrinogenic compounds. On the other hand over-production seemed more likely from the quantitative viewpoint (Goldberg and Rimington 1955).

This problem was resolved by the demonstration (Granick and Urata 1963, Granick 1963, 1966) of the induction of ALA synthetase synthesis by DDC and a wide variety of other compounds belonging to the 4 groups discussed in Section 5.2.1. Over-production of haem precursors was thus established as a common feature of the experimental porphyrias.

Among the important questions still unanswered are :

(i) how is the induction process mediated;

(ii) what determines the different excretion patterns due to different porphyrinogenic agents; and,

(iii) is there any aetiological relationship between porphyrinogenesis and various metabolic disturbances which accompany the administration of inducers?

The current theories on the mechanism(s) of induction have been discussed in Chapter 4 since, as was indicated, ideas regarding the physiological situation are largely based on work in experimental porphyria. Regarding the different haem precursor excretion patterns produced by different substances which are known to induce ALA synthetase, there is at present no substantial evidence of the existence of additional biochemical lesions or metabolic blocks which might explain these findings.

5.2.3 Associated metabolic abnormalities. Besides causing induction of ALA synthetase synthesis, porphyrinogenic compounds are known to cause a variety of associated metabolic disturbances, e.g. inhibition of mitochondrial terminal oxidation and decreased hepatic levels of ATP. These two phenomena and their possible contributions to the induction process have been dealt with in Sections 4.2.2 and 4.2.8 respectively. Schmid et al. (1955) showed that a rapid fall in liver catalase activity accompanied the induction of experimental porphyria by Sedormid in rats and rabbits and suggested that a block in the synthesis of this haem-containing enzyme was responsible, at least in part, for the raised levels of hepatic porphyrins. However, a regulatory role for catalase seems most unlikely since the demonstration that 3-amino-1,2,4-triazole lowered liver catalase in rats without influencing porphyrin output (Goldberg and Rimington 1962) and that hexachlorobenzene induced an experimental porphyria without significant alteration in hepatic catalase (Ockner and Schmid 1961). A finding, apparently completely unrelated to porphyrinogenesis, is that of increased excretion of ascorbic acid in the urine of DDC-treated rats (Ginsburg and Dowdle 1963, De Matteis 1964).

5.3 Genetic hepatic porphyria - Acute intermittent porphyria (AIP) and variegate porphyria (VP).

5.3.1 Increased ALA synthetase activity. It was not long after the demonstration by Granick and Urata (1963) of the induction of ALA synthetase in experimental porphyria that evidence for over-production of haem precursors in the genetic varieties of the hepatic porphyrias was forthcoming. Tschudy et al. (1965) found raised ALA synthetase activity in a patient with acute intermittent porphyria. This was confirmed by Nakao et al. (1966) and by Dowdle et al. (1967). The latter authors also reported similar results in variegate porphyria. In addition, studies with labelled ALA have indicated that the endogenous pool of this aminoketone is greatly expanded in these two conditions (Scott 1955, Scott and Gray 1962, Dowdle et al. 1968).

The mechanism of induction of ALA synthetase in AIP and VP remains obscure. A genetic mutation involving the repressor gene for ALA synthetase, i.e. a regulator constitutive mutation, would seem to be the logical assumption but such lesions, as pointed out by Tschudy (1965), are recessive (Monod et al. 1962), whereas AIP and VP are both dominantly inherited conditions (Waldenström 1957, Dean 1953). While an operator constitutive mutation would accord more with the genetic and biochemical picture, neither of these mutations could account for the other manifestations, e.g. abdominal and neurological symptoms. On the other hand a primary genetic defect, not directly concerned with haem biosynthesis, could conceivably lead to secondary induction of ALA synthetase and simultaneously give rise to the other manifestations of these diseases which cannot be explained on the basis of over-production of ALA (Tschudy 1965).

At present there is no concrete evidence pointing to secondary induction of liver ALA synthetase. In AIP, a partial block in the haem biosynthetic pathway beyond PBG formation might lead to derepression of ALA synthetase synthesis through decreased haem formation. In VP, on the other hand, there can obviously be no block up to the

stage of protoporphyrin formation. Dowdle et al. (1968) have recently demonstrated that hepatic haem synthesis, evaluated by 'early-labelling' of bilirubin after administration of ALA-5-^{14}C, is in fact normal in all three types of human porphyria commonly encountered in South Africa.

The evidence for a possible physiological role of sex steroids in the control of liver haem biosynthesis (Section 4.2.1) (Granick and Kappas 1967, Kappas and Granick 1968) is of particular interest in regard to the natural histories of AIP and VP and the effects of administered steroids in these conditions. Firstly, despite transmission as an autosomal dominant trait, AIP exhibits a high female preponderance (Goldberg and Rimington 1962). Secondly, AIP and VP are only rarely manifest before puberty (Goldberg and Rimington 1962, Eales 1961), and thirdly, exacerbation of both these conditions has been noted in relation to the menstrual cycle (Waldenström 1937, Lamont 1963) and to administration of sex hormones (Rimington and De Matteis 1965, Dean 1965). Over-production of haem precursors due to sex steroids has been confirmed by Levere (1966) who found markedly increased urinary URO excretion associated with elevated liver ALA synthetase activity following stilboestrol administration in a patient with cutaneous porphyria.

As indicated in Section 4.2.1, porphyrinogenesis is a property of certain free steroids, whereas the corresponding conjugated compounds are inactive. Hence the efficiency of steroid conjugation might be an important factor determining the rate of liver haem biosynthesis. In this connection it is noteworthy that glucose restriction (Perlroth et al. 1968) and total starvation (Knudsen et al. 1967), both of which could lead to decreased availability of glucuronic acid for glucuronide formation, have been reported to precipitate acute porphyria. Other possible factors which could influence the overall efficiency of steroid conjugation include enhanced intracellular hydrolysis of glucuronides and the presence of endogenous inhibitors of glucuronyl-

transferase (Kappas and Granick 1968). In respect of the latter possibility certain inducing steroids, e.g. pregnanediol, may inhibit this enzyme (Hsia et al. 1960, Arias et al. 1964) thus enhancing their porphyrinogenic effect. Kappas and Granick (1968) suggest that "patients carrying the genetic lesion (i.e., mutant operator) for hepatic porphyria would be especially susceptible to the inducing action of endogenous steroids .....".

5.3.2 Associated metabolic disturbances. A number of other biochemical abnormalities not directly associated with haem biosynthesis, such as defective alternate pathways of ALA metabolism (Section 4.2.5) and a defective acetylation system (Section 4.2.4) have been suggested as the stimuli for porphyrinogenesis in the porphyrias. There is, however, no good evidence to support any of these hypotheses.

5.4 Symptomatic porphyria.

5.4.1 Increased ALA synthetase activity. There is good evidence that symptomatic porphyria, like the genetic varieties of hepatic porphyria, is also an 'over-production disease', in that increased activity of liver ALA synthetase and indications of an enlarged endogenous ALA pool (Dowdle et al. 1968) have been found in this condition. However, there are problems. Although Dowdle et al. (1967) reported increased activity of this enzyme in all of three patients studied, by contrast, Zail and Joubert (1968) found raised values in only 5 of 12 cases compared with non-porphyric subjects with liver disease. Moreover, in the latter study no correlation was found between porphyrin excretion and ALA synthetase activity. These apparent anomalies might be explained, at least in part, by a phasic rise and fall in hepatic ALA synthetase activity and could be related to the well-known tendency to periodic clinical exacerbation and remission in this disease (Eales 1961).

The stimulus for elevation of liver ALA synthetase activity in symptomatic porphyria is unknown. No evidence of inheritance has ever

been demonstrated and the disease is commonly regarded as being related to abuse of alcoholic beverages (Section 2.2.4). Furthermore, alcohol has long been known to influence porphyrin excretion (Section 5.4.3.1). Hence it is logical to suggest that bouts of excessive drinking might be responsible in some way for increasing liver ALA synthetase activity and thus porphyrin production, while, conversely, periods of abstinence from alcohol would lead to clinical and biochemical remission.

Induction of porphyrinogenesis by unconjugated steroids (Sections 4.2.1, 5.3.1) is a distinct possibility in symptomatic porphyria since all these patients have some degree of hepatocellular pathology (Section 2.2.3.1) which could interfere with the conjugation process. It may be argued, on the other hand, that if this were the case all patients with symptomatic porphyria should exhibit hyperbilirubinaemia, whereas in fact only a minority do (Lamont et al. 1961, Eales 1963). The answer to this problem would seem to lie in the demonstration that glucuronyltransferase is probably not "one non-specific enzyme, but a family of enzymes each with its own substrate specificity" (Tomlinson and Yaffe 1966). Recently Zumoff et al. (1966, 1967) have shown that conjugation of cortisol metabolites is defective in patients with cirrhosis.

5.4.2 A metabolic defect determining disposal of ALA. In a study of ALA-5-^{14}C metabolism Dowdle et al. (1968) showed in a patient with symptomatic porphyria that, although mechanisms exist for conversion of ALA to protoporphyrin, a greater proportion of administered ALA is disposed of as URO than is the case in normal subjects and patients with AIP and VP. It therefore becomes necessary to postulate a biochemical defect, which may in fact be the basic metabolic lesion in symptomatic porphyria, whereby an abnormal proportion of the ALA pool is channelled into URO in this condition. By increasing ALA synthetase activity alcohol might be expected to aggravate the effects of such a lesion.

Sano and Granick (1961) previously pointed out that intracellular compartmentation (Section 4.2.7) may play a part in control of haem biosynthesis and on this basis Zail and Joubert (1968) suggested that a relative block in haem biosynthesis at the coproporphyrinogen oxidase stage (Fig. 3.1) with consequent derepression of ALA synthetase might explain the porphyrin excretion pattern in symptomatic porphyria. Evidence against this concept is the finding by Dowdle et al. (1968) of normal 'early-labelling' of bilirubin in a patient with symptomatic porphyria, indicating that liver haem biosynthesis was unimpaired.

Excessive intracellular oxidation of porphyrinogens to porphyrins has been suggested as the source of the excessive hepatic and excreted porphyrins in symptomatic porphyria (Heikel et al. 1958) (Section 4.2.6). Although such a mechanism could explain the porphyrin excretion pattern in this disease, there is no evidence to suggest that the intracellular milieu favours oxidation and moreover alcohol which aggravates the disease is known to lower hepatic NAD/$NADH_2$ ratios and hence favours reduction (Smith and Newman 1959, Isselbacher and Krane 1961) (Section 4.2.6).

5.4.3 The metabolic effects of alcohol in relation to symptomatic porphyria.

5.4.3.1 Effect of alcohol on porphyrin production and excretion. As long ago as 1935 (Franke and Finkentscher 1935) it was demonstrated that the administration of ethanol to man increased the urinary excretion of coproporphyrin. This observation has been confirmed both in healthy subjects and in chronic alcoholics (Brugsch 1937, Sutherland and Watson 1951, Zieve et al. 1953, Orten et al. 1963). It was thought that alcohol caused liver damage, resulting in an impaired ability on the part of this organ to excrete coproporphyrinogen which consequently 'overflowed' into the urine.

There is strong circumstantial evidence that both in South Africa and in other countries the development of symptomatic porphyria is

related to the chronic abuse of alcoholic beverages (Section 2.2.1.2). Many of these are illicit brews and it has been suggested that adulterants or contaminants which they contain may be important in the aetiology of the disease (Gelfand and Mitchell 1957, Keen et al. 1966). However, the wide range of different alcoholic beverages consumed in different places where symptomatic porphyria is encountered, coupled with the fact that purified alcohol has been shown to have a definite hepatoxic effect (Rubin and Lieber 1968), make this possibility unlikely.

The mode of action of ethanol in causing or aggravating symptomatic porphyria is unknown. Obviously the suggested explanation, mentioned above, for the coproporphyrinuria following intake of alcohol cannot apply to the effect of ethanol in this disease. Uroporphyrin, the major excreted porphyrin in symptomatic porphyria, is not excreted by the liver to any extent and appears almost exclusively in the urine. It is thus far more likely that ethanol in some way affects hepatic porphyrin synthesis and possibly storage also.

5.4.3.2 Oxidation of alcohol and the effect of increased liver $NADH_2/NAD$ ratios. Apart from a small amount (2-10%) which is excreted by the kidneys and lungs, alcohol in the body is oxidised mainly in the liver (Thompson 1956). The initial reaction involves direct transfer of hydrogen from the methylene carbon of ethanol to NAD by the enzyme, alcohol dehydrogenase (Fischer et al. 1953), which is located in the liver cell cytoplasm (Nyberg et al. 1953). As a result of this, liver $NADH_2/NAD$ ratios are increased (Smith and Newman 1959, Isselbacher and Krane 1961). "The magnitude of the change in the $NADH_2/NAD$ ratio in the soluble compartment of the cytoplasm may well represent the most characteristic change produced by the metabolism of ethanol in the liver" (Lieber 1965).

Due to the relative impermeability of the mitochondrial membrane to NAD and $NADH_2$ (Lehninger 1954, Kaufman and Kaplan 1966) the transfer

of hydrogen from cytoplasmic $NADH_2$ to the flavoprotein-cytochrome system cannot occur directly and requires an intermediate carrier, e.g. β-hydroxybutyrate or α-glycerophosphate or malate. This is supported by the finding that hepatic α-glycerophosphate increases after ethanol administration (Nikkilä and Ojala 1963) and by studies on ethanol-1-^3H metabolism during glycogen synthesis (Joubert and Shreeve 1968). Under these conditions, the disposition of tritium in the glucosyl residues of liver glycogen and the incorporation of tritium into liver fatty acids suggest that hydrogen, derived from the methylene carbon of alcohol, is cycled to the mitochondria by a 'shuttle' mechanism, and is subsequently in part 'pumped' from the mitochondria via malate and $NADPH_2$ to liver fatty acids (Joubert and Shreeve 1968).

It is therefore conceivable that during ethanol oxidation in the liver the intramitochondrial $NADH_2/NAD$ ratio could be sufficiently increased to lead to induction of ALA synthetase as postulated by Labbe (1967) (Section 4.2.2).

The production of uridine diphosphate glucuronic acid (UDPGA) which supplies the glucuronide moiety of the conjugated steroid is NAD-dependent and hence increased liver $NADH_2/NAD$ ratios might retard glucuronide formation. Consequently, if steroid induction of porphyrinogenesis is important in symptomatic porphyria, alcohol may play a part in maintaining higher than normal levels of free intracellular steroids.

5.4.3.3 Other hepatic effects of alcohol. Previously, liver damage associated with alcoholism was attributed to nutritional deficiencies. Now, however, there is evidence that alcohol itself is hepatoxic. Lieber and Rubin (Lieber and Rubin 1968, Rubin and Lieber 1968) have shown, both in alcoholics and in normal subjects, that alcohol in sufficient dosage, despite an adequate diet, rapidly induces fatty change and ultrastructural changes. Mitochondria were found to

be enlarged and distorted, including spherical, lobate and tailed forms often with disorientated cristae. The rough endoplasmic reticulum was vesiculated, with ribosomes arranged irregularly on the membranes and the smooth endoplasmic reticulum was increased in amount and vesiculated. It is suggested by these workers that "the observed mitochondrial alterations may be the morphological counterpart of impaired mitochondrial function ....." (Rubin and Lieber 1968). They also point out that mitochondria from ethanol-treated rats have exhibited increased membrane permeability and decreased oxidative properties (Rubin and Lieber 1968).

Based on the ability of antioxidants, e.g. alpha-tocopherol, to prevent liver damage by alcohol and other substances, Luzio and Hartman (1967) postulated that hepatic injury caused by alcohol is due to free radical-induced peroxidation of lipids. Mitochondria and endoplasmic reticulum which are rich in unsaturated fatty acids would be particularly liable to lipid peroxidation. The resultant changes involving structural lipids would hence account for the observed ultrastructural changes in this organelle.

If intracellular compartmentation (Section 4.2.7) is important in the control of haem biosynthesis, alcohol-induced mitochondrial membrane changes affecting permeability could obviously be of aetiological significance in symptomatic porphyria.

5.4.4 Iron metabolism in symptomatic porphyria. The high incidence of hepatic siderosis and hypersideraemia in patients of all races with symptomatic porphyria, both in South Africa (Section 2.2.3) and other countries (Ippen 1961, Filip and Berman 1957), has resulted in much speculation on the possible aetiological significance of iron overload in this disease.

Kramer (1963) doubts that excessive hepatic iron deposits play a part in the development of symptomatic porphyria since this disease is not encountered in the great majority of African patients with siderosis,

or in patients with haemochromatosis, or transfusional siderosis. He suggests that the coincidence of symptomatic porphyria and siderosis can be explained without invoking an aetiological relationship. The excessive iron absorption in these patients (Kramer 1962) is probably due mainly to the consumption of large amounts of alcoholic beverages with a high iron content. In the African population most of this iron is thought to derive from the containers used for brewing illicit liquors (Kramer 1963). Wines, particularly cheap wines, have also been shown to have a significant iron content (Saunders 1963, MacDonald 1963). Consequently, the more marked degrees of siderosis tend to occur in the heaviest drinkers (Kramer 1963). Furthermore, chronic liver disease and pancreatitis which are common in alcoholic subjects also promote increased iron absorption from the gut (Davis and Badenoch 1962).

On the other hand, Saunders (1963) and Hickman, Saunders and Eales (1967) hold the opposite view. They point out that, while siderosis is common in the African population, it is uncommon in the Cape Coloured group. In support of this view studies were conducted on the effect on porphyrin excretion of reducing the body iron stores (Saunders 1963, Hickman, Saunders and Eales 1967). Desferrioxamine, an iron-chelating agent, had no effect but repeated venesection over six months was associated in a number of patients with a fall in uroporphyrin excretion. Venesection has also been used with apparent success in PCT in other countries (Ippen 1961, Epstein and Redeker 1965). It is of course difficult to draw definite conclusions from this type of study, particularly if conducted on an out-patient basis, in view of the various other factors which are known to affect porphyrin excretion, e.g. diet (Welland et al. 1964, Perlroth et al. 1968) alcohol (Section 5.4.3) and rest in bed. Nevertheless, Hickman, Saunders and Eales (1967) claim that their patients continued drinking to excess during the period of study.

Experimental dietary iron overload has been shown to produce siderosis in rats (Gillman et al. 1959, Theron et al. 1963), histologically similar to that seen in the majority of adult African males (Bothwell and Isaacson 1962). In animals fed on a deficient diet containing 4% ferric citrate, Theron et al. (1963) were able to demonstrate ultramicroscopic changes from the ninth day of the experiment. Increased uptake of iron by the parenchymal liver cells was followed initially by localisation of iron in lysozomes. Later, mitochondria were seen to undergo a series of degenerative changes culminating in the formation of large, vacuolated, ferritin-containing structures which Richter (1957) has named 'siderosomes'.

Together with these structural changes Theron et al. (1963) found decreased mitochondrial isocitric, malic and L-glutamic dehydrogenase activity, while by contrast the activity of NADH cytochrome C reductase and NADH oxidase was increased. With respect to the increased NADH oxidase activity they suggested that ferritin or inorganic iron "may have acted as a non-enzymatic catalyst in the electron transfer between NADH and oxygen" (Theron et al. 1963).

The significance of these biochemical effects is not immediately obvious. However, the important point illustrated by this study is that iron overload can influence both the structural integrity of the mitochondria and mitochondrial enzyme activity. Hence it is quite feasible that enzymes involved in haem biosynthesis may be similarly affected, with resultant changes in porphyrin production. Thus decreased coproporphyrinogen oxidase activity could lead to accelerated porphyrin biosynthesis through defective haem formation and consequent derepression of ALA synthetase (Section 4.2.7).

THE PRESENT STUDIES

SECTION A

THE EFFECT OF ETHANOL AND OF DIETARY IRON OVERLOAD ON PORPHYRIN METABOLISM - STUDIES IN THE RAT AND IN PATIENTS WITH SYMPTOMATIC PORPHYRIA.

CHAPTER 6

THE EFFECT OF ETHANOL ON LIVER ALA SYNTHETASE ACTIVITY IN THE RAT.

6.1 Aim of the study. The evidence for the effect of ethanol on porphyrin metabolism stems mainly from the following two observations. Firstly, it has long been known that administration of alcohol to normal subjects and chronic alcoholics is followed by increased urinary excretion of COPRO (Section 5.4.3.1). Secondly, patients with symptomatic porphyria almost invariably have a history of chronic abuse of alcohol (Sections 2.2.1.2, 2.2.4, 5.4.3.1). There is, however, no information at present on how this effect is produced.

Over-production of haem precursors through increased activity of ALA synthetase, the rate-limiting enzyme for liver haem biosynthesis, has been demonstrated, not only in the experimental (Section 5.2.2) and genetic hepatic porphyrias (Section 5.3.1), but also in symptomatic porphyria (Section 5.4.1). It is therefore highly probable that alcohol promotes accelerated porphyrin biosynthesis by induction of this enzyme. The object of this study was to establish the effect of ethanol *in vivo* on the activity of hepatic ALA synthetase in normal animals (rats) before proceeding to studies in human subjects.

6.2 Materials and methods. Female Wistar rats weighing 150 - 200 g were fasted for 40 hours before intraperitoneal administration of 5 ml of 20(v/v)% ethanol in physiological saline solution or 5 ml of saline solution in the case of control animals. At the end of each time period indicated in Table 6.1 and Fig. 6.1, five alcohol-treated and five control rats were killed by decapitation, and 1 in 3 homogenates of their livers were immediately made in ice-cold buffer (0.075 M tris, 0.01 M EDTA, 0.1 M glycine, pH 7.4). ALA synthetase activity was determined in the pooled homogenate for each group as described in Section A.1.1.1, method (i).

6.3 Results. The results are summarised in Table 6.1 and Fig. 6.1. Single experiments were carried out at the 1- and 6-hour time

Table 6.1 Liver ALA synthetase activity in rats following a single intraperitoneal injection of alcohol or saline.

Time after injection (hours)	No. of animals	Mean ALA synthetase activity (mμmoles ALA formed/g liver/hour)		
		After alcohol	After saline	Difference
1	5	45.1	50.4	- 5.3
2	10	78.5	62.5	+ 16.0
3	15	80.4	56.5	+ 23.9
4	10	66.3	52.4	+ 13.9
6	5	55.4	59.3	- 3.9
8	10	44.1	49.3	- 5.2

The overall mean activity for the control (saline-treated animals was 55.1 (S.D. 5.2).

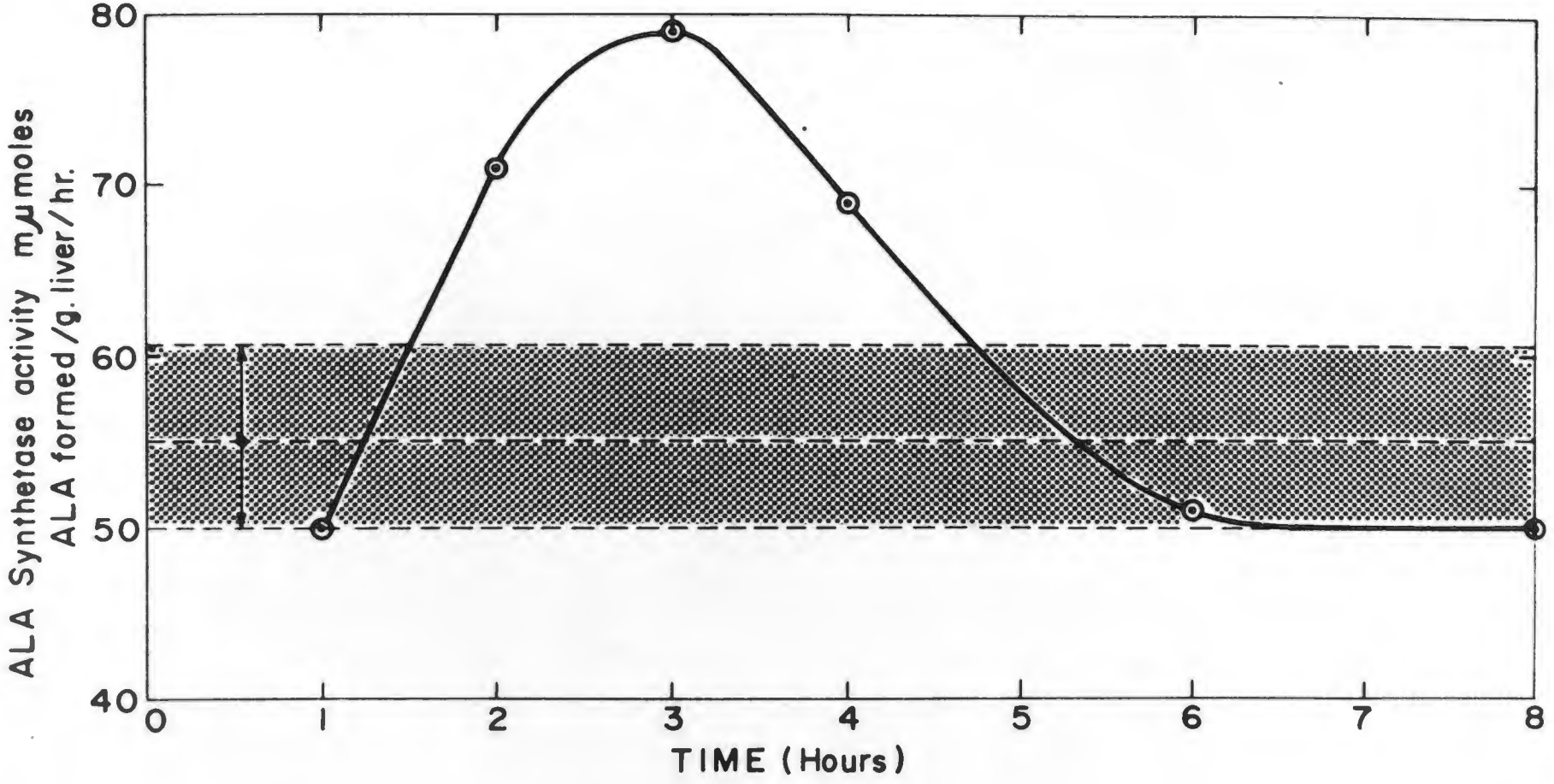
periods, while the 2-, 4- and 8-hour values each represent the mean of two experiments; the 3-hour activity is the mean of three experiments.

In the control animals (a total of 55 rats) the mean ALA synthetase activity was 55.1 (S.D. 5.2) mμmoles ALA formed/g liver/hour. By contrast, in the other animals intraperitoneal administration of a single dose of ethanol resulted in a distinctive response of liver ALA synthetase activity with time.

6.4 Discussion. Administration of a single dose of ethanol has been clearly shown in this study to produce a significant increase in hepatic ALA synthetase activity in the rat. It is reasonable to expect that the resultant endogenous overload of ALA might result in increased excretion of haem precursors. Indeed Gajdos et al. (1967) have reported that acute ethanol intoxication in the rat caused increased urinary excretion of PBG and URO, coupled with raised levels of ether-soluble porphyrins in the liver and red blood cells.

Increased ALA synthetase activity in response to a variety of porphyrinogenic drugs has been shown to be due to increased enzyme synthesis (Section 5.2.2). Marver, Collins, Tschudy and Rechcigl (1966) showed that the increase in liver ALA synthetase activity in the rat following administration of AIA can be prevented by prior treatment of the animal with an inhibitor of protein synthesis, e.g. actinomycin D or puromycin. However, in the present study no such experiments were undertaken, partly because of the cost involved, and partly because it is by no means certain that these inhibitors do not interfere with other processes in the cell. Nevertheless, there is little doubt that ethanol promotes enzyme induction rather than simple activation of preformed protein. The rate of decline of liver ALA synthetase activity from the peak value at 3 hours after administration of alcohol, shown in Fig. 6.1, is consistent with the finding of Marver, Collins, Tschudy and Rechcigl (1966) that the half-life of this enzyme in rats, treated first with AIA and later with puromycin, is between 67 and 72 minutes.

Fig. 6.1 The response of liver ALA synthetase activity in the rat to a single intraperitoneal dose of alcohol. The shaded area represents the mean activity in control animals $\pm$ S.D., while the points on the curve represent the sum of the mean control activity and the values in the last column of Table 6.1.


ALA Synthetase activity mμmoles
ALA formed/g. liver/hr.
80
70
60
50
40
0
1
2
3
4
5
6
7
8
TIME (Hours)

The findings in the present study represent the first demonstration that pure ethanol can influence the synthesis of haem precursors in the liver. Previously, fungal and chemical contaminants or additives in alcoholic beverages have often been suggested (Section 5.4.3.1) as important factors in the genesis of symptomatic porphyria. Keen et al. (1966) showed that the filtrate from cultures of the fungus, *Aspergillus fumigatus*, induces porphyrin production in the chick embryo liver cell; and subsequently Hickman, Saunders and Eales (1967) found that *iso*-amyl alcohol also induces ALA synthetase in the same cell culture system. It is pertinent to note here that the alcohol employed in the studies described in the present thesis contained only trace amounts of *iso*-amyl alcohol (less than 10 parts per million) as determined by gas chromatography (Research Dept., Natal Cane By-Products Ltd.).

In a study of ALA systhetase induction by a wide variety of porphyrinogenic compounds in chick embryo liver cell cultures, Granick (1966) used alcoholic solutions of some of these inducers. Although the point was not specifically mentioned, ethanol, in the quantities employed (1 - 3 µl per ml of growth medium), presumably did not produce porphyrin fluorescence in this system. There are several possible explanations for the apparent contradiction between the results obtained in the present study and Granick's findings.

Firstly, the concentration of alcohol presented to the liver cell in the case of the rats given a large intraperitoneal dose of ethanol (at least 4 g/kg in all cases) was undoubtedly greater than the concentration of ethanol in the culture media used by Granick (1966). Secondly, it has been pointed out (Section 5.4.3.2) that the action of ethanol on ALA synthetase might well be dependent on its dehydrogenation, and it is quite possible that the liver cells of the 16- to 17-day old chick embryo lack alcohol dehydrogenase. Thirdly, it is seldom valid to make strict comparisons between *in vitro* and *in vivo* studies,

particularly where different species are involved.

The possible mechanisms whereby alcohol could effect induction of liver ALA synthetase will not be discussed here, since this subject forms the basis of the studies described in Chapters 9 to 12 of this thesis.

6.5 Conclusions. Alcohol influences hepatic porphyrin metabolism in the rat through increased activity (probably induction) of ALA synthetase, the rate-limiting enzyme for liver haem biosynthesis.

CHAPTER 7

THE EFFECT OF ETHANOL ON LIVER ALA SYNTHETASE ACTIVITY AND URINARY PORPHYRIN EXCRETION IN SYMPTOMATIC PORPHYRIA.

7.1 Aim of the study. The common metabolic abnormality of the hepatic porphyrias appears to be over-production of haem precursors through increased activity of liver ALA synthetase, the rate-limiting enzyme for liver haem biosynthesis (Sections 5.2.2, 5.3.1, 5.4.1). Symptomatic porphyria differs from the experimental and genetic hepatic porphyrias, however, in that not all patients demonstrate this abnormality on random testing. Zail and Joubert (1968) found increased liver ALA synthetase activity in only 5 out of 12 patients showing unequivocal, clinical and biochemical evidence of symptomatic porphyria. Moreover, no correlation was found between the activity of this enzyme and porphyrin excretion. These apparently anomalous findings raise the question of whether symptomatic porphyria is in fact an 'over-production disease', i.e. whether ALA synthetase activity is related to over-production of porphyrins in this condition.

The demonstration in Chapter 6 that alcohol is capable of causing increased liver ALA synthetase activity in the rat affords an explanation. There is strong circumstantial evidence that abuse of alcohol plays an important aetiological role in symptomatic porphyria (Sections 2.2.1.2, 2.2.4, 5.4.3.1). Hence, if the response of human liver ALA synthetase to ethanol corresponds with that found in the rat, episodes of excessive intake of alcohol, alternating with periods of abstinence, might produce a pattern of phasic elevation and decline of liver ALA synthetase activity. The activity recorded in any particular patient would then depend on the timing of the enzyme assay. Patients studied immediately after admission to hospital might be expected generally to show higher liver ALA synthetase activity than those biopsied after a period of enforced abstinence from alcohol in a hospital ward.

The aim of this study was to examine the effect of administration of alcohol on liver ALA synthetase activity and urinary porphyrin

excretion in a group of hospitalised patients with symptomatic porphyria and to compare the response with that in a similar group who did not receive alcohol.

7.2 Materials and methods. Altogether 27 adult male and female African patients who were admitted to King Edward VIII Hospital, Durban, with clinical and biochemical features of symptomatic porphyria were investigated. The criteria employed were those set out in Sections 2.2.1 and 2.2.2. For diagnostic purposes casual urine and stool specimens were analysed for porphyrin precursor and porphyrin content as described in Section A.9. Total aminoketones, PBG and URO were determined in urine and COPRO and PROTO in the stool. In addition liver biopsy specimens were examined for porphyrin fluorescence under ultraviolet light.

Liver ALA synthetase activity was determined in 15 subjects while urinary porphyrin excretion was studied in another group of 12 patients. Only two cases (F. Mkize and W. Sokhela) were included in both groups.

7.2.1 Administration of ethanol. All 27 patients in the study admitted to frequent drinking of alcoholic beverages before admission to hospital. Of these, 14 randomly selected volunteers were supplied with measured volumes of purified ethanol 96(v/v)% mixed with equal volumes of water. They were also provided with orange juice or Coca Cola and allowed to mix their own drinks, the only proviso being that they should consume the quota of ethanol within a specified time (as indicated in Sections 7.2.2 and 7.2.3) under the supervision of responsible senior nursing staff. Where these conditions were not adhered to, and in a few cases where nausea or other untoward symptoms developed, the study was immediately abandoned. All patients were maintained on a normal ward diet except where fasting is specifically mentioned in Section 7.2.2.

7.2.2 Determination of ALA synthetase activity. To minimise the effect of pre-hospitalisation environmental factors, liver

biopsies for ALA synthetase determination were delayed until at least the fourth day after admission to hospital. Patients did not have access to alcohol during this period. In those cases where the prothrombin index was initially found to be less than 70%, biopsy was performed only if a satisfactory response was obtained to the administration of 10 mg vitamin K_1 daily for several days.

Of the 15 patients studied, 7 received ethanol prior to liver biopsy. Commencing in mid-morning on the preceding day, each consumed 300 ml 96(v/v)% ethanol, diluted as described in Section 7.2.1, over a period of 8 hours. After an overnight fast each consumed a further 150 ml as rapidly as possible (within 1 - 2 hours) after 7 a.m. Percutaneous liver biopsy was performed under local anaesthesia with a Menghini needle 3 to 4 hours later.

The remaining 8 patients in this group received no ethanol and served as control subjects. They were simply fasted overnight and liver biopsy was performed the next morning.

ALA synthetase activity was determined by a micro-assay method described by Zail and Joubert (1968) (Section A.1.1.2). Histological examination was carried out on a portion of the biopsy specimen in those cases where it was required for diagnostic purposes.

7.2.3 Quantitation of urinary porphyrin excretion. In 5 patients serial 24-hour urine collections were made over a period of 8 to 11 days while in a hospital ward to obtain information on the daily fluctuation in urinary porphyrin excretion following hospitalisation. Patients were selected to cover a range of URO excretion from less than 1000 µg to 8000 µg per g creatinine. These findings were compared with those in a similar group of 7 patients who consumed 300 ml of 96(v/v)% ethanol (diluted as described in Section 7.2.1) during an 8-hour period from 10 a.m. onwards on the fourth or fifth day of the study.

Longer periods of study in individual cases, while desirable,

were not possible for practical reasons. This hospital is completely devoid of facilities for long term metabolic studies. It was therefore necessary to compromise and a period of approximately 10 days was judged to be both sufficient for the purpose of the investigation and convenient for the maintenance of personal supervision of specimen collection by the author. To further obviate errors caused by incomplete 24-hour collections, porphyrin excretion was expressed as µg/g creatinine.

All urine collecting bottles were made of dark glass. It was decided not to add preservative since it is not known precisely what effects the commonly used bacterial inhibitors, e.g. toluene or chloroform, might have on the urinary porphyrins. Instead all specimens were frozen immediately on arrival at the laboratory at the end of the 24-hour period and stored at -20°C in the dark to await analysis.

The methods used for quantitation of urinary porphyrins are detailed in Section A.9. URO was determined according to Sveinsson et al. (1949) and COPRO according to Holti et al. (1958). The urinary creatinine content was determined according to Peters and Van Slyke (1932) (Section A.10). As pointed out in Section A.9, physical separation of the various porphyrins by the methods mentioned above is at best only crude. It is therefore inevitable that 'URO' and 'COPRO' thus determined represent mixtures of porphyrins and must not be regarded as true reflections of the urine content of 8- and 4-carboxyl porphyrins respectively. Nevertheless, these methods are of the type generally used for quantitating porphyrin excretion in the porphyrias, and consequently may be regarded as quite adequate for providing a broad indication as to whether or not ethanol administration aggravates porphyrin (particularly 'URO') excretion in symptomatic porphyria.

7.3 Results. The clinical and biochemical details of all patients included in this study appear in Appendix B (Tables B.2, B.3, B.4, B.5). It is apparent that the findings in these subjects conform

Table 7.1 Liver ALA synthetase activity in patients with symptomatic porphyria.

Patients		ALA synthetase activity mμmoles ALA formed /mg total Liver phosphorus/hour.	
No alcohol	After alcohol		
F. Mkize		8.6	
N. Ngubane		12.1	
I. Buthelezi		8.8	
R. Thabede		0.0	Mean 9.0
E. Ndaba		10.6	S.D. 5.9
S. Mkize		11.9	
C. Miya		18.4	
V. Pondo		1.7	
	W. Sokhela	85.8	
	M. Mthembu	50.8	
	M. Ndimande	23.9	Mean 36.8
	J. Ngubane	24.7	S.D. 28.1
	N. Hlongwane	37.5	
	Z. Majola	13.2	
	D. Mthembu	21.6	

to the pattern previously described in symptomatic porphyria (Section 2.2). Brilliant red fluorescence in ultraviolet light was exhibited by all liver biopsy speciments.

7.3.1 ALA synthetase activity. Hepatic ALA synthetase activity of control and test subjects appears in Table 7.1 and Fig. 7.1. Of the control group of 8 patients who received no ethanol while in hospital, the highest activity recorded was 18.4 mμmoles of δ-amino-laevulinic acid (ALA) formed/mg total liver phosphorus/hour. The mean value was 9.0 (S.D. 5.9).

Only one of the test subjects (Z. Majola) who received ethanol fell within this range. Activity ranging from 1.2 to 4.7 times the highest control value was recorded in the remaining 6 patients.

7.3.2 Urinary porphyrin excretion. Figs. 7.2 to 7.5 depict the daily amounts of urinary porphyrin excreted by the 12 subjects during the period of study. The 5 control subjects who did not receive ethanol covered a range of URO excretion from less than 1000 μg to 8000 μg/g creatinine. One of these (F. Mkize) showed a dramatic drop in URO output during the 10 day period whereas the other 4 patients did not demonstrate very marked variations. COPRO output, on the other hand, ranged from less than 100 μg to 400 μg/g creatinine and was more variable.

The 7 patients who received ethanol were also excreting URO in the range - less than 1000 μg to 8000 μg/g creatinine. From Fig. 7.3 it is apparent that in 6 of the 7 subjects much greater fluctuations in daily URO output occurred than in the control group (Fig. 7.2). While some of the variations preceded the administration of ethanol, there is, nevertheless, a fairly consistent pattern of transient elevation of URO output from one to several days after the dose. A similar pattern is evident in Fig. 7.5 but the picture is complicated by the fact that substantial variation in daily urinary COPRO excretion occurred in the absence of administration of ethanol (Fig. 7.4).

Fig. 7.1 Scatter diagram showing hepatic ALA synthetase activity in patients with symptomatic porphyria. The test group received ethanol prior to the study, as described in Section 7.2.1, while the control subjects did not.

ALA SYNTHETASE ACTIVITY mμ moles ALA formed/mg. total liver phosphorus/hr.
100
80
60
40
20
0
CONTROL SUBJECTS No ethanol
TEST SUBJECTS Received ethanol

Fig. 7.2 Daily urinary URO excretion in hospitalised patients with symptomatic porphyria who did not have access to alcohol.

F.M. ..... F. Mkize
H.G. ..... H. Gumede
E.Ma. .... E. Mazibuko
N.N. ..... N. Nkosi
Z.M. ..... Z. Mhlongo

Fig. 7.3 Daily urinary URO excretion in hospitalised patients with symptomatic porphyria who received ethanol. The day of administration in each case is indicated by the arrow at the top of the figure.

R.M. ..... R. Msomi
W.S. ..... W. Sokhela
S.H. ..... S. Hlongwa
J.M. ..... J. Mthembu
E.M. ..... E. Mthembu
M.M. ..... M. Mbotho
T.N. ..... T. Ndaba

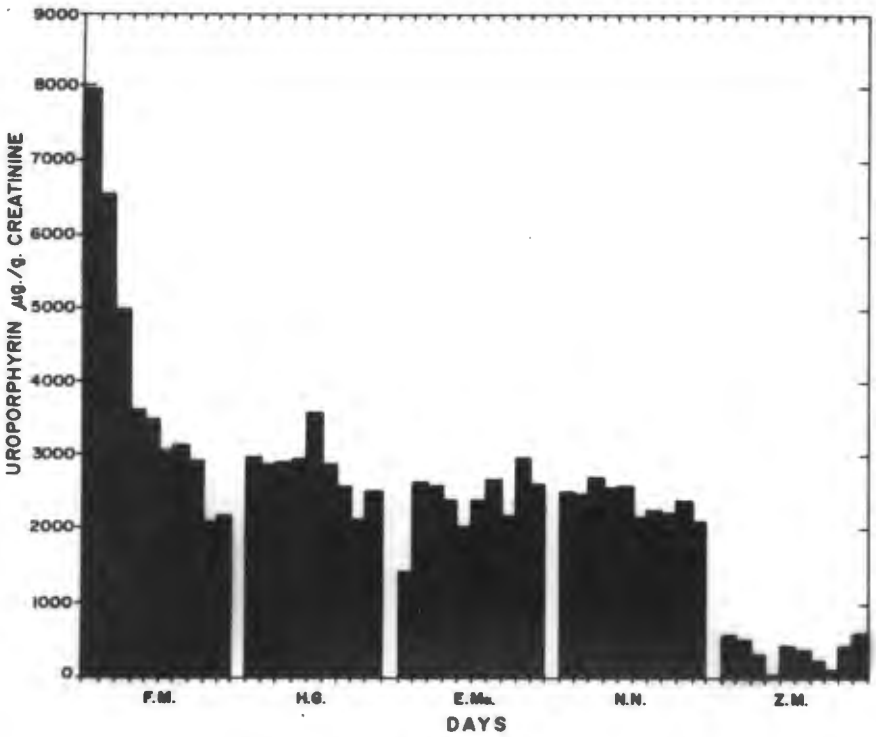


FIGURE 2

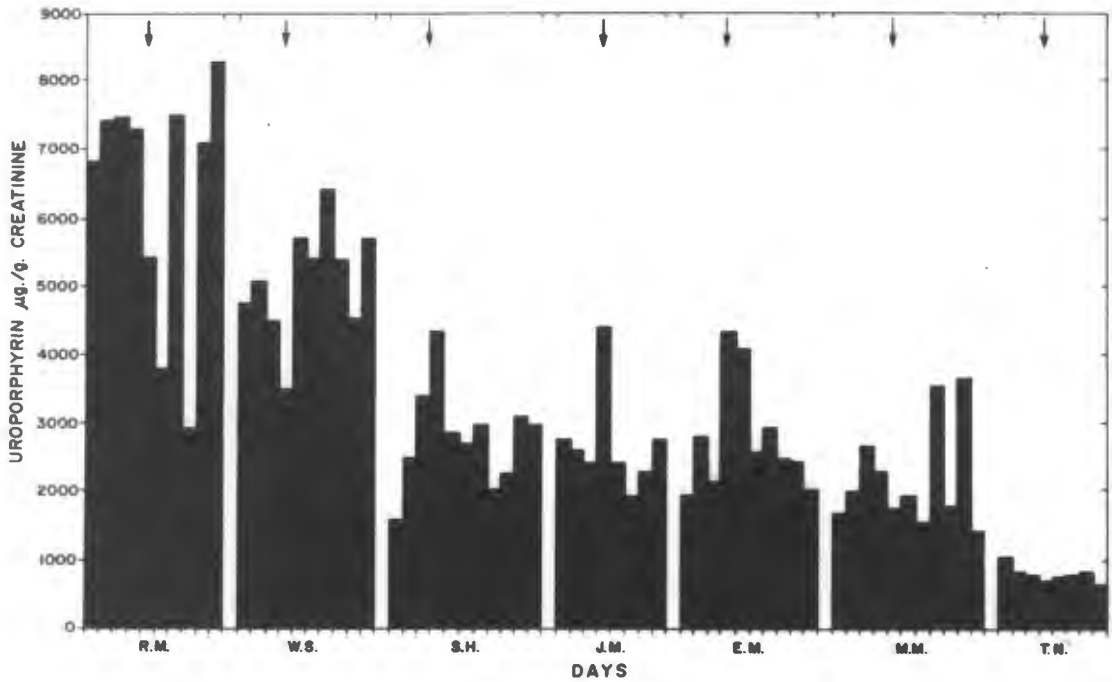


FIGURE 3

Fig. 7.4 Daily urinary COPRO excretion in hospitalised patients with symptomatic porphyria who did not have access to alcohol.

F.M. ..... F. Mkize
H.G. ..... H. Gumede
E.Ma. .... E. Mazibuko
N.N. ..... N. Nkosi
Z.M. ..... Z. Mhlongo

Fig. 7.5 Daily urinary COPRO excretion in hospitalised patients with symptomatic porphyria who received ethanol. The day of administration in each case is indicated by the arrow at the top of the figure.

R.M. ..... R. Msomi
W.S. ..... W. Sokhela
S.H. ..... S. Hlongwa
J.M. ..... J. Mthembu
E.M. ..... E. Mthembu
M.M. ..... M. Mbotho
T.N. ..... T. Ndaba

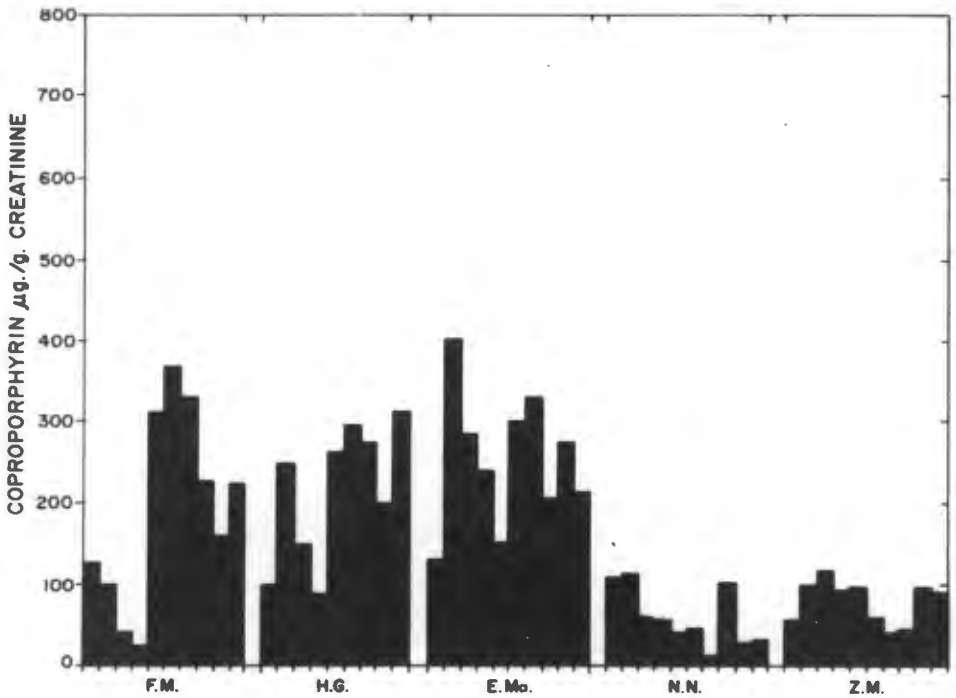


FIGURE 4

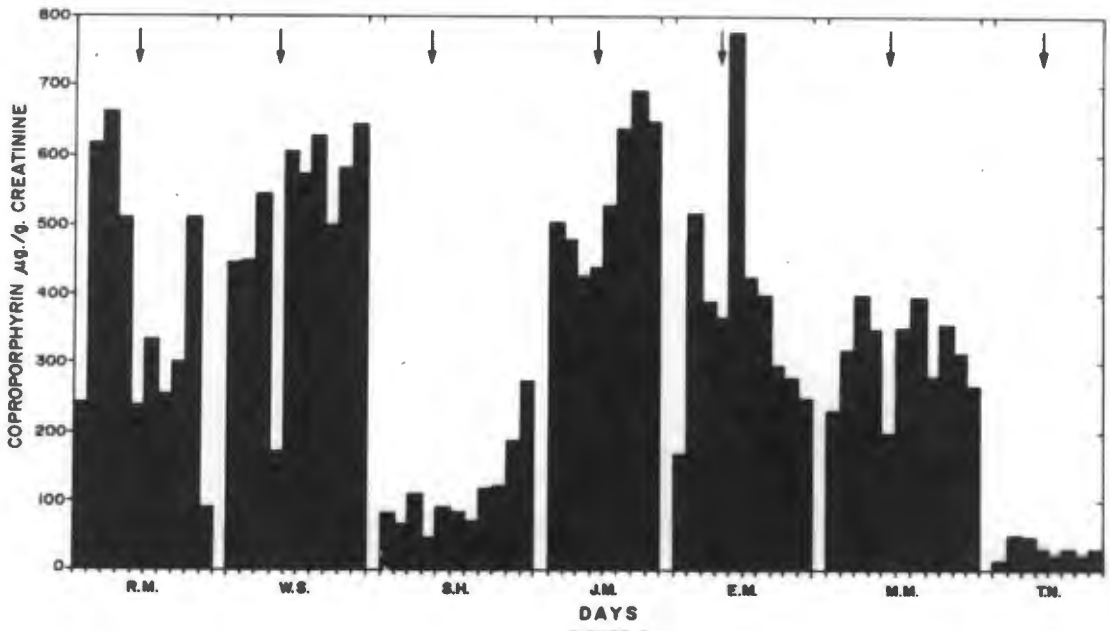


FIGURE 5

7.4 Discussion. The finding of increased liver ALA synthetase activity and enhanced URO excretion following ethanol administration in the present study is clear evidence that abuse of alcohol can aggravate symptomatic porphyria from the biochemical viewpoint. Furthermore, it is highly probable that enzyme activity and porphyrin output under these circumstances are related as cause and effect, i.e. increased excretion reflects new synthesis. It would appear, therefore, that the description of 'over-production disease' is in fact justifiable.

The suggestion that hospitalisation and withdrawal of ethanol promote a fall in liver ALA synthetase activity in symptomatic porphyria is supported in this study by the low values recorded in those patients who did not receive ethanol. It is obvious, therefore, that assay of this enzyme at varying times after admission to hospital might provide a scatter of results as found by Zaíl and Joubert (1968). Furthermore, since ALA synthetase is the rate-limiting enzyme for liver haem biosynthesis, a logical basis is provided for the impression that these measures also promote clinical remission in symptomatic porphyria together with a tendency to decreased porphyrin excretion.

These findings are entirely consistent with the concept that alcohol plays an aggravating role in symptomatic porphyria. For a number of reasons, however, it is most unlikely that induction of liver ALA synthetase by ethanol is the whole metabolic basis of this disease.

Firstly, it was shown in Chapter 6 that alcohol is a modest 'inducer' of liver ALA synthetase in normal animals. It is therefore probable that in normal human subjects this enzyme responds similarly to ethanol, although the response may well be less marked than in the case of patients with symptomatic porphyria. In normal people and chronic alcoholics, however, alcohol consumption is known to cause increased urinary excretion of COPRO (Section 5.4.3.1) but not URO as was found in the patients in the present study.

Secondly, raised ALA synthetase activity implies over-production

of porphyrin precursors yet, unlike the situation in the genetic varieties of hepatic porphyria, excretion of ALA and PBG in the urine in symptomatic porphyria is normal or only slightly increased. On the other hand, porphyrinuria may be massive.

Thirdly, substantial porphyrin excretion was encountered in a number of patients in the present study (Table B.3) at a time when liver ALA synthetase activity (Table 7.1) was comparable with or only slightly greater than values reported in a number of non-porphyric subjects with various forms of liver disease (Zail and Joubert 1968). It is conceivable that the continued excretion of porphyrin in these cases represents release from liver stores accumulated during periods of much greater liver ALA synthetase activity. Sweeney et al. (1962) have shown that the porphyrin content of the liver in symptomatic porphyria may range from 100 to 1000 times the normal concentration of about 1 µg/g wet weight. Further studies are obviously required to elucidate the relationship between synthesis, liver storage and excretion of porphyrins in this disease.

The most plausible explanation which could account for all the observations mentioned above is that ALA is handled differently in patients with symptomatic porphyria. In this connection Dowdle and co-workers (1968) using ALA-5-^{14}C showed in a patient with symptomatic porphyria that a greater proportion of administered ALA is excreted as urinary URO than is found in normal subjects or patients with AIP and variegate porphyria. It is suggested, therefore, that the biochemical lesion which determines this preferential disposal or 'metabolic sequestration' of ALA as URO is the basic defect in symptomatic porphyria. Withdrawal of alcohol, while leading to a fall in ALA synthetase activity, would not be expected to influence such a lesion. Hence even low grade overactivity of the rate-controlling enzyme, i.e. by comparison with subjects free of liver disease in whom hepatic ALA synthetase activity is usually negligible (Nakao et al. 1966, Dowdle et al. 1967, Levere 1967, Zail and Joubert 1968), could conceivably

result in substantial URO production.

At present the aetiology and nature of this basic biochemical abnormality are obscure. Nevertheless, several possibilities are suggested by the findings that hepatocellular disease is present in all patients with symptomatic porphyria and that siderosis is the most constant abnormality reported (Section 2.2.3). It is quite possible that this metabolic lesion is related to the presence of excessive deposits of iron in the liver. In Section 5.4.4 it was pointed out that experimental hepatic siderosis in the rat is associated with ultrastructural changes and various biochemical abnormalities. It may be that porphyrin production and excretion is also affected by hepatic iron overload. Under such circumstances, 'metabolic sequestration' of URO could conceivably occur, e.g. through a relative block in the haem biosynthetic pathway at a stage after the synthesis of UROgen or, alternatively, synthesis of URO might occur via a route (other than the normal haem biosynthetic sequence) which is not normally operative.

7.5 Conclusions. Alcohol causes increased liver ALA synthetase activity and increased urinary porphyrin excretion in patients with symptomatic porphyria while, conversely, hospitalisation and withdrawal of ethanol promote biochemical improvement. These findings justify the description of 'over-production disease' and define the role of alcohol in symptomatic porphyria as an aggravating factor. It is suggested that the basic lesion in this disease is one which determines the altered handling of ALA, leading to 'metabolic sequestration' of abnormal amounts of haem precursors chiefly as URO.

CHAPTER 8

THE EFFECT OF CHRONIC DIETARY IRON OVERLOAD, ALONE AND IN COMBINATION WITH ETHANOL, ON

- URINARY PORPHYRIN EXCRETION,
- LIVER ALA SYNTHETASE ACTIVITY AND
- LIVER MITOCHONDRIAL COPROPHYRINOGEN OXIDASE ACTIVITY IN THE RAT.

8.1 Aim of the study. In Chapter 7, it was suggested that the role of alcohol in symptomatic porphyria is to aggravate the effects of an underlying biochemical lesion, the nature and aetiology of which remain unknown at present. This lesion, it was postulated, is responsible for the 'metabolic sequestration' and subsequent excretion of abnormal amounts of haem precursors chiefly as URO.

Apart from alcohol, the most likely exogenous pathogenic agent in symptomatic porphyria is iron. Hepatic siderosis due to dietary iron overload is encountered in all these patients, irrespective of racial group. While siderosis is common in the African population at large in this country, it is encountered much less frequently among the Cape Coloureds and Whites (Sections 2.2.3.1, 2.2.4, 5.4.4). There is however no direct experimental evidence that iron overload affects porphyrin metabolism.

On the other hand, there is good evidence that experimental dietary iron overload in the rat leads to hepatic siderosis which resembles that commonly seen in the African population of South Africa (Section 5.4.4). This experimental siderosis is accompanied by profound ultrastructural changes in the liver cell, including mitochondrial abnormalities which are associated with altered activity of mitochondrial enzymes (Section 5.4.4). It is conceivable, therefore, that iron overload of the liver cell directly or indirectly affects mitochondrial enzymes concerned in haem biosynthesis.

Previously, it has been suggested that a relative block in liver haem biosynthesis at the stage of conversion of COPROgen to PROTOgen could explain the characteristic porphyrin excretion pattern in sympto-

Table 8.1 Experimental regimes for the six groups of rats.

Group	Diet	Drinking fluid
1	Normal	Water
2	White maize meal	"
3	White maize meal + 4% ferric citrate	"
4	Normal	20% ethanol (v/v)
5	White maize meal	"
6	White maize meal + 4% ferric citrate	"

matic porphyria, through derepression of ALA synthetase (Sections 4.2.7, 5.4.4). Coproporphyrinogen oxidase is located in the mitochondrial membrane and hence structural changes affecting the functional integrity of the mitochondrion could conceivably inactivate this enzyme or prevent access of substrate due to altered membrane permeability.

Accordingly, the aim of this study was to investigate the effect in the rat of chronic dietary iron overload, alone and in combination with ethanol, on urinary porphyrin excretion and on the activity of ALA synthetase and coproporphyrinogen oxidase.

8.2 Materials and methods. Six groups of young female Wistar rats, closely matched for age and weight within the range 150-200 g, were maintained for a period of 48 weeks on the regimes shown in Table 8.1.

The normal diet was a powdered rat food supplied by United Oil and Cake Mills, Durban, and consisted of yellow maize meal, bran, maize germ meal, fish meal, ground nuts, vitamins and minerals.

Second grade white maize meal was obtained from H. Thomas and Co. Ltd., Durban. Ferric citrate scales (British Drug Houses) were finely ground before being thoroughly mixed (4% by weight) with white maize meal. This mixture was employed by Gillman et al. (1959) who showed that hepatic siderosis is much more readily induced in rats by feeding an iron-enriched, otherwise deficient (viz. maize meal) diet, than by providing an iron-enriched normal diet. Dietary supplementation with iron was preferred to parenteral administration since it was considered desirable to ascertain what effect concomitant drinking of alcohol might have on the rate of development and degree of siderosis. All groups were supplied with their respective diets ad libitum. In addition, supplementation with milk (approximately 15 ml per rat) was provided once a week in order to obviate rapid deterioration of those groups on the deficient diets. Despite this measure, a gradual fall in weight and loss of condition in these animals necessitated discontinuation of the experimental diets for varying periods as indicated

in Figs. 8.1, 8.2. During these intervals a normal diet was provided and the study was temporarily suspended, i.e. no urine collections were made. In those groups on ethanol 20%(v/v), this solution was provided in lieu of drinking water throughout the experiment.

Urinary porphyrin excretion was determined on 24-hour urine samples collected at intervals as shown in Figs. 8.4, 8.5. For this purpose, 3 rats per group were used. Except where the death of an animal prevented it, the same 3 rats were selected on each successive occasion. The animals were kept for 24 hours in a cage suspended over a glass collecting system which separated urine and faeces. Food but not fluid was withheld during the 24-hour period to obviate undue contamination of the specimen. At the end of this time the animals were returned to the group cage. The collecting system was then washed down with a small volume of distilled water and the washings added to the collective urine specimen which was thereafter diluted to 15 ml with distilled water and stored at $-20^{\circ}C$ to await analysis.

No special measures were taken to preserve porphyrin precursors during the collection period, since for practical reasons it was not possible to refrigerate the urine at this time. Nevertheless, estimation of PBG and total aminoketones was carried out in addition to urinary URO and COPRO in order to detect any major increase in the excretion of these precursors should this occur. The methods employed are described in Section A.9.

At 7, 14 and 48 weeks after the commencement of the experiment a representative sample of animals from each group was sacrificed by decapitation. Livers were immediately removed for biochemical and histological examination. ALA synthetase activity was determined at all three time periods as described in Section A.1.1.1, method (ii); coproporphyrinogen oxidase activity was assayed only at 48 weeks as described in Section A.1.2. At this stage, portions of all livers were examined for porphyrin fluorescence under ultraviolet light. In

order to assess the degree of siderosis and associated pathology accompanying the biochemical changes, all livers removed were examined histologically. Special stains were employed to demonstrate the presence of iron (Perl's reaction) fat (Sudan III) and fibrous tissue (van Gieson).

8.3 Results.

8.3.1 Growth. The only group of animals which remained in good condition throughout the experiment were those on a normal diet and water regime (Group 1). From Fig. 8.1, it is apparent that the mean weight for this group increased by approximately 20% during the course of the experiment. By contrast, all other groups began to lose weight at some stage which necessitated substitution of normal rat food and water for the experimental regimes at various intervals (Figs. 8.1, 8.2) in order to avert undue deaths. Despite this measure, the mortality was particularly high in those groups drinking alcohol instead of water (Figs. 8.1, 8.2). The animals which lost weight most dramatically were those in Groups 3 and 6 receiving iron-enriched diets.

8.3.2 Histological findings. The development of hepatic siderosis in the two groups of rats subjected to dietary iron overload is depicted in Fig. 8.3. No significant difference was detected between the livers of animals on the maize plus iron plus water regime (Group 3) and those fed maize plus iron plus ethanol (Group 6).

As early as the seventh week there was a considerable amount of histochemically demonstrable iron in the parenchymal liver cells. Haemosiderin, visible as discrete granules, was almost confined to the periphery of the lobule. At fourteen weeks iron was present throughout the lobule although the distribution remained chiefly periportal. Individual parenchymal cells were more heavily laden and in addition clumping of pigment was visible within phagocytes around the central veins. By the end of the experiment (48 weeks) the deposition of haemosiderin throughout the lobule was still more intense but as in the

Fig. 8.1 Weight progress charts for rats on experimental regimes:-

group 1 ..... normal diet + water

group 2 ..... maize diet + water

group 3 ..... maize + Fe + water

Each point represents the mean body weight for the group.

⊖ = normal diet substituted for experimental diet

⊕ = experimental diet recommenced

Group	Mortality (%)
1	0
2	6.0
3	13.2
4	26.4
5	33.3
6	33.3

Fig. 8.2 Weight progress charts for rats on experimental regimes:-

group 4 ..... normal diet + alcohol

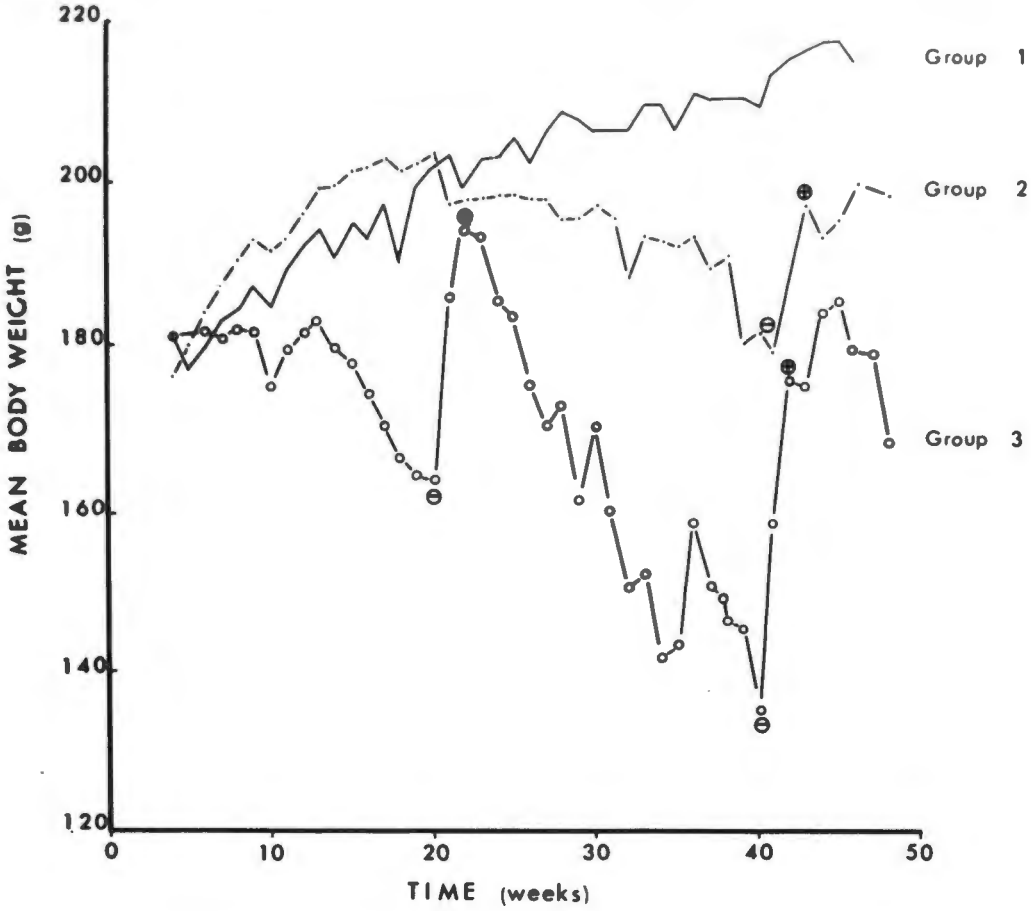
group 5 ..... maize diet + alcohol

group 6 ..... maize + Fe + alcohol

Each point represents the mean body weight for the group.

⊖ = normal diet substituted for experimental diet

⊕ = experimental diet recommenced


220
200
180
160
140
120
MEAN BODY WEIGHT (g)
0
10
20
30
40
50
TIME (weeks)
Group 1
Group 2
Group 3

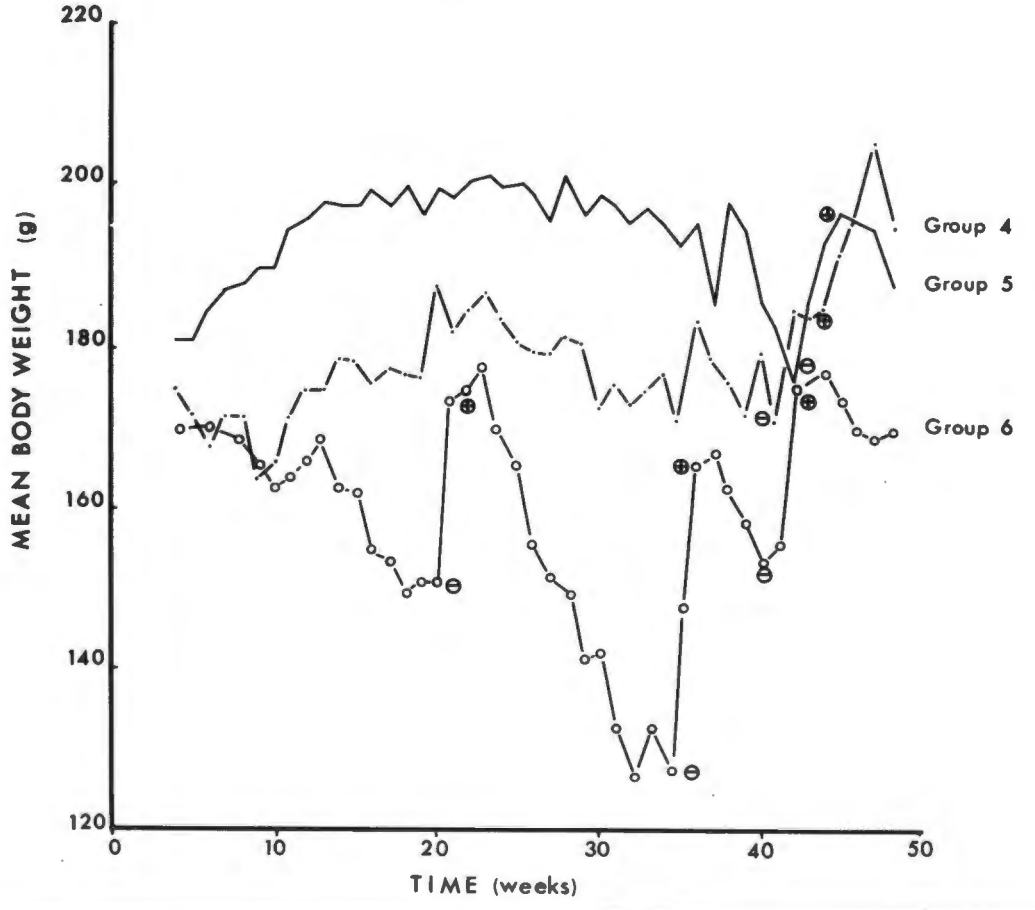

220
200
180
160
140
120
MEAN BODY WEIGHT (g)
0
10
20
30
40
50
TIME (weeks)
Group 4
Group 5
Group 6

Fig. 8.3 Photomicrographs showing distribution of iron in rat livers following an iron-enriched maize diet. No differences were detected between the group on the maize + Fe + water regime and the group on the maize + Fe + alcohol regime.

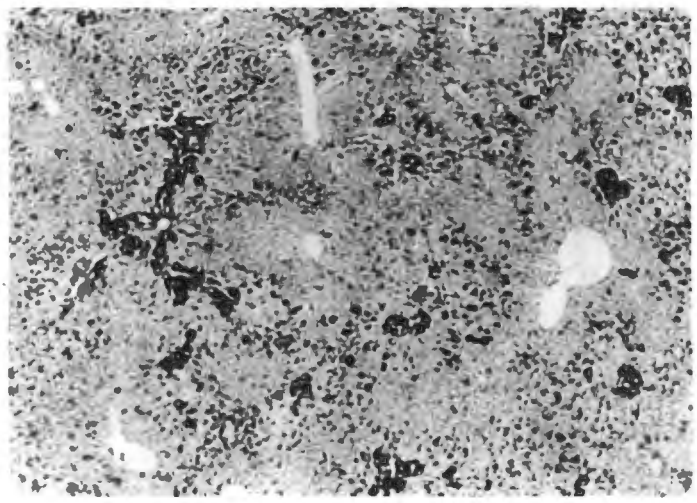
(Perl's reaction; magnification x 25)

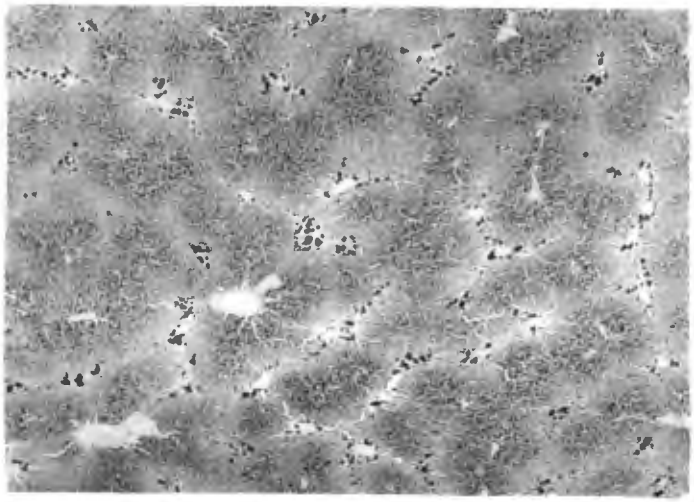
8.3a (top left) After 7 weeks.

8.3b (top right) After 14 weeks.

8.3c (bottom) After 48 weeks.

For full descriptions see Section 8.3.2.





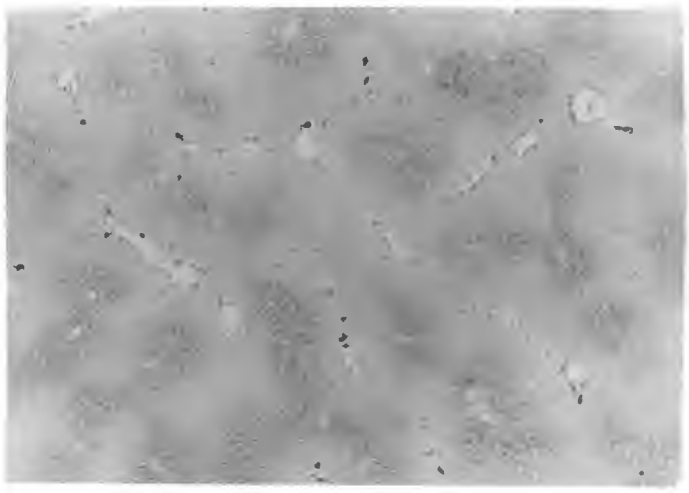


Fig. 8.4 Urinary URO excretion in the 6 groups of rats on experimental regimes:-

group 1 ..... normal diet + water

group 2 ..... maize diet + water

group 3 ..... maize + Fe + water

group 4 ..... normal diet + alcohol

group 5 ..... maize diet + alcohol

group 6 ..... maize + Fe + alcohol

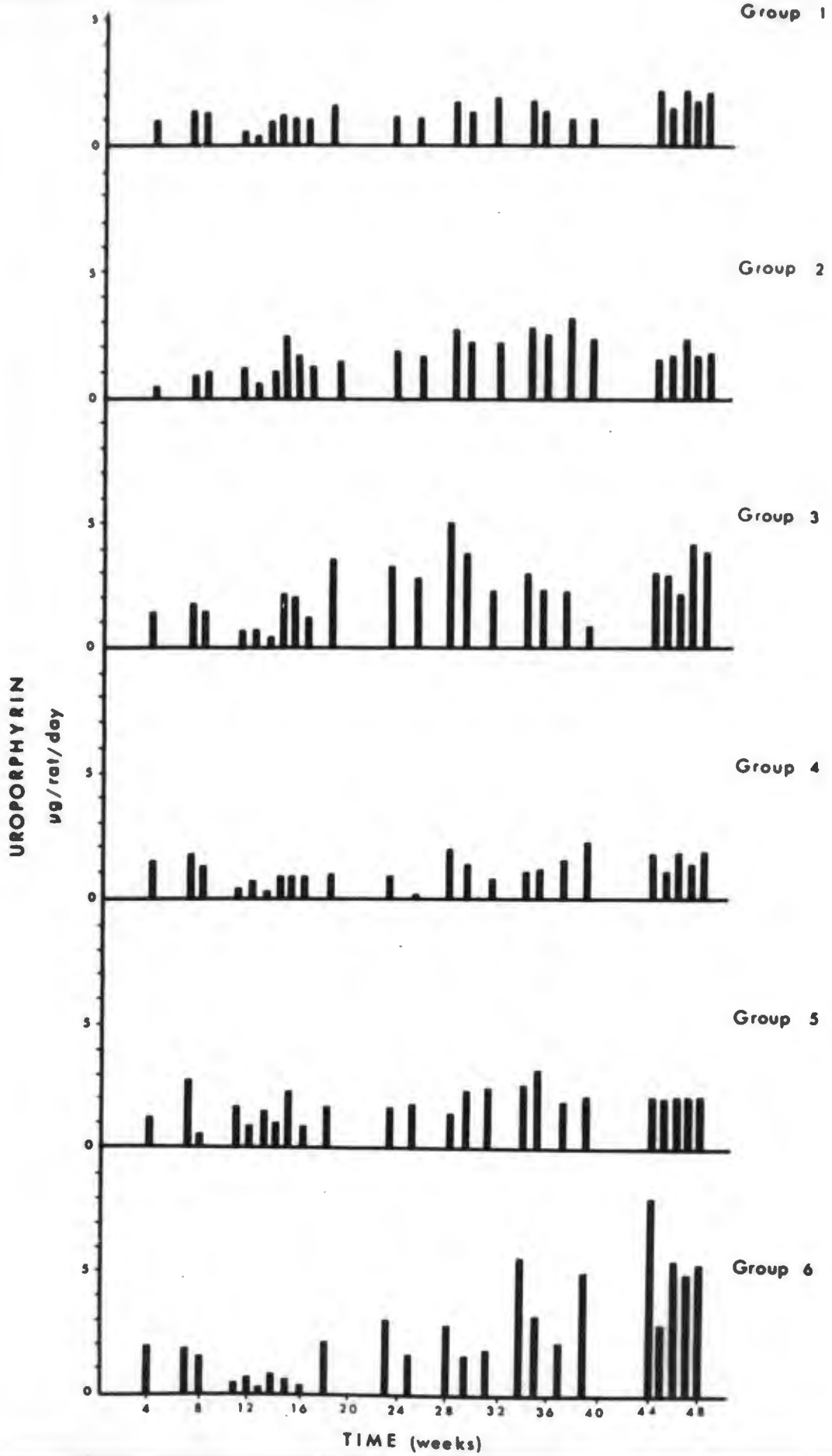

Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
5
0
UROPORPHYRIN
µg/rat/day
4
8
12
16
20
24
28
32
36
40
44
48
TIME (weeks)

Fig. 8.5 Urinary coproporphyrin excretion in the 6 groups of rats on experimental regimes:-

group 1 ..... normal diet + water

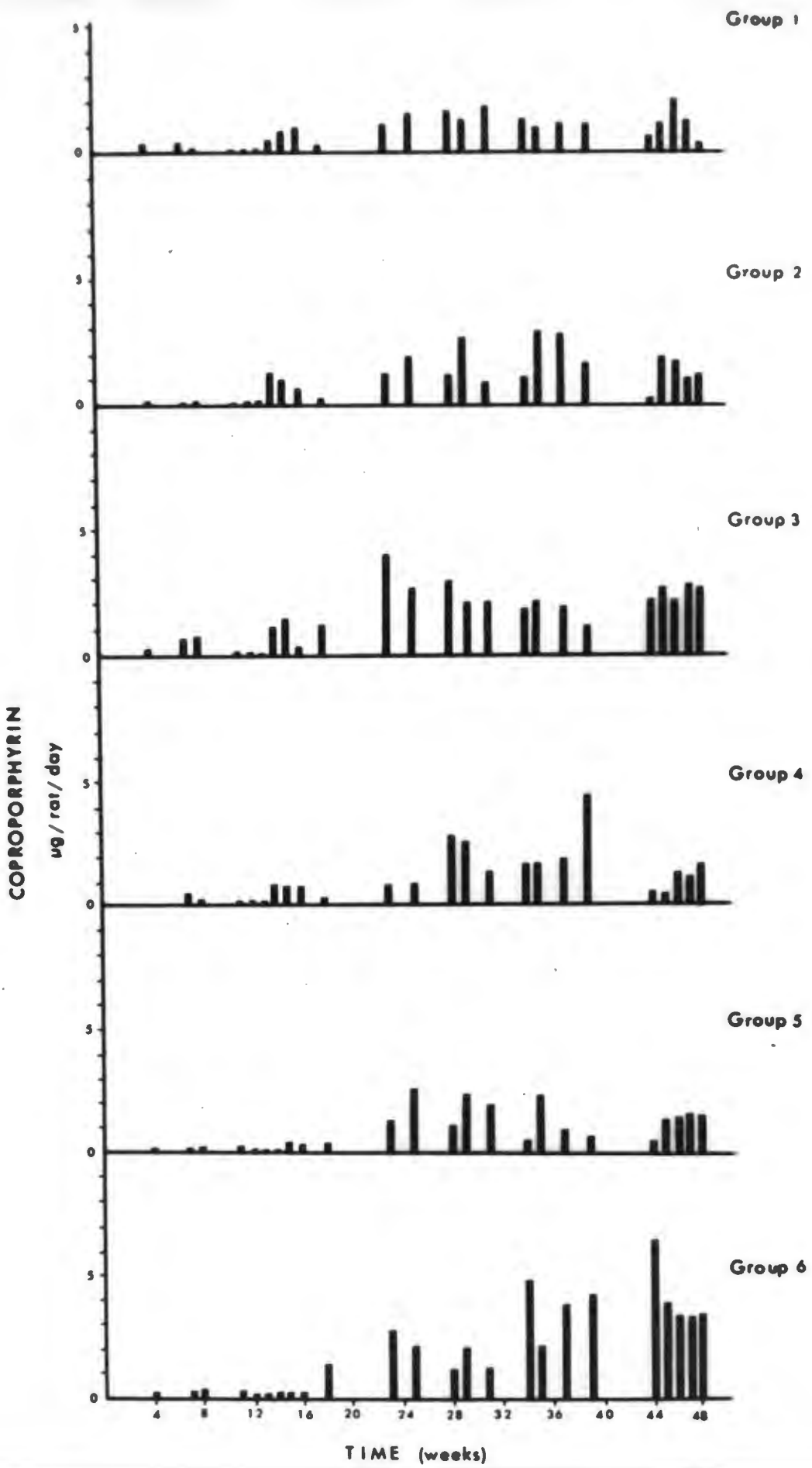
group 2 ..... maize diet + water

group 3 ..... maize + Fe + water

group 4 ..... normal diet + alcohol

group 5 ..... maize diet + alcohol

group 6 ..... maize + Fe + alcohol


Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
COPROPORPHYRIN
ug / rat / day
5
0
4
8
12
16
20
24
28
32
36
40
44
48
TIME (weeks)

earlier phases the concentration in the periphery was never obscured. The most striking feature at this stage was the marked clumping of iron pigment within phagocytes mainly in the vicinity of the portal tracts.

In the groups not receiving an iron-enriched diet, histochemichally demonstrable iron was present in trace amounts only within Kupffer cells. No significant fatty change, fibrosis or other histological abnormality was evident in any of the livers.

8.3.3 Liver fluorescence. No evidence of porphyrin fluorescence was detected in any of the livers examined.

8.3.4 Urinary total aminoketones, PBG, URO and COPRO. Figs. 8.4 and 8.5 illustrate graphically the mean daily urinary output of URO and COPRO respectively, expressed as µg/rat/24 hours, for the six groups of animals at the various time periods indicated.

It is obvious that in the early part of the experiment no consistent effect was produced by any of the treatments. After about 20 weeks, however, differences began to appear. The combination of a maize diet with iron plus ethanol (Group 6) clearly produced a significant increase in the urinary excretion of both URO and COPRO in the latter part of the experiment. In addition it appears that a maize diet with iron, in the absence of alcohol, also increased urinary URO and COPRO excretion significantly but to a lesser extent. On the other hand, none of the other treatments produced any consistent increase in porphyrin excretion. Statistical analysis of the results over the last 5 weeks was carried out; analysis of variance according to Scheffé (1959) as set out in Appendix C (Section C.1.1) confirmed that the foregoing impressions are correct. It will be noted that in Section C.1.1 it is stated that alcohol of itself significantly affected urinary URO excretion. From Table C.1, however, it appears that this effect was not consistent and hence may be discarded.

With regard to PBG and total aminoketones, no significant increase in the urinary excretion of these substances was found in any

Table 8.2 Liver ALA synthetase and coproporphyrinogen oxidase activity in rats on experimental regimes.

Group	ALA Synthetase Activity mμmoles ALA formed/g liver/hr.						Coproporphyrinogen Oxidase Activity mμmoles protoporphyrin formed/mg mitochondrial protein	
	7 weeks		14 weeks		48 weeks		48 weeks	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	37.1	12.0	15.9	14.9	33.3	13.9	2.27	0.14
2	16.9	12.8	9.7	4.1	32.8	11.6	2.61	0.15
3	11.3	5.5	17.5	3.6	39.5	12.5	2.76	0.77
4	29.7	19.8	28.4	9.8	59.5	--	3.05	0.12
5	30.8	15.4	13.7	15.0	69.5	20.1	2.03	0.20
6	18.7	10.5	16.9	4.6	76.8	2.2	2.98	0.35

of the groups at any stage of the experiment.

8.3.5 Hepatic ALA synthetase and coproporphyrinogen oxidase activity.

The results of these enzyme assays are summarised in Table 8.2. Although some differences between groups are evident for ALA synthetase activity at 7 and 14 weeks, none of the treatments gave values greater than those recorded for normal rats (Group 1). At the end of 48 weeks, on the other hand, it appears that Groups 4, 5 and 6, receiving alcohol, all showed considerably increased ALA synthetase activity compared with Groups 1, 2 and 3, not receiving ethanol.

Statistical analysis of these results (at 48 weeks) according to Scheffé (1959) as set out in Appendix C (Section C.1.3) confirmed that alcohol alone had a highly significant effect on ALA synthetase activity whereas the diets alone had no significant effect. The only significant interaction was between the maize diet and alcohol.

Coproporphyrinogen oxidase activity was obviously not impaired by a maize and iron diet, either alone or in combination with ethanol. On the contrary, statistical analysis according to Scheffé (1959) as set out in Appendix C (Section C.1.2) revealed minor differences in the opposite direction.

8.4 Discussion.

8.4.1 Effect of chronic dietary iron overload on urinary porphyrin excretion. It is apparent from the present study that chronic dietary iron overload resulting in hepatic siderosis can affect porphyrin excretion in rats. The urinary output of both URO and COPRO was increased in these animals, an observation which cannot be attributed to non-specific liver damage. The pattern of urinary porphyrin excretion obtained in these rats was not unlike that found in human subjects with symptomatic porphyria. It must be stressed, however, that iron overload did not produce 'experimental symptomatic porphyria' in this study, although it is conceivable that over a still longer period on iron-enriched diets rats may become frankly porphyric. In

extrapolating from these findings to symptomatic porphyria certain problems must be considered.

Firstly, it may be argued that the present experiment was unphysiological in that the dietary iron overload was far greater than is experienced by siderotic patients. This is undoubtedly true. However, the object of the study was not to determine whether dietary iron overload leads to hepatic siderosis since this is well established (Section 5.4.4). The purpose was rather to determine whether this experimental siderosis is associated with disturbed porphyrin metabolism. For practical purposes, therefore, it was necessary to use unphysiological concentrations of iron in the diet to expedite deposition of haemosiderin in the liver cells. In the event, the degree of siderosis produced in these animals (Fig. 8.3) was in no instance as severe as is often encountered in human subjects, both porphyric and non-porphyric (Sections 2.2.3.1, 2.2.4).

Secondly, is experimental siderosis in the rat comparable with the human condition as encountered in South Africa? Siderosis in the African begins as a peripheral or periportal deposition of haemosiderin and progresses to the centre of the lobule until all the parenchymal cells contain iron pigment. The periportal areas, however, remain more heavily laden with haemosiderin throughout. Later, clumps of pigment begin to appear chiefly in the portal tracts which eventually are packed with iron pigment. The distribution of haemosiderin in the parenchyma at this stage becomes more variable, probably due to degeneration and regeneration of liver cells, the newly formed cells containing much less pigment than the older ones. Haemosiderin liberated by disintegrated cells is swept up by phagocytes and taken to the portal tracts (Wainwright 1957).

The histological pattern observed in the rat livers in the present study is identical with that reported in rats by Gillman et al. (1959) employing the same experimental diets, and in general resembles the picture seen in African subjects with hepatic siderosis.

Points of difference are the intensity of iron deposition as mentioned above and the distribution of iron-laden phagocytes. Whereas these cells are congregated in the portal tracts in human cases, in the livers examined in the present experimental study and those described by Gillman et al. (1959) deeply stained phagocytes were also prominent near the central veins. These differences may be related to (i) the comparatively short duration of the experiment in the rats as opposed to the period of many years of dietary iron overload in most human siderotic subjects, and (ii) possible anatomical differences between rat and human liver with regard to the distribution of phagocytes.

The third problem is : if excessive iron in the liver cell plays an aetiological role in the pathogenesis of symptomatic porphyria, why is this disease not much more common? Wainwright (1957) and Bothwell and Bradlow (1959) found that 80% of adult male Africans exhibited siderosis which was severe in at least 24% of cases (Wainwright 1957). It has been suggested that the presence of siderosis in patients with symptomatic porphyria is incidental to the development of the disease and is related instead to the excessive intake of iron-containing alcoholic beverages (Kramer 1963). There is, however, further strong circumstantial evidence which makes this suggestion unlikely. Uys and Eales (1963) reported that "siderosis constituted the most constant single abnormality" presented by the livers of patients with symptomatic porphyria. They found excessive iron in all 40 cases examined and in 95% this was classified as grade - C (Section 2.2.3.1). When it is considered that only 8 of these patients were African while 30 were Coloured and 4 were White, these findings assume particular significance since siderosis is relatively uncommon in the latter 2 population groups. Uys et al. (1960) found at autopsy an incidence of 29.1% among the Cape Coloureds and 30.1% in the White group. Hence, on the basis of the findings in the present study, it seems reasonable to suggest that siderosis may play an important part in the development

of abnormal hepatic porphyrin metabolism in human subjects, culminating in acquired, symptomatic porphyria. It may be that individual idiosyncrasy or some other predisposing factor determines whether excessive iron in the liver exerts a porphyrinogenic effect or not and thus accounts for the fact that not all siderotic subjects become porphyric.

8.4.2 The mode of action of excessive hepatic iron on porphyrin metabolism. It is apparent from the present study that iron overload alone did not affect liver ALA synthetase activity (Table 8.2) and therefore porphyrin precursor production. In some other way, however, the presence of excessive iron in the liver cell determined the loss of greater than normal amounts of haem precursors as URO and COPRO.

At what stage in haem biosynthesis might such an effect occur? It has been suggested that iron might produce a relative block in haem biosynthesis at the stage of conversion of COPROgen to PROTOgen through an effect on coproporphyrinogen oxidase activity (Section 5.4.4). This enzyme is located in the mitochondrial membrane and gross abnormalities of this organelle have been reported in experimental siderosis (Theron et al. 1963). In the present study coproporphyrinogen oxidase activity in siderotic liver mitochondria was found to be unimpaired, indicating that excessive iron does not denature this enzyme. On the other hand mitochondrial membrane permeability to COPROgen was not tested since solubilised mitochondria were employed in the assay. However, damaged mitochondria are inclined to be more permeable than normal to substrates and therefore it does not seem likely that decreased activity of this enzyme *in vivo* might result from inability of COPROgen to reach the active site. As far as symptomatic porphyria is concerned, evidence against a block in liver haem biosynthesis is the finding of Dowdle et al. (1968) (Section 5.4.2) of normal early-labelling of bilirubin in this disease. An alternative explanation is that, while haem biosynthesis is not decreased, greater than normal amounts of haem precursors are being 'drawn off' at some stage in the biosynthetic

sequence and are being 'metabolically sequestered' as URO and COPRO.

Excessive intracellular oxidation of porphyrinogens to porphyrins has been suggested as the source of the excessive hepatic and excreted porphyrins in symptomatic porphyria (Heikel et al. 1958, Sweeney 1963) (Section 5.4.2). Such a mechanism could explain the porphyrin excretion pattern in this disease but there is no evidence to suggest that the intracellular milieu favours oxidation and, moreover, alcohol which aggravates the disease is known to increase hepatic $NADH_2/NAD$ ratios and hence favours reduction (Smith and Newman 1959, Isselbacher 1961).

Yet another concept which must be given serious consideration is that in symptomatic porphyria there may be an alternative pathway, other than the accepted haem biosynthetic sequence, which synthesises porphyrins from ALA. It may be that excessive iron in the liver stimulates such a pathway.

8.4.3 The combined effect of ethanol and iron on porphyrin metabolism.

In the absence of iron overload, voluntary consumption of alcohol by animals in the present study did not cause increased urinary excretion of porphyrins. However, when ethanol was combined with an iron-enriched diet there was a significant increase in the output of both URO and COPRO. This finding is particularly interesting, suggesting that the basic abnormality was caused by the excessive iron in the liver and that ethanol simply aggravated the situation. This accords well with the concept that abuse of alcohol is not the prime cause of symptomatic porphyria but is an important exacerbating factor.

The aggravating effect of alcohol on porphyrin excretion is reflected in the results of the ALA synthetase assays. At the end of the study (48 weeks) liver ALA synthetase activity was significantly increased in all groups drinking alcohol. Hence it seems reasonable to suggest that alcohol produced a situation of chronic endogenous overproduction of ALA which in the siderotic animals could not be

handled in the normal way and was disposed of as URO and COPRO. The fact that liver ALA synthetase activity was not increased in the alcohol-consuming rats at the 7 and 14 week intervals appears anomalous but is probably due to the fact that rats do not readily take to drinking alcohol. In the early part of the experiment they were drinking minimal quantities, whereas latterly they drank the 20% solution as readily as normal rats drink water.

8.5 Conclusions. Experimental siderosis in the rat due to dietary iron overload leads to increased urinary excretion of URO and COPRO which is further aggravated by concurrent drinking of alcohol in accordance with the demonstrated increase in liver ALA synthetase activity. Unlike ethanol, iron apparently does not cause increased liver ALA synthetase activity but rather seems to lead to increased disposal of available haem precursors as URO and COPRO. The mechanism of this effect is unknown. It does not appear to be mediated via an effect on hepatic mitochondrial coproporphyrinogen oxidase. It is suggested that excessive iron in the liver may promote the synthesis of porphyrins from ALA via a pathway other than the normal haem biosynthetic sequence.

On the basis of these findings, it seems reasonable to suggest that the high incidence of hepatic siderosis in symptomatic porphyria, irrespective of race, is more than incidental. It is highly likely that the presence of excessive iron in the liver cells is an important factor determining the development of symptomatic porphyria. A poor diet (i.e. maize meal) in itself appears to have no effect on ALA synthetase activity or urinary porphyrin excretion.

THE PRESENT STUDIES

SECTION B

THE MODE OF ACTION OF ALCOHOL ON LIVER ALA SYNTHETASE - STUDIES IN EXPERIMENTAL AND SYMPTOMATIC PORPHYRIA.

INTRODUCTION

It was concluded from the studies described in Section A that the effect of ethanol in symptomatic porphyria is mediated via increased activity of hepatic ALA synthetase, the rate-limiting enzyme for liver haem biosynthesis. The second section of this thesis is concerned with the problem of how alcohol might induce this enzyme.

Of the various hypotheses discussed in Chapter 4 regarding the mode of induction of liver ALA synthetase by porphyrinogenic agents, that which seems most applicable to alcohol is Labbe's (Section 4.2.2). AIA and many other porphyrinogenic drugs are inhibitors of mitochondrial terminal oxidation and Labbe has postulated that their inducing potential stems from this property.

Under these conditions a raised intramitochondrial $NADH_2/NAD$ ratio, he suggests, is the stimulus for the reductive synthesis of succinate from fumarate. In the livers of mice made porphyric with AIA, Labbe and co-workers have demonstrated $NADH_2$-dependent fumarate reductase activity and the presence of a non-constitutive succinyl CoA synthetase. Succinyl CoA, thus generated in a separate intramitochondrial 'compartment' from constitutive succinyl CoA of the tricarboxylic acid cycle, might then act as a derepressor of ALA synthetase synthesis by substrate induction.

It is further proposed by Labbe that pyruvate is the carbon source which augments the fumarate pool via a reductive pathway involving oxaloacetate and malate, and thereby supports a net increase in succinate synthesis.

The influence of ethanol in increasing the liver cytoplasmic $NADH_2/NAD$ ratio and the possible mechanisms whereby this effect might be reflected in the intramitochondrial redox state have been fully discussed in Section 5.4.3.2. It was concluded that during ethanol oxidation in the liver the intramitochondrial $NADH_2/NAD$ ratio might be sufficiently increased to lead to the induction of ALA synthetase as postulated by Labbe.

Since alcohol has been shown to be a relatively poor 'inducer' of

ALA synthetase, it was decided to test the validity of Labbe's hypothesis, initially, with regard to a potent inducing agent, viz., AIA. This hypothesis may be tested in several ways and in the present thesis two experimental approaches, using rats treated with AIA, were employed. Firstly, the aim in Chapter 9 was to determine whether pyruvate could in fact be the carbon source augmenting the fumarate pool in experimental porphyria. Secondly, in Chapter 10 it was intended to demonstrate whether hydrogen transfer via NAD to succinate and ultimately liver haem is increased in experimental porphyria as would be expected if Labbe's proposals were valid.

If the effect of alcohol on ALA synthetase in symptomatic porphyria is a function of its influence on the redox state of the liver, it would be logical to expect a correlation between these two parameters. In Chapter 11 a study is described in which liver biopsy for ALA synthetase assay was performed on patients with symptomatic porphyria together with an intravenous galactose tolerance test which, under certain conditions, provides a measure of the liver cytoplasmic $NADH_2/NAD$ ratio.

An explanation for the action of alcohol in inducing ALA synthetase was also sought in terms of the proposals of Granick regarding steroid control of haem biosynthesis (Section 4.2). Granick showed that certain steroids including aetiocholanolone in the free (unconjugated) form are potent inducers of liver porphyrin synthesis and postulated that these compounds may play a role in the normal control of liver haem biosynthesis (Section 4.2.1). In view of this, induction of porphyrinogenesis by unconjugated steroids in symptomatic porphyria is a distinct possibility, since all these patients have some degree of hepatocellular pathology which could result in defective conjugation (Section 5.4.1). In this event, alcohol could conceivably have an aggravating effect by further interfering with the conjugation process. The production of uridine diphosphate glucuronic acid (UDPGA) is linked to the reduction of NAD (Fig. 12.2) and it seems reasonable to suggest that this process

may be impaired in the presence of an increased $NADH_2/NAD$ ratio resulting from the hepatic dehydrogenation of alcohol. In Chapter 12 the object was to gauge the efficacy of conjugation of the steroid, aetiocholanolone, in patients with symptomatic porphyria and to ascertain how this is affected by administration of alcohol.

CHAPTER 9

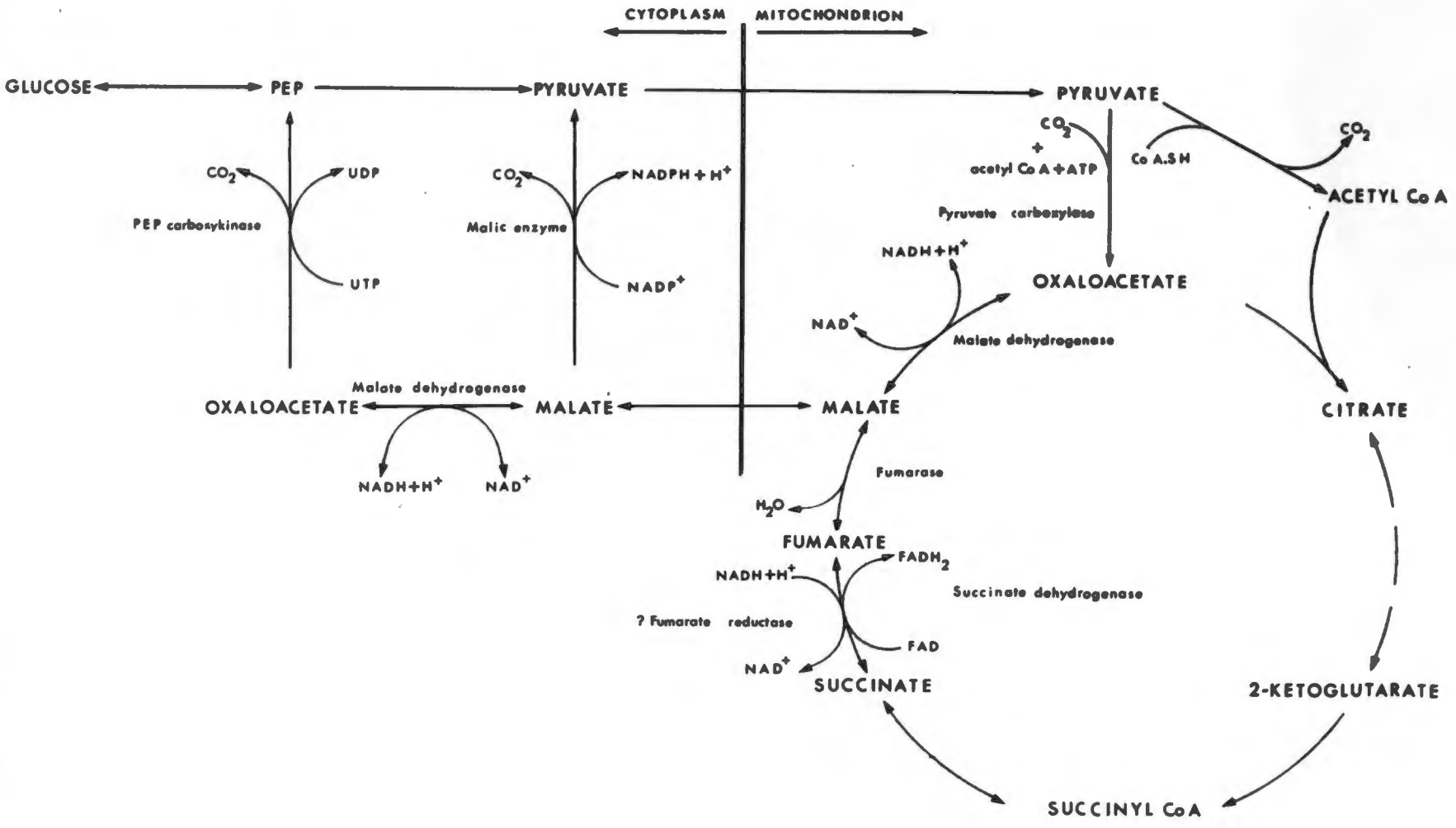
CARBON SOURCES FOR AUGMENTED HEAM SYNTHESIS IN EXPERIMENTAL PORPHYRIA.

9.1 Aim of the study. If there is a net increase in reductive synthesis of succinate from fumarate by an NAD-dependent fumarate reductase as a prelude to the induction of ALA synthetase in AIA-induced porphyria, as proposed by Labbe (Section 4.2.2), it is obvious that the fumarate pool must be augmented from some source.

Labbe has suggested that the source is pyruvate (Section 4.2.2) which after carboxylation to either oxaloacetate or malate can be converted to fumarate by the sequence of reactions shown in Fig. 9.1. If this hypothesis is valid it should be possible to demonstrate increased carboxylation of pyruvate accompanying increased activity of fumarate reductase and ALA synthetase in the livers of rats treated with AIA. It is the aim of this study to test Labbe's hypothesis by showing whether or not this association exists.

9.2 Experimental approach. Pyruvate may be carboxylated to oxaloacetate or malate by the enzymes pyruvate carboxylase (Utter and Keech 1963) and malic enzyme (Ochoa et al. 1948) respectively. Under physiological conditions, however, malic enzyme operates in the reverse direction, catalysing the oxidative decarboxylation of malate to pyruvate (Utter, Keech and Scrutton 1964). In effect, therefore, increased carboxylation of pyruvate must be accomplished by pyruvate carboxylase. This enzyme is rate-limiting for a number of pathways and is inducible, e.g. by starvation (Freedman and Kohn 1964). Hence, in accordance with Labbe's views, it would be reasonable to expect increased pyruvate carboxylase activity in the livers of AIA-treated animals at a time when ALA synthetase was undergoing induction. Fumarate reductase activity should likewise be concurrently increased. Consequently, in this study the activity of these 3 enzymes was compared in the livers of normal rats and those of rats sacrificed 3 hours after a single large dose of AIA. Fed animals were used since pyruvate carboxylase is maximally repressed by feeding and it was felt that under these conditions any

Fig. 9.1 Schematic representation of the metabolic fate of pyruvate. (Conversion of pyruvate and oxaloacetate to aminoacids has been omitted.)


CYTOPLASM
MITOCHONDRION
GLUCOSE
PEP
PYRUVATE
PYRUVATE
CO_2
UDP
PEP carboxykinase
UTP
CO_2
NADPH + H^+
Malic enzyme
$NADP^+$
CO_2
+
acetyl Co A + ATP
Co A.S H
CO_2
ACETYL Co A
Pyruvate carboxylase
$NADH+H^+$
OXALOACETATE
NAD^+
Malate dehydrogenase
Malate dehydrogenase
OXALOACETATE
MALATE
MALATE
CITRATE
$NADH+H^+$
NAD^+
Fumarase
H_2O
FUMARATE
$FADH_2$
$NADH+H^+$
Succinate dehydrogenase
? Fumarate reductase
FAD
NAD^+
SUCCINATE
2-KETOGLUTARATE
SUCCINYL CoA

increase in activity would be more easily demonstrated. Two further determinations were carried out in the course of this study, viz., liver acetyl coenzyme A (acetyl CoA) content and malic enzyme activity. The reasons are outlined below.

The obligatory requirement by pyruvate carboxylase for acetyl CoA led Keech and Utter (1963) to propose that the concentration of this coenzyme in the liver may exert a controlling effect on the activity of the enzyme. It has since been shown that conditions which lead to increased pyruvate carboxylase activity, e.g. ketosis (Freedman and Kohn 1964), also lead to an increase in liver acetyl CoA content (Wieland and Wiess 1963). Hence in the present experiments the liver content of this coenzyme was also estimated in normal and porphyric animals.

Intramitochondrially synthesized oxaloacetate, the product of pyruvate carboxylase activity, can be metabolised by several pathways, apart from reductive conversion to succinate. These alternative routes shown in Fig. 9.1 include (1) oxidation in the TCA cycle, or after reduction to malate and diffusion into the cytoplasm, (2) gluconeogenesis or (3) reconversion to pyruvate through the action of malic enzyme. The possibility that one or more of these pathways might be inhibited by AIA, and thereby render greater amounts of oxaloacetate available for net reductive synthesis of fumarate and succinate, despite unchanged pyruvate carboxylase activity, must therefore be considered.

With respect to oxidative TCA cycle activity there is good evidence that this is unaltered by AIA (Tschudy et al. 1962, Labbe et al. 1965). Similarly, there is no evidence that AIA affects gluconeogenesis and, in any event, inhibition of this process would be reflected in decreased activity of pyruvate carboxylase which is rate-limiting (Krebs 1964). On the other hand, malic enzyme which under physiological conditions oxidatively decarboxylates malate to pyruvate (Utter, Keech and Scrutton 1964), and which is not a rate-limiting enzyme, could con-

ceivably be inhibited by AIA (while pyruvate carboxylase activity remained unchanged), thus leading to increased availability of oxaloacetate for reductive synthesis. Hence malic enzyme activity was measured in the livers of the normal and AIA-treated rats.

It may be argued that measurement of substrate concentrations in addition to these enzyme assays would yield more decisive information, However, it is not practicable to measure intramitochondrial substrate concentrations and determination of substrates in whole liver would not be helpful in the present context.

9.3 Materials and methods. Allyl*iso*propylacetamide dissolved in physiological saline solution (at a concentration of 20 mg/ml) was administered by the intraperitoneal route to groups of fed adult female Wistar rats weighing between 150 and 200 g. In each experiment animals were closely matched for age and weight. Rats were used instead of mice, as employed by Labbe and co-workers (Section 9.1), since they were more readily available. The dosage of AIA was in all cases 400 mg/kg body weight. Physiological saline was administered alone (in equivalent volume) by intraperitoneal injection to similar groups of control animals.

Separate experiments were conducted for the assay of each different enzyme and for the determination of acetyl CoA content. Three hours after the commencement of the experiment, control and test groups were sacrificed by decapitation and the livers immediately removed and cooled as indicated in the following methods. ALA synthetase activity was determined according to the method for whole homogenate of rat liver described in Section A.1.1.1, method (ii), pyruvate carboxylase according to Utter and Keech (1963) (Section A.1.3), malic enzyme according to Ochoa (1955) (Section A.1.4), fumarate reductase according to Kurumada and Labbe (1966) (Section A.1.5), and acetyl CoA according to Wieland and Weiss (1963) (Section A.3). As a further check on the pyruvate carboxylase assay system, the activity of this enzyme was also

Table 9.1 ALA synthetase, pyruvate carboxylase, malic enzyme and fumarate reductase activity plus acetyl CoA concentration in livers of fed female rats three hours after administration of AIA (400 mg/kg).

ESTIMATION	UNITS	No. of animals per group	Preparation used	RESULTS			
				Control animals		AIA-treated animals	
				Mean	S.D.	Mean	S.D.
ALA synthetase activity	mμmoles ALA formed/g liver per hour	3	whole homogenate	39.3	3.1	178	0
Pyruvate carboxylase activity	mμmoles $NaHCO_3$ fixed/mg mitochondrial protein (in 5 mins at 30°C)	5	fresh mitochondria	49.0	3.4	55.0	10.0
		3	mitochondrial acetone powders	82.0	16.5	67.0	14.5
		2*		222.0	35.3		
Acetyl CoA	mμmoles/g liver	5	whole homogenate	38.5	7.5	39.6	6.1
Malic enzyme activity	mμmoles malate decarboxylated /minute/mg protein	4	105,000 *g* supernatant	3.6	1.1	3.7	0.8
Fumarate reductase activity	mμmoles hydroxamate formed/hour/mg protein	3	fresh mitochondria and mitochondrial acetone powders	inconsistent results			
		* fasted for 40 hours					

assayed in the livers of several control animals, fasted for 40 hours.

9.4 Results. The results are summarised in Table 9.1. Three hours after AIA administration liver ALA synthetase activity was between 4 and 5 times greater than in control animals which received a saline injection. At this time, on the other hand, there was no significant difference between AIA-treated and control groups with respect to mean activity of liver pyruvate carboxylase and malic enzyme. In animals simply fasted for 40 hours, however, pyruvate carboxylase activity was markedly increased. The liver acetyl CoA content in control and test groups was not significantly different.

It proved impossible to demonstrate fumarate reductase activity with any consistency in the livers of either AIA-treated or control rats. Since it was suspected that there might be a species difference with regard to this enzyme, liver mitochondria from normal and AIA-treated white mice (as used by Labbe) were also assayed, with equally unsuccessful results. In 65% of cases no activity could be demonstrated, while in the remaining instances values which bore no relationship to the experimental conditions and which ranged from 4 - 74 mμmoles hydroxamate formed/hour/mg mitochondrial protein were obtained.

9.5 Discussion. In the present study the levels of liver ALA synthetase activity recorded in the AIA-treated rats clearly demonstrate that induction of this enzyme occurred within 3 hours. This is consistent with the data of Marver, Collins, Tschudy and Rechcigl (1966) who showed that in rats, following a single injection of AIA (400 mg/kg body weight), hepatic ALA synthetase activity increased almost immediately and reached a peak 12 to 16 hours later.

After 3 hours pyruvate carboxylase activity was unaffected by administration of AIA, irrespective of whether fresh mitochondria or mitochondrial acetone powders were used in the assay. By contrast, markedly increased values were obtained in normal fasted animals, indicating that this enzyme is readily inducible by starvation. The finding of a similar acetyl CoA content in the normal and porphyric

rat livers is to be expected if the concentration of this coenzyme does exert a regulatory effect on pyruvate carboxylase activity (Section 9.2).

The absence of any influence of AIA on pyruvate carboxylase activity in fed animals is particularly significant since this enzyme which is rate-controlling for conversion of pyruvate to dicarboxylic acids by the reductive pathway shown in Fig. 9.1, is maximally repressed by feeding (Table 9.1). It must therefore be concluded that, if pyruvate is the source augmenting the fumarate pool in experimental porphyria as prcposed by Labbe, this augmentation is not accomplished by induction of pyruvate carboxylase. Under these circumstances the only possible alternative mechanism is inhibition of one or more of the other pathways of oxaloacetate disposal (Section 9.2) which are discussed below.

The first possibility is decreased oxidative activity of the TCA cycle. Several workers have produced evidence that TCA cycle activity is unchanged by AIA. Tschudy et al. (1962) found that normal and porphyric liver slices formed $^{14}CO_2$ from α-ketoglutarate-5-^{14}C at the same rate. Labbe et al. (1965) found the same difference between normal and porphyric mice with respect to the incorporation of radioactivity into liver haem from succinate-1-^{14}C and from succinate-2,3-^{14}C when the results were expressed as counts/minute/ labelled carbon atom. Since succinate-1-^{14}C becomes unlabelled during an oxidative turn of the TCA cycle, it can be incorporated into liver haem only via direct conversion to succinyl CoA. By contrast succinate-2,3-^{14}C can label haem both via direct conversion to succinyl CoA and after traversing the TCA cycle. Hence, if TCA cycle activity were altered after administration of AIA, these two labelled precursors should have behaved differently with respect to labelling of haem. Labbe et al. (1965) also reported that both α-ketoglutarate-5-^{14}C and acetate-2-^{14}C labelled haem to the same extent in control and porphyric livers. On the basis of these various findings it seems reasonable to discount the possibility

of inhibition of oxidative TCA cycle activity in experimental porphyria.

The second alternative route of oxaloacetate disposal is conversion to phosphoenolpyruvate (PEP) in the gluconeogenic pathway (Fig. 9.1). However, as pointed out in Section 9.2, there is no evidence that AIA affects gluconeogenesis; and the fact that pyruvate carboxylase activity was unaltered in the present study confirms that AIA neither inhibits nor promotes gluconeogenesis.

Thirdly, the possibility of altered malic enzyme activity must be considered. The mitochondrial membrane is relatively impermeable to oxaloacetate, whereas malate can diffuse freely in either direction (Lardy et al. 1965). In rat liver, pyruvate carboxylase is located in the mitochondrion (Utter and Keech 1963) and hence oxaloacetate synthesized by this enzyme cannot enter the cytoplasm. According to the view of Lardy et al. (1965) oxaloacetate is reduced intramitochondrially to malate which then diffuses into the cytoplasmic compartment where it may become substrate for malic enzyme. Utter, Keech and Scrutton (1964) have shown that, under physiological conditions, this enzyme operates in the direction of oxidative decarboxylation of malate to pyruvate, with the production of $NADPH_2$. It has been proposed (Shrago and Lardy 1966, Krebs et al. 1967) that the malate-oxaloacetate system, operating in this fashion, is a device to 'pump' intramitochondrial hydrogen to the cytoplasm. Here, through the action of malic enzyme, hydrogen may be transferred from malate to NADP and the resultant $NADPH_2$ may be used in reductive synthesis (Lowenstein 1961, Young et al. 1964, Wise and Ball 1964). If this pathway of malate disposal were inhibited by AIA, the supply of oxaloacetate for reductive synthesis of fumarate might be increased. In the event, malic enzyme activity was unaffected in the porphyric animals. Consequently, the present findings do not accord with the concept that pyruvate is the carbon source supplying additional substrate for increased haem precursor and haem synthesis under the influence of AIA.

The failure to demonstrate fumarate reductase activity with any consistency in liver mitochondria of rats or mice (the animal used by Labbe), despite the most careful attention to experimental detail, caused considerable concern. At first, it was thought that variations in the permeability of the mitochondrial preparations to $NADH_2$ might be the explanation. It is well known that carefully isolated mitochondria are relatively impermeable to reduced or oxidised NAD (Lehninger 1954, Kaufman and Kaplan 1966, Purvis and Lowenstein 1961). However, no improvement was effected by the use of mitochondrial acetone powders where no such permeability barrier exists. Alternatively, those instances where 'fumarate reductase activity' was found may represent the formation of some non-specific chromogen, other than hydroxamate. It is also noteworthy that the assay measures other acyl CoA compounds besides succinyl CoA, although it is claimed that these contribute little under the particular experimental conditions (Kurumada and Labbe 1966).

In reply to queries regarding experimental procedures, Labbe (1968) indicated that he and his co-workers were experiencing considerable difficulty in obtaining consistent results in further work on the pathway from pyruvate to succinyl CoA in experimental porphyria. It seems reasonable to conclude, therefore, that fumarate reductase probably does not exist either in normal or porphyric mammalian liver. This aspect will be dealt with in more detail in Chapter 10.

9.6 Conclusions. Pyruvate does not appear to be the carbon source supplying additional substrate for increased haem precursor and haem synthesis in AIA-induced porphyria. It is doubtful whether reductive synthesis of succinate from fumarate is mediated by a fumarate reductase enzyme, either in normal or porphyric rat liver. These findings do not support Labbe's hypothesis on induction of porphyrinogenesis.

CHAPTER 10

10.1 Aim of the study. The failure to demonstrate 'fumarate reductase' activity in Chapter 9 does not rule out the possibility that $NADH_2$-linked reduction of fumarate, nevertheless, occurs in rat liver mitochondria and is accelerated after administration of AIA.

The experiments described in this chapter represent an attempt to test Labbe's hypothesis from the aspect of hydrogen transfer. It was reasoned that if reductive synthesis of succinate from fumarate is $NADH_2$-dependent, then it should be possible to demonstrate transfer of hydrogen by the use of hydrogen isotope tracer techniques which result in the labelling of intramitochondrial $NADH_2$.

The aim of this study, therefore, was to investigate the effect in the rat of administration of AIA on the transfer of ^3H from suitable ^3H-labelled precursors via succinate to liver haem.

10.2 Experimental approach. Vennesland and co-workers (Fisher, Conn, Vennesland and Westheimer 1953) demonstrated many years ago that, in NAD-dependent oxidation-reduction reactions, hydrogen is transferred directly from the donor substrate via NAD to the acceptor molecule. According to Labbe's hypothesis, therefore, a tritiated hydrogen donor, oxidised in an NAD-dependent intramitochondrial reaction, may be expected to label liver succinate and therefore haem to a greater extent in AIA-treated than in normal animals. An advantage of the use of the tritiated precursors listed below is that none of them can augment the radioactivity of liver succinate, and consequently of liver haem, through oxidative TCA cycle activity. In each case ^3H is completely removed in one turn of the cycle, so that, while malate and oxaloacetate remain labelled, α-ketoglutarate is unlabelled (Fig. 10.2) (Hoberman and Prosky 1967). This prediction can be conveniently tested, after the administration of one of these tracers, by the determination of the specific activity of liver aspartate and glutamate which are in equilibrium *in vivo* with oxaloacetate and α-ketoglutarate respectively.

The tritiated compounds used in this study are listed in Table 10.1.

Table 10.1 Details of radioactive precursors used in tracer experiments.

PRECURSOR	SUPPLIER	SPECIFIC ACTIVITY
Succinic acid-2,3-^{3}H	The Radiochemical Centre, Amersham, U.K.	2750 mc/mM
Sodium propionate-2,3-^{3}H	" "	477 mc/mM
DL-Glutamic acid-2-^{3}H	New England Nuclear Corp., Boston, Massachusetts, USA.	3050 mc/mM
Ethanol-1-^{3}H	" "	25 mc/mM
DL-Malic acid-2-^{3}H	" "	30.8 mc/mM
Glycine-2-^{14}C	" "	7.81 mc/mM
Water-^{3}H	" "	1 mc/g

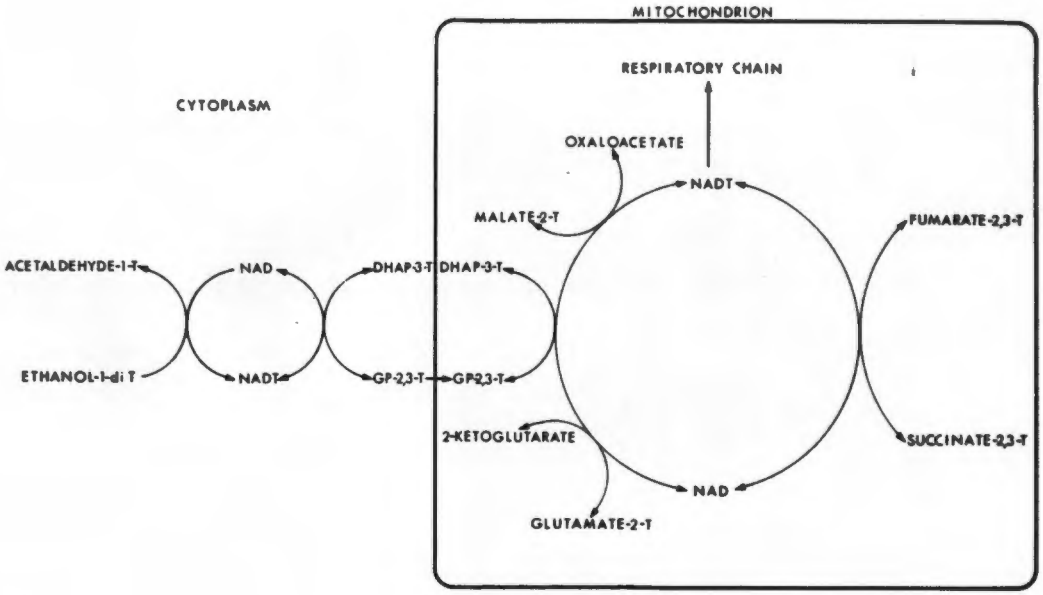
Succinate-2,3-^{3}H which labels haem by direct incorporation via succinyl CoA was selected in order to provide a standard of comparison for the other tritiated compounds which can only label haem by prior labelling of succinate. Factors determining the choice of these other ^{3}H-labelled hydrogen donors were, (i) the existence of information on hydrogen transfer from these compounds, and (ii) the fact that the appropriate dehydrogenases differ in intracellular location and steric specificity with regard to reduction of NAD.

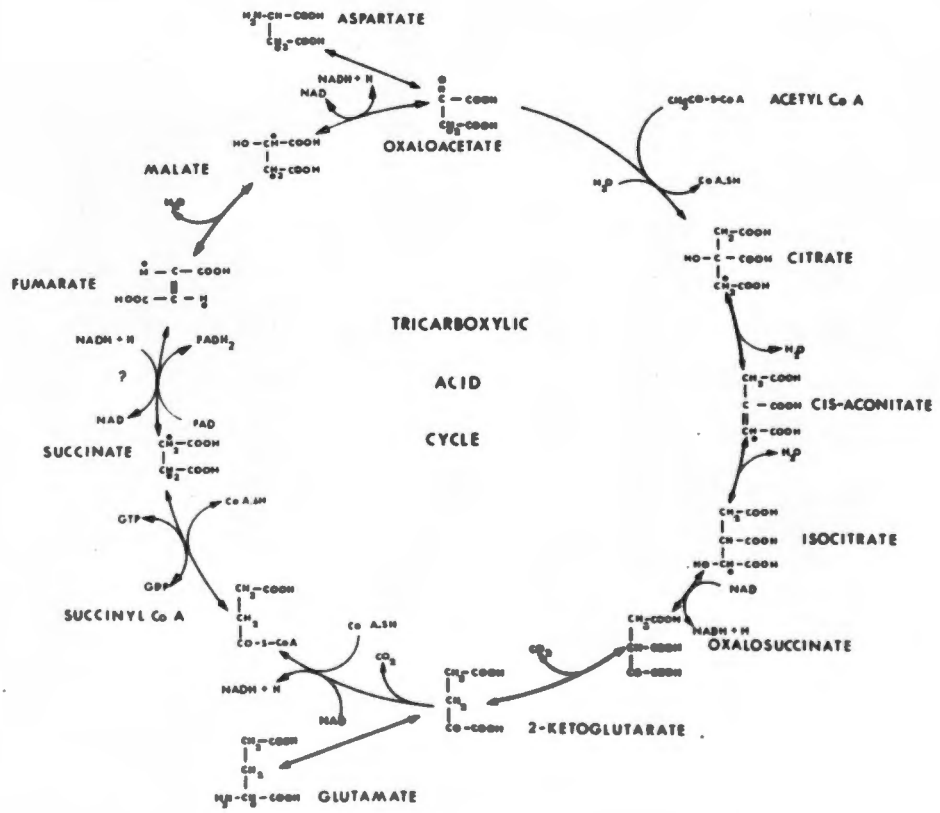
Fig. 10.1 represents diagrammatically the manner in which the three tracers, glutamate-2-^{3}H, malate-2-^{3}H and ethanol-1-^{3}H could label intramitochondrial $NADH_2$, involved in the reduction of fumarate. In rat liver, glutamate dehydrogenase is exclusively intramitochondrial (Delbrück et al. 1959), alcohol dehydrogenase is essentially extramitochondrial (Nyberg et al. 1953), and malate dehydrogenase is present in both compartments (Thorne 1961). Hence, dehydrogenation of both glutamate-2-^{3}H and malate-2-^{3}H will label intramitochondrial NAD. Whether ^{3}H is transferred to succinate in the subsequent reduction of fumarate would be influenced by the degree of coupling of glutamate and malate dehydrogenases, respectively, with 'fumarate reductase'; this is, of course, unknown. With regard to the use of ethanol-1-^{3}H, there is evidence that oxidation of ethanol in the cytoplasm is tightly coupled to the reduction of dihydroxyacetone phosphate (DHAP), and that α-glycerophosphate, so formed, diffuses into the mitochondrion where NAD becomes labelled as indicated (Fig. 10.1) (Joubert and Shreeve 1968, Haeckel and Haeckel 1968). It has also been shown that in rat liver mitochondria α-glycerophosphate can act as a reductant of fumarate (Hoberman and Prosky 1967).

Apart from oxidative or reductive TCA cycle turnover, the succinyl CoA pool in the liver in experimental porphyria may be augmented from another source, viz., propionate via the methylmalonate cycle (Section 3.10.2). For this reason the incorporation of ^{3}H from propionate-2,3-^{3}H

Fig. 10.1 Schematic representation of the reactions whereby glutamate-2-^{3}H, malate-2-^{3}H and ethanol-1-^{3}H could transfer tritium via intramitochondrial NAD to succinate. Extramitochondrial dehydrogenation of malate has not been shown. (T = ^{3}H)

Fig. 10.2 Diagram showing schematically (1) the removal of tritium from succinate-2,3-^{3}H or malate-2-^{3}H in one oxidative turn of the tricarboxylic acid cycle with the result that aspartate but not glutamate becomes labelled; and (2) the complete removal of tritium from glutamate-2-^{3}H on conversion to 2-ketoglutarate (α-ketoglutarate). Dehydrogenation of isocitrate may be NAD- or NADP-linked. In this figure only the mitochondrial NAD-linked reaction is represented. Labelling of $NADH_2$ by tritium has not been indicated. ($\overset{\bullet}{H}$ = tritium)


MITOCHONDRION
RESPIRATORY CHAIN
CYTOPLASM
OXALOACETATE
NADT
MALATE-2-T
FUMARATE-2,3-T
ACETALDEHYDE-1-T
NAD
DHAP-3-T
DHAP-3-T
ETHANOL-1-di T
NADT
GP-2,3-T
GP2,3-T
2-KETOGLUTARATE
SUCCINATE-2,3-T
NAD
GLUTAMATE-2-T


ASPARTATE
NADH + H
NAD
OXALOACETATE
ACETYL Co A
MALATE
FUMARATE
CITRATE
TRICARBOXYLIC
ACID
CYCLE
NADH + H
FADH2
?
NAD
FAD
SUCCINATE
CIS-ACONITATE
ISOCITRATE
NAD
NADH + H
OXALOSUCCINATE
GTP
GDP
SUCCINYL Co A
NADH + H
NAD
2-KETOGLUTARATE
GLUTAMATE

into liver haem was also determined in normal and porphyric animals.

Tritiated water was used to obtain a measure of the labelling of haem resulting from exchange reactions between carbon-bound hydrogen and body water.

Glycine-2-^{14}C was employed as an independent check on the experimental procedures since ^{14}C incorporation into liver haem from this precursor has been shown to be increased after administration of AIA (Labbe et al. 1965).

10.3 Materials and Methods. Details of the radioactive compounds used in this study appear in Table 10.1. In all cases radiochemical purity was stated by the manufacturer to be not less than 99%. Solutions for injection were prepared by dilution with distilled water to an activity of 1 mc/ml, except in the case of glycine-2-^{14}C when 0.1 mc/ml was employed.

Groups of adult female Wistar rats, closely matched for age and weight (within the range 150-200 g), were used in all experiments. To facilitate the induction of the porphyric state, food was withheld throughout the experiment. After 24 hours of starvation, test animals received a single intraperitoneal dose of AIA in saline (400 mg per kg body weight). Control groups received an equivalent volume of saline by the same route.

Sixteen hours later, at a time of peak ALA synthetase activity (Marver, Collins, Tschudy and Rechcigl 1966), radioactive precursors were administered, also by the intraperitoneal route. In all instances the dose employed was 1 µc/g body weight, except in the case of glycine when 0.1 µc/g body weight was administered. After a further three hours the animals were sacrificed by decapitation. Blood was collected in beakers containing heparin for the determination of radioactivity of body water after lyophilization; and livers were immediately removed for isolation of haemin with the aid of carrier according to Labbe and Nishida (1957) (Section A.4).

To ascertain that liver ALA synthetase activity was markedly raised at the time of injection of the radioactive precursors, this enzyme was assayed, as described in Section A.1.1.1, method (ii), in a separate group of 4 rats treated in the same way as the test animals but sacrificed at 16 hours after the dose of ALA.

As mentioned in Section 10.1, none of the radioactive substances employed in this study can theoretically give rise to tritiated succinate by oxidative tricarboxylic acid cycle activity, since in all cases the label should be removed in one turn of the cycle (Fig. 10.2). For this reason, the specific activity of liver glutamate following administration of these tracer compounds (except of course glutamic acid-2-^{3}H) should not be significantly different from that due to exchange reactions with ^{3}H-labelled body water. On the other hand, the specific activity of liver aspartate should be much greater following injection of tritiated succinate, propionate, malate or ethanol than after administration of tritiated water.

Accordingly, normal adult female rats weighing 150-200 g were starved for 40 hours prior to injection of the labelled compounds shown in Table 10.1, with the exception of glycine-2-^{14}C. The dose employed was again 1 µc/g body weight. Three hours later animals were sacrificed and livers immediately removed for isolation and purification of aspartic and glutamic acids as described in Section A.5. The specific activity of the pure compounds was determined as described in Sections A.5, A.6.

10.4 Results. The results of the radioactive tracer experiments appear in Tables 10.2 and 10.3 ^{3}H incorporation into body water (Table 10.2) was by no means uniform among the various precursors, ranging from about 20% with succinate-2,3-^{3}H to over 70% with propionate-2,3-^{3}H. From Table 10.3 it appears that ethanol-1-^{3}H and malate-2-^{3}H did not contribute significantly to the specific activity of liver glutamic acid whereas succinate-2,3-^{3}H and propionate-2,3-^{3}H labelled this amino-acid

slightly more than did HO^3H. By contrast the specific activity of liver aspartic acid was significantly greater than that due to HO^3H in the case of all the other tritiated compounds administered.

Incorporation of radioactivity from propionate-2,3-^3H, succinate-2,3-^3H, malate-2-^3H and glycine-2-^{14}C into liver haem in normal animals was significant but in the case of glutamate-2-^3H and ethanol-1-^3H was little greater than from HO^3H. Administration of AIA almost doubled the radioactivity of liver haem when glycine-2-^{14}C was used as a precursor. On the other hand, a consistent decrease in ^3H-labelling of haem by succinate-2,3-^3H, propionate-2,3-^3H and malate-2-^3H occurred in the porphyric animals.

The mean hepatic ALA synthetase activity of the group of 4 animals sacrificed at 16 hours after a single dose of AIA was 243 mμmoles ALA formed/g liver/hour which is about 4 times the values obtained in normal fasted rats (Section 6.3).

10.5 Discussion. Before discussing the results obtained in the present experiments, some aspects of the use of ^3H as a tracer in general, and those tritiated compounds employed in this study in particular, will be considered.

10.5.1 Mechanisms of ^3H transfer in the intact cell.

i) It has been assumed that ^3H incorporated into the compounds isolated in this study was stably bound to carbon. S-,O- and N-^3H bonds, formed by exchange with tritiated body water, are labile in strongly acid solutions and hence non-specific isotope incorporation of this nature would undoubtedly be removed in the course of the isolation procedures employed (Sections A.4, A.5).

ii) It is also possible for stable C-^3H bonds to be formed by non-oxidative exchange with tritiated body water. This phenomenon is well exemplified by the fate of H in position 1 of fructose-6-phosphate, when the latter is transformed by hexose phosphate isomerase to glucose-1-phosphate (Topper 1957, Bloom and Foster 1964). Hydrogen or

^3H in this position can exchange with body water so that the molecule becomes labelled or unlabelled, depending on conditions. Many other examples of this type are known. The results obtained with tritiated water in the present experiments, however, indicate that such non-oxidative transfer of tritium to the tritiated compounds isolated was small.

iii) From Fig. 10.3, which is based on the work of Hoberman (1958), Hoberman and D'Adamo (1960), and Joubert and Shreeve (1968), it is apparent that transfer of ^3H from malate-2-^3H and ethanol-1-^3H in the intact animal leads to labelling of a considerable number of intermediates. For the purposes of the subsequent discussion the important information is that both malate-2-^3H and ethanol-1-^3H equilibrate with fumarate, resulting in randomisation of ^3H between carbons 2 and 3 of fumarate and malate. Hence, incorporation of ^3H into succinate and therefore haem from both these tracers can be expected, whether ^3H-labelling of intramitochondrial NAD occurs or not.

iv) On the other hand, transfer of ^3H from glutamate-2-^3H by glutamate dehydrogenase is strictly to intramitochondrial NAD and the product of oxidative deamination of this amino-acid, α-ketoglutarate, is unlabelled (Fig. 10.3). Hence, ^3H incorporation into succinate and therefore liver haem following administration of glutamate-2-^3H must involve transfer via NAD.

v) As indicated previously, succinate can be expected to label haem by direct incorporation via succinyl CoA. With regard to labelling of intramitochondrial NAD, Hoberman et al. (1964) have shown that succinate-2,3-^3H cannot transfer ^3H directly to NAD. Nevertheless, ^3H in succinate-2,3-^3H may be transferred to intramitochondrial NAD by operation of the TCA cycle and dehydrogenation of malate-2,3-^3H so formed (Fig. 10.3).

vi) Propionate-2,3-^3H may be converted via methylmalonate to succinate-2,3-^3H and hence the subsequent transfer of ^3H will be as for the latter compound.

Fig. 10.3 Schematic representation of proposed routes of transfer of hydrogen in the cytoplasmic compartment of the liver cell by coupled NAD-dependent reactions. The evidence relating to the various substrates concerned is derived from the work of the following authors :

lactate ..... Hoberman (1958)

fumarate .... Hoberman and D'Adamo (1960)

ethanol ..... Joubert and Shreeve (1968).

(Reproduced by kind permission of Joubert and Shreeve)

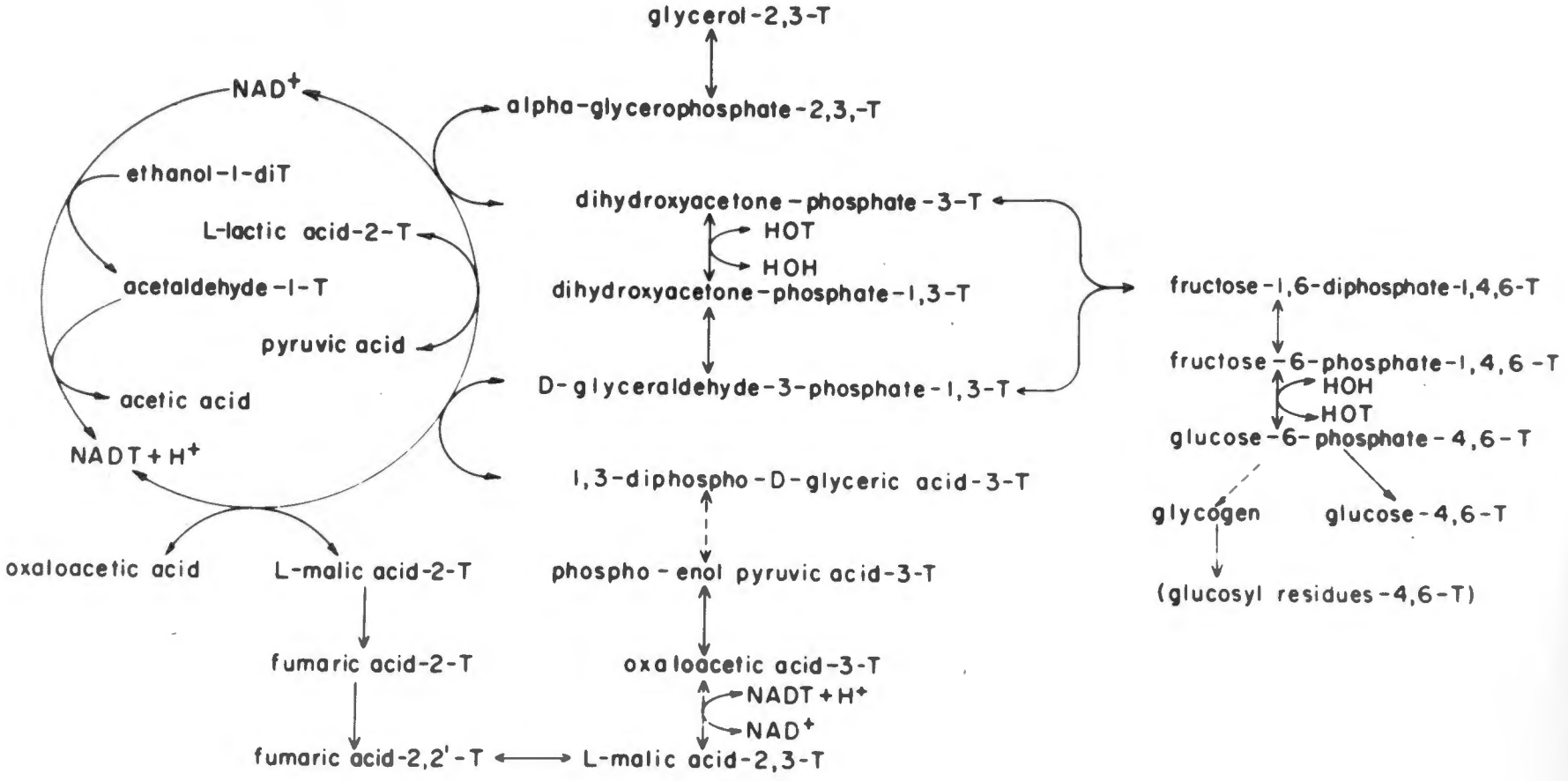

glycerol-2,3-T
NAD+
alpha-glycerophosphate-2,3,-T
ethanol-1-diT
dihydroxyacetone-phosphate-3-T
HOT
HOH
L-lactic acid-2-T
dihydroxyacetone-phosphate-1,3-T
fructose-1,6-diphosphate-1,4,6-T
acetaldehyde-1-T
pyruvic acid
fructose-6-phosphate-1,4,6-T
HOH
HOT
D-glyceraldehyde-3-phosphate-1,3-T
acetic acid
glucose-6-phosphate-4,6-T
NADT+H+
1,3-diphospho-D-glyceric acid-3-T
glycogen
glucose-4,6-T
oxaloacetic acid
L-malic acid-2-T
phospho-enol pyruvic acid-3-T
(glucosyl residues-4,6-T)
fumaric acid-2-T
oxaloacetic acid-3-T
NADT+H+
NAD+
fumaric acid-2,2'-T
L-malic acid-2,3-T

Table 10.2 Incorporation of radioactivity from selected tritiated precursors into body water and liver haem in normal and porphyric rats.

Precursor	No. of rats per group	Mean percentage of radioactive dose in body water		Mean radioactivity of liver haem (d.p.m./mg)	
		Normal rats	Porphyric rats	Normal rats	Porphyric rats
Tritiated water	3	-	-	11	15
Succinate-2,3-^{3}H	3	21.5	20.4	373	258
	3	-	-	267	225
	3	-	-	280	182
	3	-	-	234	141
Glutamate-2-^{3}H	3	43.5	42.7	15	13
	3			24	29
Ethanol-1-^{3}H	1	-	-	24	7
	1	-	-	27	5
	1	-	-	14	5
	1	-	-	18	4
Malate-2-^{3}H	3	46.2	46.5	190	117
	3			85	60
Propionate-2,3-^{3}H	3	74.4	69.9	322	254
	3	-	-	498	254
	3	-	-	279	179
	3	-	-	442	229
Glycine-2-^{14}C	3	-	-	199	377

Table 10.3 Specific activity (S.A.) of liver aspartic and glutamic acids following administration of selected tritiated precursors to normal rats.

Precursor	S.A. Aspartic acid muc/mM	S.A. Glutamic acid muc/mM
Tritiated water	0.9	4.2
Succinate-2,3-^{3}H	9.8 15.5	10.5 10.9
Glutamate-2-^{3}H	11.9	552.5
Ethanol-1-^{3}H	27.8	4.4
Malate-2-^{3}H	55.8	6.2
Propionate-2,3-^{3}H	24.8	9.6

10.5.2 Incorporation of ^3H into body water and liver aspartate, glutamate and haem in control animals.

i) Incorporation of ^3H into body water. The markedly different percentage incorporation of administered ^3H into body water, achieved by the various precursors used, is probably a reflection of differential membrane permeability to these tritiated compounds. From Table 10.2 it is apparent, for example, that oxidation of ^3H in succinate to HO^3H during the experiment was only about one-third that of propionate. It is therefore not possible to make strictly quantitative comparisons between the various precursors in respect of ^3H incorporation into the other compounds isolated, i.e. aspartate, glutamate and haem. With respect to haem another element which invalidates strict comparisons is the fact that different amounts of carrier haem were used in different experiments (Section A.4).

ii) Incorporation of ^3H into liver glutamate and aspartate. The prediction, that the ^3H-labelled precursors used in this study could not augment the radioactivity of the succinate pool in the intact animal through an oxidative turn of the TCA cycle, is borne out by the specific activity of liver aspartate and glutamate following the administration of ethanol-1-^3H and malate-2-^3H. In each case the labelling of glutamate was not significantly greater than that due to triated water, while on the other hand, aspartate was well labelled by these precursors compared with HO^3H. These findings confirm those of Hoberman and Prosky (1967) in perfused rat liver.

Incorporation of ^3H into glutamate from succinate-2,3-^3H and propionate-2,3-^3H was slightly greater than expected. A possible explanation is that both substances equilibrate with α-ketoglutarate as a result of Shemin cycle activity, thus giving rise to α-ketoglutarate-3,4-^3H which in turn equilibrates with glutamate.

Labelling of aspartate by glutamate-2-^3H was some 12 times greater than that achieved by an equivalent radioactive dose of HO^3H. Since α-ketoglutarate formed from glutamate-2-^3H is completely unlabelled, ^3H

could not have been incorporated into the dicarboxylic acids via an oxidative turn of the TCA cycle. It is suggested that this transfer was mediated by intramitochondrial $NADH_2$, labelled by oxidative deamination of glutamate-2-^3H. Reduction of oxaloacetate by ^3H-labelled $NADH_2$ would give rise to malate-2-^3H, followed by randomisation of ^3H between carbon atoms 2 and 3 of malate and fumarate via the fumarase reaction. Malate-2,3-^3H, so formed, would then yield labelled oxaloacetate and aspartate as shown in Fig. 10.2.

The low specific activity of aspartate after administration of succinate-2,3-^3H was unexpected but was confirmed in repeat experiments. As suggested previously to account for the similarly low percentage incorporation of ^3H from this precursor into body water, the explanation is probably that succinate did not penetrate the cell and/or mitochondrion as efficiently as did propionate, for example. Another possibility which must be considered is that kinetic isotope effects operate with regard to ^3H removal by liver succinic dehydrogenase, as demonstrated for soluble beef heart succinic dehydrogenase (Huffner and Hollocher 1968). In other words, the steric orientation of ^3H in succinate-2,3-^3H determines the rate of isotope removal and oxidation. However, if this were the case in the present experiments, it is inconceivable that both aspartate and body water would be poorly labelled.

iii) Incorporation of ^3H into liver haem. The results of ^3H incorporation from succinate-2,3-^3H into liver haem accord with the fact that succinate is a known precursor of haem. The finding that propionate-2,3-^3H was apparently the most efficient haem precursor in this study must be related to the fact that ^3H enrichment of body water by this tracer was much greater than with any of the other ^3H donors used. Nevertheless, it does indicate that propionate, via methylmalonate (Section 3.10.2), could be an appreciable carbon source for increased liver haem biosynthesis in experimental porphyria. At one stage it was also considered possible that propionyl CoA, apart from conversion to

succinyl CoA, might be involved in the formation of a heterologous pyrrole which could condense to form coproporphyrinogen directly (Section 3.3). The subsequent work of Nandi and Shemin (1968) on the mechanism of ALA dehydratase action, however, precludes this possibility (Section 3.3).

Both malate-2-^{3}H and ethanol-1-^{3}H were much less efficient ^{3}H sources with respect to haemin-^{3}H. This is probably due to dilution of radioactivity in a large number of different pools since, as indicated earlier (Section 10.5.1), malate, and ethanol via malate, lead to labelling of many different compounds (Fig. 10.3). Nevertheless, these results contain important information. It was previously pointed out that both of these substances will label fumarate in positions 2 and 3 (Section 10.5.1) and that ^{3}H in these positions will be removed by an oxidative turn of the TCA cycle (Section 10.2). Consequently, labelling of succinate and hence of haem by malate-2-^{3}H and ethanol-1-^{3}H is evidence that reduction of fumarate must have occurred, whether or not the mechanism involved transfer of ^{3}H by intramitochondrial NAD.

If fumarate reduction were $NADH_2$-dependent, it would be reasonable to expect substantial ^{3}H-labelling of haem by glutamate-2-^{3}H. It has been suggested that the labelling of aspartate by glutamate-2-^{3}H was mediated by tritiated $NADH_2$. In the event, incorporation of radioactivity into liver haem from this tracer was, at best, little greater than after administration of H^{3}O. These findings, therefore, do not support the proposal that intramitochondrial $NADH_2$ is a reductant of fumarate in normal rat liver. As suggested by Hoberman and Prosky (1967), it would seem more likely that succinic dehydrogenase rather than an $NADH_2$-dependent 'fumarate reductase' catalyses this reaction.

This reasoning may, however, be criticised on the grounds that the various dehydrogenases differ in steric specificity for $NADH_2$. Any one of these enzymes can transfer either the α- or the β-oriented H in position 4 of $NADH_2$ but not both, thus some dehydrogenases are α-specific

and others β-specific. If, therefore, the steric specificity of glutamate dehydrogenase were opposite to that of 'fumarate reductase', ^3H would not be transferred via $NADH_2$ although H would. On the other hand, as Hoberman (1958a) pointed out with respect to deuteriolactate, in the intact animal, where NAD is 'pooled' among dehydrogenases of both steric specificities, administration of a tritiated hydrogen donor would rapidly lead to replacement of both α- and β-oriented hydrogens by tritium.

Furthermore, there is evidence that in rat liver α-glycerophosphate can reduce fumarate (Hoberman and Prosky 1967). Since ethanol oxidation appears to be closely linked to the reduction of dihydroxyacetone to α-glycerophosphate (Joubert and Shreeve 1968, Haeckel and Haeckel 1968), it seems reasonable to expect that labelling of haem by ethanol-1-^3H should have been more effective, if reduction of fumarate were NAD-dependent.

Finally, it may be argued that, since mitochondrial NAD is linked via $NADH_2$ dehydrogenase to the electron transport chain, any ^3H incorporated into $NADH_2$ may be oxidised to HO^3H long before it could be utilised in any reductive synthesis. From the present study, the only evidence against this is the labelling of aspartate by glutamate-2-^3H which was considered to be mediated by $NADH_2$.

10.5.3 Transfer of ^3H to liver haem in AIA-treated rats. The evidence for increased liver haem biosynthesis under the influence of AIA is two-fold:-

i) marked induction of hepatic ALA synthetase, the rate-limiting enzyme for this pathway, occurs (Tschudy et al. 1964, Marver, Collins, Tschudy and Rechcigl 1966); and

ii) incorporation of radioactivity from ^{14}C-labelled precursors, e.g. glycine-2-^{14}C, into liver haem is increased (Labbe et al. 1965).

In the present study both of these findings were confirmed and it therefore seems reasonable to conclude that haem biosynthesis in the

AIA-treated animals was increased.

The finding in these AIA-treated animals of decreased incorporation of ^{3}H from all the tritiated precursors used (except $HO^{3}H$ and glutamate-2-^{3}H was consequently most unexpected. Even if no transfer of ^{3}H occurred via NAD, greater labelling of haem should have been effected, at least when succinate-2,3-^{3}H was employed.

It is obvious, therefore, that AIA must stimulate some mechanism or pathway which leads to less of the administered ^{3}H being incorporated into liver haem. One such possibility is that the succinate pool is diluted with unlabelled succinate which arises, e.g. from increased oxidative turnover of the TCA cycle, or from propionate via methylmalonate (Section 3.10.2), and which does not equilibrate with the administered tritiated precursors. However, if this were the case, incorporation of radioactivity from succinate-^{14}C should also be decreased, whereas Labbe et al. (1965) have shown that both succinate-1-^{14}C and succinate-2,3-^{14}C label haem considerably better in AIA-treated animals than in normals. Furthermore, there is good evidence that oxidative TCA cycle activity is unaffected by AIA (Tschudy et al. 1962, Labbe et al. 1965, Section 9.5.).

A more feasible explanation of the present results is that they reflect an isotope effect. Isotope discrimination in studies with tritiated compounds is well known. ^{3}H has an atomic weight almost 3 times that of ^{1}H and consequently the energy of the C-^{3}H bond is different from that of the C-^{1}H bond. This difference may render the tritiated compound a less suitable substrate (thermodynamically) for a particular enzyme than the corresponding non-radioactive compound, i.e. the enzyme may discriminate against the ^{3}H-labelled molecules. However, isotope discrimination can only be observed in a given experiment when incorporation of the isotope in question is compared with a marker against which discrimination does not occur, e.g. if suitable intermolecularly (say ^{14}C- and ^{3}H-)labelled precursors are used. To interpret

the results in the present experiments, therefore, it becomes necessary to postulate an alternative mechanism.

It may be that AIA stimulates a pathway of haem biosynthesis, different from that which normally operates in the liver, and that one (or more) of the enzymes or iso-enzymes involved discriminates to a much greater extent against C-^{3}H bonds than the corresponding enzymes of the normal haem biosynthetic sequence. Alternatively, it is possible that AIA stimulates, at some stage in the synthetic sequence leading from succinate to haem, a mechanism which promotes the exchange of ^{3}H in haem precursors (or haem itself) for ^{1}H.

In Section 10.5.2 it was concluded from the poor labelling of haem by glutamic acid-2-^{3}H that reduction of fumarate in normal rat liver was probably not $NADH_2$-dependent. Unfortunately, in the case of the AIA-treated animals it is impossible to evaluate the results of ^{3}H incorporation from glutamate-2-^{3}H into haem in view of the problems discussed above.

10.6 Conclusions. It has been demonstrated that reduction of fumarate to succinate occurs normally in the intact rat. The significant transfer of ^{3}H from glutamate-2-^{3}H to aspartate in normal rats was interpreted as evidence of ^{3}H transfer via intramitochondrial NAD. If this interpretation is correct, it would appear that fumarate reduction under normal circumstances is not NAD-dependent. This accords with evidence obtained in the perfused rat liver that succinate dehydrogenase, under suitable conditions, may catalyse reduction of fumarate.

Contrary to expectations AIA administration led to decreased rather than increased incorporation of ^{3}H from various tritiated precursors into liver haem. This was true of substances which could be directly incorporated as well as those expected to transfer ^{3}H by indirect routes. It was therefore concluded that an isotope effect was probably responsible for the unexpected results following administration of AIA. To account for the present findings it was suggested that AIA might stimulate a

pathway of liver haem biosynthesis which is not normally operative, and that one (or more) of the enzymes involved exercises greater discrimination against ^{3}H-labelled molecules than the corresponding enzymes in the normal biosynthetic sequence. These observations rendered it impossible to evaluate the results of ^{3}H transfer via intramitochondrial NAD to liver haem after administration of AIA.

CHAPTER 11

THE REDOX STATE OF THE LIVER CELL IN SYMPTOMATIC PORPHYRIA - CORRELATION WITH LIVER ALA SYNTHETASE ACTIVITY.

11.1 Aim of the study. If the effect of alcohol on hepatic ALA synthetase activity in symptomatic porphyria is a function of its influence on the $NADH_2/NAD$ ratio of the liver cell cytoplasm, as suggested in Section 5.4.3.2, it would be logical to expect a correlation between these two parameters. The aim of this study is to determine whether such a correlation exists.

Measurement of the total NAD and $NADH_2$ content of whole liver by direct means is unsatisfactory since this gives no information on the relative amounts of free and bound nucleotides or on their intracellular distribution which is heterogenous (Borst 1963). Methods employing indirect measurements are far more useful, e.g. determination of "the ratio of the concentration of the oxidised and reduced metabolites of suitable NAD-linked dehydrogenase systems that are located in different cell compartments and, on account of their high activity, are in equilibrium with the nucleotides ....." (Williamson et al. 1967). As far as the cytoplasmic compartment is concerned the lactate-pyruvate system gives the most reliable results (Bücher and Rüssmann 1963). Since lactate and pyruvate in the liver cell are in equilibrium with hepatic venous blood, measurement of these metabolites in specimens obtained by catheterisation of the hepatic vein has also been used as a measure of the redox state of the cytoplasmic compartment. However, none of these methods was either suitable or feasible for use in the present study.

The most convenient approach was the use of the galactose tolerance test as recommended by Forsander (1966). Clearance of galactose from the blood is performed mainly by the liver (Roe and Cogwill 1935) and this function has been shown to be redox sensitive. Four stages are involved in the conversion of galactose to glucose in the liver:-

galactose + ATP → galactose-1-phosphate + ADP (1)

galactose-1-phosphate + UTP → UDP-galactose + pyrophosphate (2)

UDP-galactose $\xrightarrow{\text{UDP-galactose-4-epimerase}}$ UDP-glucose (3)

UDP-glucose + pyrophosphate → glucose-1-phosphate + UTP (4)

Of these four reactions the UDP-galactose-4-epimerase reaction is NAD-dependent and is inhibited by $NADH_2$ (Maxwell 1957, Isselbacher and Krane 1961). Robinson et al. (1963) showed that the ratio of oxidised to reduced NAD rather than the absolute amounts of these nucleotides was important with regard to inhibition. Under reducing conditions, therefore, reaction 3 becomes rate-limiting for the conversion of galactose to glucose. This is well illustrated by the effect of alcohol which has been shown to inhibit oxidation of galactose *in vitro* (Isselbacher and Krane 1961) and the elimination of administered galactose *in vivo* (Stenstam 1946, Tygstrup and Winkler 1958).

In a reaction sequence such as that shown above it is obvious that a number of other factors may become rate-limiting under pathological conditions. Under these circumstances the rate of galactose elimination and the redox state of the liver cell cytoplasm may not be related. It was realised that this might apply in symptomatic porphyria since a large proportion of these patients have hepatocellular pathology (Section 2.2.3.1) which is often associated with impaired galactose tolerance (Stenstam 1946, Tengstrom 1966). Nevertheless, since nothing is known of the redox state of the liver cell cytoplasm in symptomatic porphyria and no other convenient approach was possible, it was considered worthwhile to examine the relationship between galactose tolerance and liver ALA synthetase activity in these patients.

11.2 Materials and Methods. The subjects studied were eleven adult male and female African patients with symptomatic porphyria who were included in the investigation described in Chapter 7. Details of the preparation of these patients for determination of liver ALA synthetase activity are recorded in Section 7.2.2. Eight were members of the control group which did not have access to alcohol, while the remaining

three belonged to the group which received ethanol in the prescribed manner (Section 7.2.2).

In all cases an intravenous galactose tolerance test was performed immediately after the liver biopsy. ALA synthetase activity was assayed as described in Section A.1.1.2.

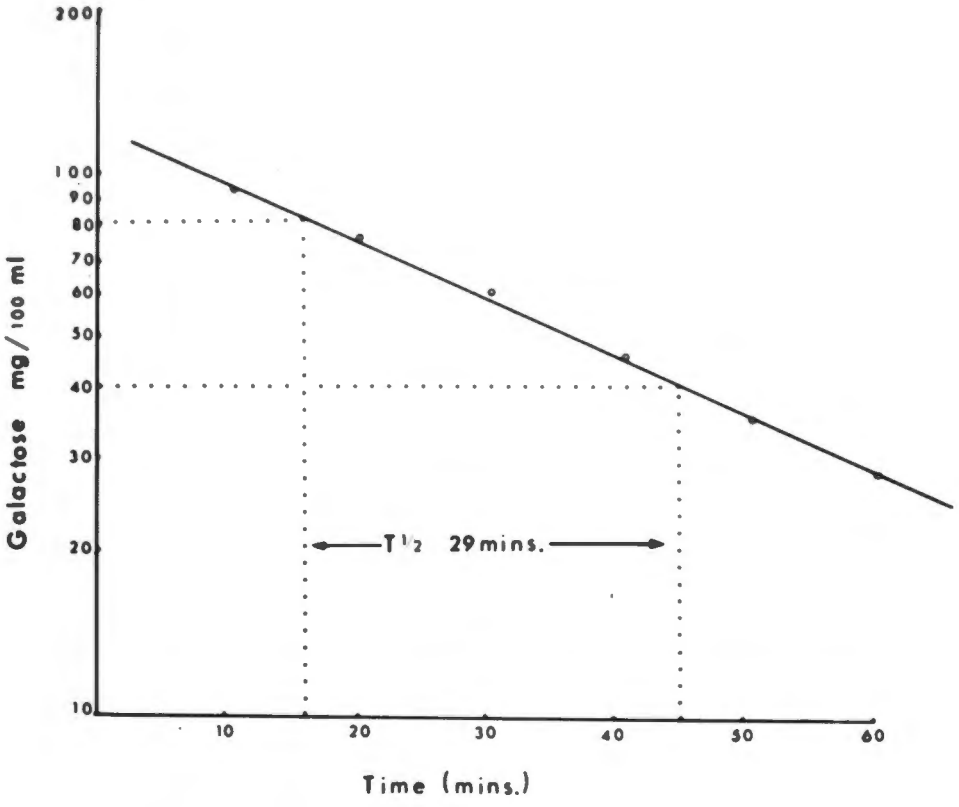
11.2.1 Intravenous galactose tolerance test. The single-dose intravenous galactose tolerance test described by Tengstrom (1966) was employed. Galactose was obtained from AB Kabi, Stockholm, as a sterile 30% solution containing not more than 0.3% glucose and not more than 1.5% of carbohydrates other than galactose.

Patients who had been fasted overnight received an intravenous infusion of 350 mg galactose per kg body weight over a period of one to three minutes. Timing by stop watch was commenced when half of the calculated volume had been administered. Venous blood was collected into tubes containing EDTA as anticoagulant at 10-minute intervals for 60 minutes. 0.1 ml of this blood was then transferred as rapidly as possible to previously marked test tubes containing 2 ml 0.025 N NaOH to prevent further metabolism of galactose. Determination of galactose by an enzymatic (galactose oxidase) method was performed as described in Section A.7. The values thus obtained were plotted against time on semilogarithmic paper and the $T_{\frac{1}{2}}$ for galactose was determined graphically from the straight line best fitting the curve as shown in the example (Fig. 11.1).

The use of this method as an accurate measure of hepatic elimination of galactose may be criticised in several respects. Firstly, no account is taken of urinary excretion of galactose. Tengstrom (1966), however, states that only about 7% of the administered dose is lost in the urine during the test and this amount is surprisingly constant irrespective of the $T_{\frac{1}{2}}$. Secondly, although extrahepatic metabolism of galactose is small under normal conditions, it is not known whether this is true in the presence of liver dysfunction. Nevertheless, by

Fig. 11.1 Semilogarithmic plot of the galactose elimination curve in a patient with symptomatic porphyria (N. Hlongwa). The $T_{\frac{1}{2}}$ for galactose was determined graphically as shown.

Fig. 11.2 The relationship between liver ALA synthetase activity and galactose $T_{\frac{1}{2}}$ in patients with symptomatic porphyria.


200
100
90
80
70
60
50
40
30
20
10
Galactose mg/100 ml
T½ 29mins.
10
20
30
40
50
60
Time (mins.)

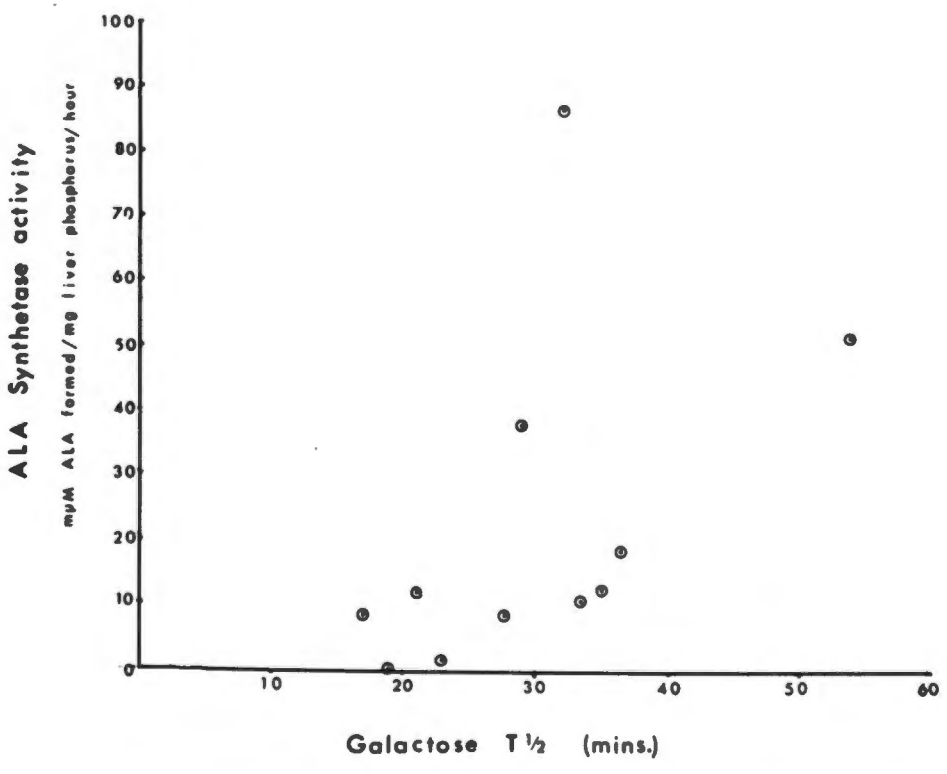

ALA Synthetase activity
mμM ALA formed/mg liver phosphorus/hour
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
Galactose T½ (mins.)

Table 11.1 Hepatic ALA synthetase activity and galactose tolerance in patients with symptomatic porphyria.

Patient	ALA synthetase activity mμmoles ALA formed/mg total liver phosphorus/hour	Galactose $T_{\frac{1}{2}}$ (minutes)
E. Ndaba	10.6	33.5
V. Pondo	1.7	23.0
C. Miya	18.4	36.5
I. Buthelezi	8.8	17.0
S. Mkize	11.9	21.0
R. Thabede	0	19.0
N. Ngubane	12.1	35.0
F. Mkize	8.6	27.5
W. Sokhela*	85.8	32.0
M. Mthembu*	50.8	54.0
N. Hlongwana*	37.5	29.0

* Received ethanol prior to liver biopsy and galactose tolerance test.

comparison with other diagnostic aids in hepatobiliary diseases, Tengstrom (1966) obtained satisfactory results in well over 100 tests on normal subjects and patients with various forms of liver disease. "The $T\frac{1}{2}$ value of the semilogarithmically plotted curve is an empirical parameter which was found to have certain correlations with different liver disorders, correlations which indeed depend essentially on the elimination capacity of the liver" (Tengstrom 1966). Hence, it was felt that, if any correlation existed between liver ALA synthetase activity and the rate of hepatic elimination of galactose, it would be revealed by this single-dose intravenous galactose tolerance test.

In 74 normal subjects Tengstrom (1966) found the mean galactose $T\frac{1}{2}$ to be 12.0 mins. (S.D. 2.6) and he therefore considered the upper limit of the normal range to be 17 minutes.

11.3 Results. The clinical and biochemical details of all patients studied appear in Appendix B (Tables B.2, B.3).

Table 11.1 and Fig. 11.2 depict the results of liver ALA synthetase determinations and galactose tolerance tests in the eleven subjects. Statistical analysis of these values, as outlined in Appendix C (Section C.2) revealed that there was no significant correlation at the 5% level between the two parameters. This was true whether the results in all eleven cases were considered together, or whether those appertaining to the patients who received no ethanol (Table 11.1) were analysed separately.

Administration of ethanol (last 3 patients in Table 11.1) produced a considerable increase in liver ALA synthetase activity in all 3 cases compared with the levels obtained in the first 8 patients. By contrast, in only one case was the galactose $T\frac{1}{2}$ outside the range for the group not given ethanol.

11.4 Discussion. The finding of no significant correlation between galactose $T\frac{1}{2}$ and hepatic ALA synthetase activity does not support the hypothesis that the induction of this enzyme may be the consequence

of an increased $NADH_2/NAD$ ratio in the cytoplasmic compartment of the liver cell. More difficult to decide is the question: may such a correlation between ALA synthetase activity and the redox state, nevertheless, exist?

It has been pointed out that galactose tolerance is a measure of the redox potential of the liver, only as long as UDP-galactose-4-epimerase remains rate-limiting for the conversion of galactose to glucose (Section 11.1). Under pathological conditions some other factor, e.g. hepatic blood flow, may become crucial. It has been shown that liver circulation is decreased by approximately one third in cirrhosis and that this reduction is associated with a marked fall (about 50%) in hepatic bromsulphthalein extraction (Bradley et al. 1952). Hence abnormal galactose tolerance in cirrhotic patients (Stenstam 1946, Tengstrom 1966) may, similarly, be attributable to diminished liver circulation. Since cirrhosis is frequently encountered in patients with symptomatic porphyria, and some degree of hepatocellular pathology is found in all cases (Section 2.2.3.1), it is therefore quite likely that diminished hepatic blood flow contributed significantly to the abnormal values for galactose $T_{\frac{1}{2}}$ obtained in the majority of patients in the present study. In other words, the galactose half-life in many of these patients was possibly not a reflection of the redox state of the liver cell cytoplasm.

Another factor meriting consideration is the concentration in the liver of ATP, the nucleotide which is required for the first step in the sequence of reactions converting galactose to glucose (Section 11.1). O'Donnel et al. (1962) found decreased hepatic levels of ATP in patients with various types of hepatocellular disease and Gajdos (Section 4.2.8) postulated that a decrease in hepatic ATP level is the stimulus for porphyrinogenesis both in experimental and human porphyria. Consequently, it is quite conceivable that in some patients with symptomatic porphyria reaction (1) of the sequence shown in Section 11.1

and not reaction (3) is the rate-limiting one for conversion of galactose to glucose.

The values obtained in those patients who received ethanol point to a dissociation between the two parameters, since markedly increased liver ALA synthetase activity was accompanied in 2 of the 3 cases, by a galactose $T_{\frac{1}{2}}$ which was only slightly greater than the mean value for the 8 patients who did not receive ethanol (26.5 mins.). However, interpretation of these results is again complicated by the difficulty that factors other than the UDP-galactose-4-epimerase reaction may be rate-limiting for hepatic galactose elimination in these subjects. Indeed, Stenstam (1946) found that, unlike the effect on normal subjects, alcohol administered to patients with liver disease (acute and subacute hepatitis) did not further impair oral galactose tolerance.

In view of these various contingencies, therefore, it is impossible to exclude entirely the possibility that hepatic ALA synthetase activity and $NADH_2/NAD$ ratios in symptomatic porphyria are correlated.

11.5 Conclusions. No correlation was found between hepatic galactose elimination and liver ALA synthetase activity in patients with symptomatic porphyria. While these findings do not support the hypothesis that an increase in the reducing potential of the liver cell cytoplasm leads to induction of the rate-controlling enzyme for liver haem biosynthesis, they do not entirely exclude the possibility.

CHAPTER 12

THE EFFICACY OF STEROID (AETIOCHOLANOLONE) CONJUGATION IN SYMPTOMATIC PORPHYRIA.

12.1 Aim of the study. As discussed in Section 4.2.1, a number of physiological steroids have been shown to have a porphyrinogenic effect in chick embryo liver cells. It was found that only the free steroid exhibited this property, the conjugates being completely inactive. On the basis of these findings Granick and Kappas (1967) postulated a physiological role for these compounds in the normal control of liver haem biosynthesis. In terms of this hypothesis the unconjugated steroid competes with haem (the natural corepressor) for a site on an aporepressor molecule, thereby blocking normal repression of ALA synthetase. At any time "the concentration of active steroid inducers in the hepatic cell would depend, among other factors, on the rate of their conversion by UDP-glucuronyltransferase to the inactive glucuronides ....." (Kappas and Granick 1968). It has further been suggested that failure of such control mechanisms could be an important cause of increased porphyrin production in the hepatic porphyrias, both genetic and acquired (Kottra and Kappas 1967, Kappas and Granick 1968).

Since all patients with symptomatic porphyria have some degree of hepatocellular pathology (Section 2.2.3.1), it is a distinct possibility that failure to conjugate endogenous steroids in the normal manner may be important in the pathogenesis of this disease. Furthermore, if this were the case, it is conceivable that alcohol, through its well-known effect on the liver $NADH_2/NAD$ ratio, might retard glucuronide formation since the production of uridine diphosphate glucuronic acid (UDPGA) which supplies the glucuronide moiety of the conjugated steroid is NAD-dependent (Fig. 12.2). This provides another possible explanation for the mode of action of ethanol in inducing liver ALA synthetase and thereby aggravating symptomatic porphyria. The aims of this study are, therefore, (i) to assess the efficacy of steroid conjugation in symptomatic porphyria and (ii) to determine whether alcohol exerts a retarding effect on this process.

12.2 Experimental approach. The neutral steroid hormones are metabolised in 3 successive stages : (i) reduction of the double bond in ring A, (ii) reduction of the carbonyl group in ring A and (iii) conjugation (Dorfman and Ungar 1953, Tomkins 1957). Hence disappearance of administered free steroid from the plasma depends on all 3 processes. In the case of the reduced steroids such as aetiocholanolone, on the other hand, only stage (iii) is applicable. Consequently, it is reasonable to expect that the rate of clearance of free aetiocholanolone from the circulation following an intravenous injection would reflect the rate of glucuronide formation in the liver.

In a study of the fate of testosterone metabolites, Slaunwhite and Sandberg (1958) employed aetiocholanolone-^{14}C by intravenous injection in order to determine the rate of disappearance of this steroid from the plasma in patients with normal liver function. For the purposes of the present investigation the ^{14}C-labelled compound was not readily available and hence aetiocholanolone-1,2-^{3}H which is commercially obtainable was used in a similar clearance study in 10 patients with symptomatic porphyria. These results were compared with those obtained in a control group of non-porphyric subjects with various forms of liver disease. In order to assess the influence of ethanol on glucuronide conjugation of this steroid, in 4 of the porphyric patients the study was carried out both before and after administration of ethanol.

12.3 Materials and Methods.

12.3.1 Patients. The subjects included in this study were 17 adult male and female African patients who were admitted to King Edward VIII Hospital, Durban. Ten of these presented clinical and biochemical features of symptomatic porphyria, while the remaining seven were non-porphyric patients who exhibited various types of hepatocellular pathology. The criteria employed for the diagnosis of symptomatic porphyria were those set out in Sections 2.2.1 and 2.2.2. For diagnostic

purposes casual urine and stool specimens were analysed for porphyrin precursor and porphyrin content as described in Section A.9. Total aminoketones, PBG and URO were determined in urine and COPRO and PROTO in the stool. In the porphyric group liver biopsy was performed for diagnostic purposes in only 2 cases. On the other hand, 5 out of 7 control subjects underwent liver biopsy to establish the nature of their hepatic pathology. A battery of standard 'liver function' tests was also carried out on all patients (Section A.11).

12.3.2 Determination of aetiocholanolone half-life. Aetiocholanolone-1,2-^{3}H of specific activity 10 c/mM was purchased from New England Nuclear Corporation, Boston, Mass. A solution in absolute alcohol containing 10 µc/ml was prepared. Each patient received 1 ml of this solution, diluted with 19 ml of sterile physiological saline, by intravenous injection over a period of one to two minutes. The final preparation consisted of a fine stable suspension of the steroid in saline which presented no problems with regard to injection.

In order to time the collection of specimens a stop-watch was started midway during the injection. Samples of 30-40 ml of venous blood were withdrawn at 15, 30 and 60 minutes later. Conical flasks containing a few drops of heparin solution were used for collection. All samples were centrifuged immediately afterwards and the plasma was stored at -20°C to await extraction. At the end of the hour, the patient was requested to empty his/her bladder into a receptacle provided. The volume of this specimen was measured and an aliquot was stored at -20°C.

The procedure employed for the extraction and determination of free aetiocholanolone-1,2-^{3}H in plasma are described in Sections A.8 and A.6. The radioactivity of the injected solution and of the urine was determined according to the procedures discussed in Section A.6.2.

The content of free aetiocholanolone-1,2-^{3}H in each plasma sample was expressed as d.p.m./ml. On the assumption that plasma volume represented 5% of the body weight, the percentage of the injected dose of radio-

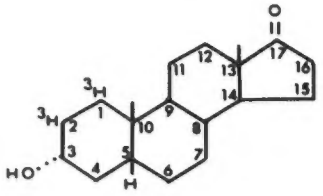
Fig. 12.1 Aetiocholanolone-1,2-^{3}H

Fig. 12.3 The clearance of unconjugated aetiocholanolone-1,2-^{3}H from the plasma in a patient with symptomatic porphyria (D. Khumalo). Semilogarithmic plot of % injected dose remaining in total plasma volume against time. The $T\frac{1}{2}$ was calculated graphically as shown.

Fig. 12.2 Schematic representation of steroid glucuronide formation.

UDPG = uridine diphosphate glucose

UDPGA = uridine diphosphate glucuronic acid



Aetiocholanolone–1,2–^{3}H

$$UDPG + 2\,NAD^{+} \xrightarrow{\text{UDPG dehydrogenase}} UDPGA + 2NADH + 2H^{+}$$

$$UDPGA + \text{Steroid} \xrightarrow{\text{Glucuronyl transferase}} \text{Steroid Glucuronide} + UDP$$

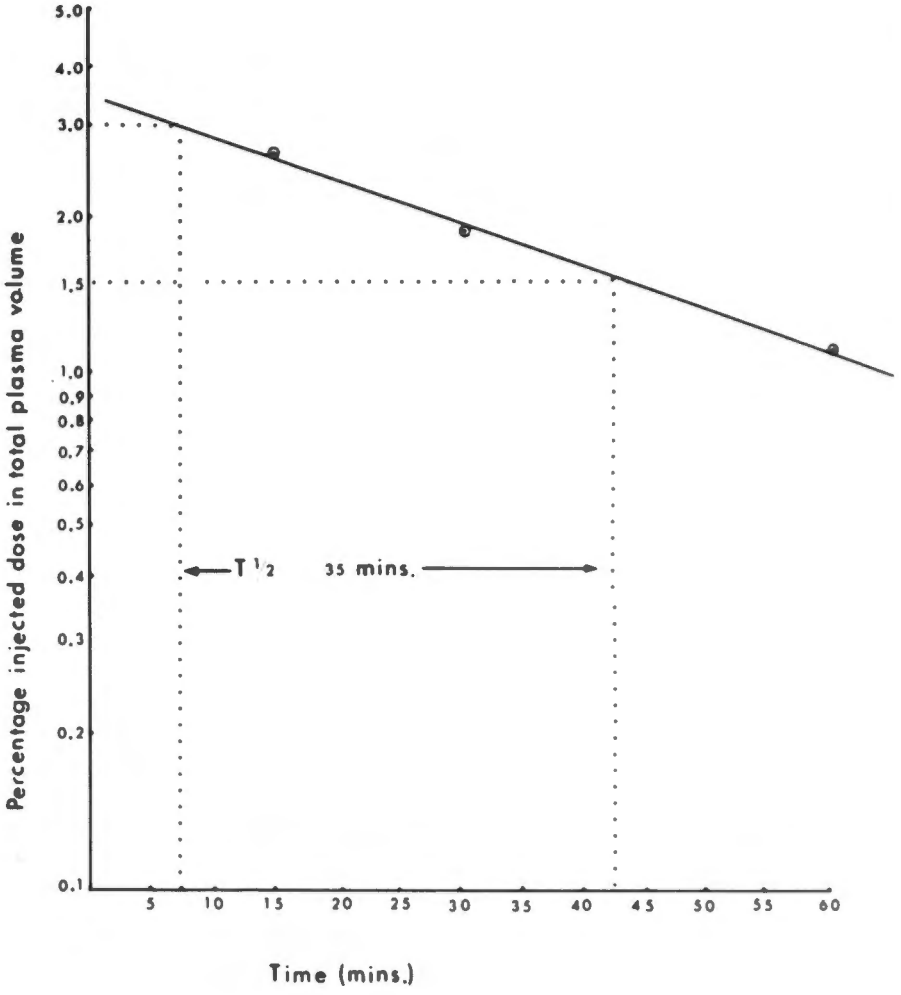


Table 12.1 Aetiocholanolone $T\frac{1}{2}$ in patients with symptomatic porphyria compared with non-porphyric subjects with liver disease. Urinary URO, serum bilirubin, SGOT and SGPT values are included for purposes of correlation.

Patient	Wt. (kg)	Diagnosis	Aetiocholanolone $T\frac{1}{2}$ (mins.)		Urinary URO (ug/100 ml)	Serum bilirubin (mg/100 ml)	SGOT (Karmen Units)	SGPT (Karmen Units)
			Observations	Mean				
Chonco, A.	66.4		29.0		186	3.8	65	32
Mkize, H.	50.0		37.5		314	0.6	61	32
Mokhantso, J.	60.5		38.5		46	0.8	58	36
Kuzwayo, C.	47.0		18.5		39	0.6	58	36
Ndlovu, F.	41.8	Symptomatic	25.0	32.3	313	0.6	68	86
Mbele, E.	46.4	porphyria	20.5	S.D.8.8	551	0.7	25	14
Ngubane, S.	64.5		35.5		3522	1.8	149	177
Mcetshwa, G.	70.0		36.0		68	0.5	>300	69
Sibiya, N.	73.0		47.0		65	1.2	45	22
Khumalo, D.	63.6		35.0	—	107	0.6	44	-
Mbambo, P.	55.9	Cirrhosis	33.5		12	1.3	67	67
Khubisa, M.	36.8	Cirrhosis & Siderosis	30.0		0	0.8	47	40
Thusi, E.	61.8	Cirrhosis	24.0		19	0.5	46	17
Mtetwa, P.	56.4	Marked siderosis	27.5		3	0.4	34	61
Ngcobo, M.	51.0	Marked siderosis & septal fibrosis	25.5	27.5 S.D.3.4	23	0.8	48	-
Shusha, A.	54.5	Hepato-cellular carcinoma	28.0		3	1.1	28	28
Mabaso, M.	46.8	Amoebic liver abscess	24.0		2	0.3	25	-

activity remaining in the total plasma volume at each time period was calculated. The values thus obtained were plotted against time on semilogarithmic paper and the $T\frac{1}{2}$ for aetiocholanolone was determined graphically from the straight line best fitting the curve as shown in the example (Fig. 12.3). The average percentage of injected dose of radioactivity remaining in the total plasma volume for all patients was 2.3% at 15 minutes and 0.8% at 60 minutes. Likewise, the percentage of the injected dose of radioactivity appearing in the urine during the hour of the test was also calculated.

12.3.3 Administration of ethanol. The test was repeated in 4 of the porphyric subjects after an interval of 5-7 days. On this occasion each patient voluntarily drank 200 ml of ethanol 96(v/v)%, diluted in the manner described previously in Section 7.2.1, within 1-2 hours after 7 a.m. The injection of aetiocholanolone was then administered 3-4 hours later.

12.4 Results. The clinical and biochemical details of all patients studied appear in Appendix B (Tables B.8, B.9). Table 12.1 summarises the results of the aetiocholanolone clearance tests in the two groups of subjects. The mean $T\frac{1}{2}$ for the porphyric patients was 32.3 minutes (S.D. 8.8) and that for the non-porphyric subjects was 27.5 minutes (S.D. 3.4). Statistical analysis as outlined in Appendix C (Section C.3) revealed that there was no significant difference at the 5% level between these two mean values.

It is also apparent from Table 12.1 that no correlation was found between aetiocholanolone $T\frac{1}{2}$ and any of the following:- body weight, urinary URO concentration, serum bilirubin level, SGOT and SGPT.

The results obtained in the 4 porphyric patients in whom the aetiocholanolone $T\frac{1}{2}$ was determined on 2 occasions are tabulated in Table 12.2. In only one of these subjects was the aetiocholanolone $T\frac{1}{2}$ prolonged after consumption of ethanol whereas in the other three the clearance of this steroid was more rapid on the second occasion.

Table 12.2 Aetiocholanolone $T\frac{1}{2}$ in 4 patients with symptomatic porphyria, before and after ethanol.

Patient	Aetiocholanolone $T\frac{1}{2}$ (mins.)	
	Before ethanol	After ethanol
Ngubane, S.	35.5	24.0
Mcetshwa, G.	36.0	34.0
Sibiya, N.	47.0	34.0
Khumalo, D.	35.0	42.5

The percentage of radioactivity appearing in the urine in the first hour after the injection was not related in either group of patients to the rate of clearance of free aetiocholanolone from the plasma. The mean value in the case of the porphyric subjects was 18.2% and in the case of the non-porphyric patients it was 17.7%. Less than 1% of this radioactivity was extractable with chloroform before hydrolysis. After hydrolysis, on the average 50% of the total urine count was then extractable with chloroform.

12.5 Discussion.

12.5.1 Metabolism of aetiocholanolone-1,2-^{3}H compared with aetiocholanolone-^{14}C. Intravenously injected aetiocholanolone-1,2-^{3}H appears to be metabolised in the same manner as aetiocholanolone-^{14}C as described by Slaunwhite and Sandberg (1958). In the present study the percentage of the injected dose of radioactivity remaining in the total plasma volume at the various time periods following intravenous administration was of the same order as the figures given by these authors for the ^{14}C-labelled compound. Likewise, the percentage of the administered dose of ^{3}H appearing in the urine during the first hour after injection was very similar to that found for aetiocholanolone-^{14}C in normal subjects. The fact that less than 1% of the urinary radioactivity was extractable with chloroform before hydrolysis confirms that the free steroid is not excreted in the urine. Following hydrolysis, on the average only 50% of the urinary radioactivity was extractable with chloroform. Slaunwhite and Sandberg (1958) similarly found that 35% of the counts in the urine "appeared either as conjugates not hydrolysable by present procedures, or as extremely hydrophilic metabolites". In the case of aetiocholanolone-1,2-^{3}H non-oxidative exchange of ^{3}H with body water (Section 10.5.1) must be considered as a possible additional factor contributing to the low percentage of urinary radioactivity recoverable by these procedures.

12.5.2 The half-life of free aetiocholanolone in symptomatic porphyria and in non-porphyric liver disease. Normal subjects were not included in the present study because of the frequency of liver pathology, in the general adult African population of Southern Africa (Section 2.2.4, Gelfand 1957), and the consequent difficulty of establishing normality without recourse to liver biopsy. The findings in the present study, therefore, have been compared with those of Slaunwhite and Sandberg (1958). These authors found an average $T\frac{1}{2}$ for aetiocholanolone of 19 minutes in 5 adult (presumably White) patients who had no evident renal or hepatic dysfunction. In the present study patients with symptomatic porphyria (mean $T\frac{1}{2}$ 32.3 mins.) and those with hepatocellular pathology but who were not porphyric (mean $T\frac{1}{2}$ 27.5 mins.), on the average, cleared free aetiocholanolone from the plasma at a slower rate. These results suggest that aetiocholanolone glucuronide formation by the liver was defective in both groups of patients. This is not unexpected since steroid conjugation has previously been shown to be defective in patients with liver disease. (Zumoff et al. (1966, 1967) using cortisol-^{14}C found that steroid glucuronide formation in patients with cirrhosis was between 41 and 87% of normal.

No significant difference, however, was found between the porphyric and non-porphyric groups with respect to aetiocholanolone clearance from plasma. This evidence does not support the proposal that defective conjugation of porphyrinogenic steroids is important in the pathogenesis of symptomatic porphyria but at the same time does not exclude the possibility. Several contingencies merit consideration.

Firstly, it may be argued, since patients with symptomatic porphyria tend to improve both from the clinical and biochemical viewpoint after hospitalisation, that the porphyric subjects in the present study might have manifested even less efficient glucuronide formation before admission. Consumption of ethanol has been shown to increase hepatic

Table 12.3 Potent inducing 5β-H steroid metabolites of physiological origin in man (Granick and Kappas 1967).

	Trivial name	Systematic name
1.	Aetiocholanolone	5β-Androstane-3α-ol,17-one
2.	Aetiocholandiol	5β-Androstane-3α,17β-diol
3.	Aetiocholanolone-3β	5β-Androstane-3β-ol,17-one
4.	Aetiocholandione	5β-Androstane-3,17-dione
5.	Aetiocholanolone-17β	5β-Androstane-3-one,17β-ol
6.	11-ketoaetiocholanolone	5β-Androstane-3α-ol,11,17-dione
7.	Pregnandiol	5β-Pregnane-3α,20α-diol
8.	Pregnandiol-20β	5β-Pregnane-3α,20β-diol
9.	Pregnanolone	5β-Pregnane-3α-ol,20-one
10.	11-ketopregnanolone	5β-Pregnane-3α-ol,11,20-dione
11.	Pregnandione	5β-Pregnane-3,20-dione
12.	Pregnantrione	5β-Pregnane-3,11,20-trione
13.	17α-OH-pregnandione	5β-Pregnane-3,20-dione,17α-ol
14.	17α-OH-pregnanolone	5β-Pregnane-3α,17α-diol,20-one
15.	17α-OH-11-ketopregnanolone	5β-Pregnane-3α,17α-diol,11,20-dione
16.	17α-OH-pregnandiol	5β-Pregnane-3α,17α,20α-triol

ALA synthetase activity and to aggravate urinary porphyrin excretion (Chapters 6, 7 and 8). Furthermore, ethanol through increasing hepatic $NADH_2/NAD$ ratios could conceivably interfere with glucuronide formation as proposed in Section 12.1. Hence it seems quite feasible that abuse of alcohol which is almost invariably associated with symptomatic porphyria could lead to intermittent further impairment of steroid glucuronide conjugation. However, the results obtained in the 4 patients tested before and after ethanol administration do not support this hypothesis. Despite the consumption of 200 ml 96(v/v)% ethanol over a period of 1-2 hours, which represents a dose in excess of 2 g/kg body weight for each of these subjects, the aetiocholanolone $T\frac{1}{2}$ was shortened rather than prolonged in 3 patients. In only one case was there an increase. According to Leevy (1967) this dose produces a significant increase in the liver $NADH_2/NAD$ ratio in about 4 hours. If alcohol acted in the manner suggested above, a significant prolongation of the aetiocholanolone $T\frac{1}{2}$ should have been found in this experiment. Hence it seems reasonable to assume that the mechanism whereby alcohol induces ALA synthetase is not related to interfering with the conjugation of inducing steroids.

Secondly, the possibility must be considered that a broader study employing a number of other potent porphyrinogenic steroids (Table 12.3) might still reveal a specific conjugation defect for one or more of these compounds in symptomatic porphyria. It has been shown that glucuronide formation by UDP-glucuronyltransferase differs with respect to different substrates. In rabbit liver Tomlinson and Yaffe (1966) found marked differences in the biochemical properties and relative activities of this enzyme(s) for the conjugation of bilirubin and p-nitrophenol. They have postulated that glucuronyltransferase is probably not "one non-specific enzyme, but a family of enzymes each with its own substrate specificity". Supposing that different steroids were conjugated by different glucuronyltransferases, nevertheless, it

seems unlikely that only one or a few of these enzymes might be selectively affected on the basis of hepatocellular pathology in symptomatic porphyria. Furthermore, aetiocholanolone is one of the more potent 5β-H inducing steroids (Granick and Kappas 1967) and is also one of the most abundant 17-ketosteroids found in human urine (Kellie and Wade 1957, Joubert 1960). It is therefore unlikely that a similar study, employing other steroids listed in Table 12.3, would yield more useful information.

The third and most likely possibility is that in symptomatic porphyria, as in the genetic varieties, steroids play an aggravating rather than an aetiological role. Various authors have stressed the importance of endocrine factors in the precipitation of attacks of acute intermittent porphyria. Thus, the preponderance of females over males presenting with symptoms and signs of this disease is well recognised (Priestly 1894, Garrod and Hopkins 1896, Waldenström 1937, Goldberg and Rimington 1962), it is uncommon for the disease to become manifest before puberty (Goldberg and Rimington 1962), and exacerbations in association with the menstrual cycle and with pregnancy have been noted (Waldenström 1937, Vine et al. 1957, Zimmerman et al. 1966). Furthermore, administration of sex hormones both to patients with AIP and variegate porphyria (VP) has frequently been reported to lead to biochemical and clinical deterioration (Watson et al. 1962, Redeker 1963, Wetterberg 1964, Dean 1965). Paradoxically, some patients with AIP obtained benefit from the use of oral progestational agents and it is suggested that in these cases suppression of the production of endogenous steroids with potent porphyrinogenic effects may be the mechanism involved (Perlroth et al. 1965).

The onset of cutaneous porphyria (porphyria cutanea tarda) has also been reported following prolonged stilboestrol therapy (Watson 1960, Becker 1965, Levere 1966). Levere's patient (Levere 1966) exhibited markedly elevated urinary uroporphyrin levels associated with

raised liver ALA synthetase activity. This was the first demonstration in man of the induction of the rate-limiting enzyme for liver haem biosynthesis by an oestrogenic substance. Stilboestrol, however, was shown by Granick (1966) to be a relatively weak inducer of porphyrin synthesis in the chick embryo liver cell system and hence Levere (1966) suggested that subjects manifesting porphyria precipitated by treatment with this drug probably have a latent predisposition.

Hence it seems logical to suggest that in symptomatic porphyria endogenously produced steroids may also have an aggravating effect on the manifestations of the disease by induction of liver ALA synthetase as has been shown for ethanol. The resultant clinical effects and uroporphyrinuria under these circumstances would then be dependent on the basic metabolic defect proposed in Chapters 7 and 8. Furthermore, this would explain why impaired steroid conjugation might be significant in symptomatic porphyria whereas the same degree of impairment in non-porphyric subjects does not lead to increased URO excretion.

12.6 Conclusions. Conjugation of aetiocholanolone appears to be abnormal not only in patients with symptomatic porphyria but also in non-porphyric subjects with various forms of hepatocellular disease. While these findings do not support the hypothesis that defective conjugation of porphyrinogenic steroids is an exclusive aetiological factor in symptomatic porphyria, such a lesion could be an important aggravating factor.

Prior administration of alcohol to patients with symptomatic porphyria did not adversely affect aetiocholanolone clearance from the plasma. This is interpreted as evidence that the influence of ethanol on liver ALA synthetase activity is not mediated through impairment of the conjugation of potent porphyrinogenic steroids.

SUMMARY AND GENERAL CONCLUSIONS

Symptomatic porphyria is generally regarded as an acquired disorder of hepatic porphyrin metabolism associated with abuse of alcohol. Some degree of parenchymal liver pathology, of which siderosis is the most constant feature, is present in all cases. It has therefore been suggested that excessive deposits of iron in the liver cell are also causally related to the development of this type of porphyria.

Accordingly, the aims of this thesis, as outlined in the preface, were :

(1) to determine the effect of ethanol on porphyrin biosynthesis and excretion, both in animals and in African patients with symptomatic porphyria,

(2) to attempt an evaluation of the relationship between hepatic siderosis and disordered porphyrin metabolism by means of an experimental model,

and (3) to investigate possible mechanisms whereby alcohol could influence porphyrin biosynthesis.

To provide a background to these studies, relevant aspects of the literature, dealing with the porphyrias (symptomatic porphyria in particular) and porphyrin and haem biosynthesis and metabolism, were critically reviewed in Chapters 1 to 5.

In Section A of the present studies (Chapters 6, 7 and 8) it was shown that administration of pure ethanol to rats and to African patients with symptomatic porphyria caused increased activity of hepatic ALA synthetase, the rate-limiting enzyme for liver haem and porphyrin biosynthesis. Ethanol was also demonstrated to produce increased urinary porphyrin excretion in porphyric patients. Conversely, withdrawal of ethanol was associated with a tendency to decreased urinary porphyrin excretion and lowered hepatic ALA synthetase activity. It was suggested that these findings justify the description of symptomatic porphyria as an 'over-production disease' and define the role of alcohol in this contion as an aggravating rather than a prime, aetiological factor.

Experimental hepatic siderosis in the rat, produced by dietary

iron overload, was shown to lead to increased urinary excretion of URO and COPRO, aggravated by drinking of alcohol. Siderosis *per se* was not associated with increased ALA synthetase activity, whereas oral consumption of alcohol was. Accordingly, it was postulated that, in these animals and in patients with symptomatic porphyria, the presence of excessive amounts of iron in the liver, in some way, determines the disposal of abnormal amounts of available haem precursors as URO and COPRO. Alcohol would then aggravate this situation by promoting the synthesis of ALA. The nature of the metabolic defect determining the preferential 'sequestration' of haem precursors, chiefly as URO, remains unknown. No block in hepatic haem synthesis at the level of coproporphyrinogen oxidase (which could theoretically explain the porphyrin excretion pattern in symptomatic porphyria) could be demonstrated. It was suggested that in symptomatic porphyria alternative pathways of porphyrin synthesis, other than the generally accepted haem biosynthetic sequence, may operate.

In Section B of the present studies a number of investigations (in animals and in porphyric subjects) which were aimed at providing an explanation for the effect of ethanol on liver ALA synthetase, were described. In view of the well-known effect of alcohol on the liver $NADH_2/NAD$ ratio, Labbe's hypothesis was considered to afford the most plausible explanation. This hypothesis states that the stimulus for porphyrinogenesis, both in experimental and human porphyria, is inhibition of terminal oxidation, with a resultant increase in the intramitochondrial $NADH_2/NAD$ ratio, thereby promoting reductive synthesis of succinate from fumarate. The carbon source supplying the increased amounts of fumarate required under these circumstances is held by Labbe to be pyruvate. Two approaches were employed to test this hypothesis.

In the study described in Chapter 9 the rate of conversion of pyruvate to succinate, via the reductive pathway proposed by Labbe, was investigated by means of liver enzyme assays in animals, made porphyric

by administration of AIA. No evidence was found to support Labbe's proposals. It was concluded : (1) that pyruvate does not appear to be the carbon source supplying additional substrate for increased haem precursor synthesis in experimental porphyria, and (2) that $NADH_2$-dependent reduction of fumarate probably does not occur either in normal or porphyric animals.

Hydrogen isotope (^3H) tracer techniques were employed (Chapter 10) to ascertain whether increased transfer of ^3H via intramitochondrial $NADH_2$ to liver haem occurs in experimental porphyria, as would be expected if Labbe's proposals were valid. While it was demonstrated that reduction of fumarate to succinate occurs normally in the intact rat, and evidence was obtained which indicates that intramitochondrial transfer of ^3H via $NADH_2$ occurs in normal animals, it would appear that fumarate reduction is not normally $NADH_2$-dependent. Reduction of fumarate by $NADH_2$ in porphyric animals could not be evaluated due to the unexpected finding that AIA depressed the incorporation of ^3H into liver haem from all the labelled precursors employed (except HO^3H and glutamate-2-^3H). It was concluded that this phenomenon could only be explained in terms of discrimination by enzymes against the C-^3H bond, operating in the porphyric animals but not in the normal rats. It was suggested that AIA might stimulate an alternate pathway of haem synthesis where one or more of the enzymes or iso-enzymes involved discriminates against ^3H to a greater extent than the corresponding enzyme(s) of the normal synthetic sequence, or that AIA promotes a mechanism for the exchange of ^3H in haem precursors or haem itself for non-radioactive H. This study focuses further attention on the concept that in porphyria it may well be that excessive synthesis of haem precursors occurs via alternate pathways, other than the commonly accepted sequence discussed in Chapter 3.

In Chapter 11 an attempt to correlate hepatic ALA synthetase activity with the redox state of the liver cell cytoplasm in patients

with symptomatic porphyria, using an intravenous galactose tolerance test, was described. The reaction which is normally rate-limiting for hepatic conversion of galactose to glucose is redox sensitive. No significant correlation between galactose tolerance and liver ALA synthetase activity was found.

From the results of the studies described in Chapters 9. 10 and 11, therefore, it was concluded that Labbe's hypothesis is probably invalid and hence does not provide an explanation for the mode of action of alcohol on ALA synthetase.

The final study, described in Chapter 12, was designed to test the proposal that certain endogenously produced steroids, shown by Granick to be porphyrinogenic in their free, unconjugated forms, might play an important part in the pathogenesis of symptomatic porphyria, and that the effect of alcohol on ALA synthetase may be mediated via interference with the conjugation of these steroids. No difference in the rate of conjugation of free aetiocholanolone, one of the more powerful inducing steroids, was found between patients with symptomatic porphyria and non-porphyric patients with various types of hepatocellular pathology. In both cases, however, the mean half-life of aetiocholanolone was greater than that reported for normal subjects. It was concluded, therefore, that steroid conjugation is probably impaired, not only in symptomatic porphyria but also in liver disease. On this basis it was suggested that, like alcohol, steroids might play an aggravating role in symptomatic porphyria through induction of ALA synthetase, while the basic lesion, mentioned previously, would determine the characteristic porphyrin excretion pattern which is not encountered in non-porphyric subjects with liver disease. Alcohol was found not to impair the rate of aetiocholanolone conjugation in patients with symptomatic porphyria and hence an effect on this mechanism cannot be the basis of the action of ethanol on liver ALA synthetase.

In essence, therefore, the findings in the present thesis have

led to the following conclusions :

(1) symptomatic porphyria is, in fact, an 'over-production' disease,

(2) the role of alcohol in symptomatic porphyria is that of an aggravating and not a prime aetiological factor,

(3) the basic lesion in symptomatic porphyria is one which, in some way, determines the 'metabolic sequestration' of abnormal amounts of haem precursors, chiefly as URO,

(4) the development of this basic lesion may be related to the presence of abnormal amounts of iron in the liver,

(5) the effect of ethanol in 'inducing' ALA synthetase cannot be explained, either in terms of an influence on the mitochondrial redox state, or on the conjugation of porphyrinogenic steroids,

(6) unconjugated steroids may also play an important aggravating role in symptomatic porphyria, and

(7) alternate pathways of porphyrin and haem biosynthesis, other than the commonly accepted synthetic sequence, may operate in human and experimental porphyria.

ACKNOWLEDGEMENTS

I wish to record my appreciation of the generous assistance of various staff members of the Sub-Department of Chemical Pathology and the Department of Pathology, University of Natal. In particular I am very grateful to Prof. S.M. Joubert for giving me the opportunity of doing this work and for his interest, valuable advice and helpful criticism throughout its course. My thanks are also due to Dr. S.S. Zail, formerly Senior Lecturer in Chemical Pathology, for his guidance during the early stages of these studies.

I am indebted to Mr. W.M. Deppe, Lecturer in Chemical Pathology, and to Miss G. Reynolds for their generous technical assistance in many of the studies, to the staff of King Edward VIII Hospital Laboratory for the routine chemico-pathological and haematological investigations, to Mr. D. Wickham for the histological preparations, to Mrs. L. Attenborough and Mrs. V. Morgan for assistance with typing, and to Mr. R. Stuart for the photographs.

I wish also to thank Dr. S. Kallichurum, Senior Lecturer in the Department of Pathology, for advice on the histological findings and Mr. L. Troskie of the Department of Mathematics, University of Natal, for assistance with the statistical analyses.

I want to thank the Superintendent of King Edward VIII Hospital for access to patients, and various members of the medical and nursing staff for their co-operation.

I gratefully acknowledge the financial support of the Council for Scientific and Industrial Research, the South African Atomic Energy Board, the Rockefeller Foundation and the United States Atomic Energy Commission.

Finally, I wish to thank my wife for her patience, understanding and encouragement and for her assistance in proof-reading.

REFERENCES

ARIAS, I. M., GARTNER, L.M., SEIFTER, S. and FURMAN, M. (1964) J. clin. Invest. 43, 2037.

BAGADASARAIN, M. (1958) Nature 181, 1399.

BAILEY, N.T.J. (1959) Statistical Methods in Biology, London, The English Universities Press Ltd.

BARNES, H.D. (1945) Clin. Proc. 4, 269.

BARNES, H.D. (1951) S. Afr. J. clin. Sci. 2, 117.

BARNES, H.D. (1955) S. Afr. med. J. 29, 781.

BARNES, H.D. (1958) S. Afr. med. J. 32, 680.

BARNES, H.D. (1959) S. Afr. med. J. 33, 274.

BARNES, H.D., FROOTKO, J. and PARNELL, J.L. (1957) S. Afr. med. J. 31, 342.

BAUMSTARK, F. (1874) cited by Goldberg and Rimington 1962.

BECKER, F.T. (1965) Arch. Derm. 92, 252.

BERMAN, J. and BIELICKÝ, T. (1956) Dermatologia 113, 78.

BERNSTEIN, W. and BALLENTINE, R. (1950) cited by Sinex, Van Slyke et al, 1955.

BLOOM, B. and FOSTER, D.W. (1964) J. biol. Chem. 239, 967.

BOGORAD, L. (1955) Science 121, 878.

BOGORAD, L. (1958a) J. biol. Chem. 233, 501.

BOGORAD, L. (1958b) J. biol. Chem. 233, 510.

BOGORAD, L. (1958c) J. biol. Chem. 233, 516.

BOGORAD, L. (1961) cited by Mauzerall 1964.

BOGORAD, L. (1963) Ann. N.Y. Acad. Sci. 104, 676.

BOGORAD, L. and GRANICK, S. (1953) Proc. nat. Acad. Sci. (Wash.) 39, 1176.

BORST, P. (1963) cited by Williamson, Lund and Krebs 1967.

BOTHWELL, T.H. and BRADLOW, B.A. (1959) S. Afr. J. med. Sci. 24, 149.

BOTHWELL, T.H. and ISAACSON, C. (1962) Brit. med. J. 1, 552.

BRADLEY, S.E., INGELFINGER, F.J. and BRADLEY, G.P. (1952) Circulation 5, 419.

BROWN, E.G. (1958a) Biochem. J. 70, 313.

BROWN, E.G. (1958b) Nature 182, 313.

BROWN, E.G. (1958c) Nature 182, 1091.

BRUGSCH, J.T. (1937) Proc. Mayo Clin. 12, 609.

BRUNSTING, L.A. (1954) Arch. Derm. Syph. 70, 551.

BÜCHER, Th. and RÜSSMAN, W. (1963) cited by Williamson, Lund and Krebs 1967.

BURNHAM, B.F. and LASCELLES, J. (1963) Biochem. J. 87, 462.

CAMPBELL, J.A.H., EALES, L. and DOWDLE, E.B.D. (1965) S. Afr. med. J. 39, 1025.

CETINGIL, A.I. and ÖZEN, M.A. (1960) Blood 16, 1002.

CONFERENCE ON THE PORPHYRIAS, CAPE TOWN (1963) S. Afr. J. Lab. clin. Med. 9, 301.

COOKSON, G.H. and RIMINGTON, C. (1953) Nature 171, 875.

COWGER, M.L. and LABBE, R.F. (1965) Lancet 1, 88.

DAVIS, A.E. and BADENOCH, J. (1962) Lancet 2, 6.

DEAN, G. (1953) Brit. med. J. 2, 1291.

DEAN, G. (1956) S. Afr. med. J. 30, 377.

DEAN, G. (1963) The Porphyrias, London, Pitman Medical Publishing Co.

DEAN, G. (1965) S. Afr. med. J. 39, 278.

DEAN, G. (1968) Brit. med. J. 1, 443.

DEAN, G. and BARNES, H.D. (1955) Brit. med. J. 2, 89.

DEAN, G. and BARNES, H.D. (1958) Brit. med. J. 1, 298.

DEAN, G. and BARNES, H.D. (1959) S. Afr. med. J. 33, 246.

De MATTEIS, F. (1964) Biochim. biophys. Acta 82, 641.

De MATTEIS, F. and RIMINGTON, C. (1962) Lancet 1, 1332.

De MATTEIS, F., SLATER, T.F. and WANG, D.Y. (1963) Biochim. biophys. Acta 68, 100.

DELBRÜCK, A., SCHIMASSEK, H., BARTSCH, K. and BÜCHER, Th. (1959) cited by Williamson, Lund and Krebs 1967.

DENT, C.E. (1968) Brit. med. J. 1, 311.

DOBRSCHANSKY, M. (1906) cited by Goldberg and Rimington 1962.

DORFMAN, R.I. and UNGAR, F. (1953) cited by Slaunwhite and Sandberg 1958.

DOWDLE, E.B. (1963) S. Afr. J. Lab. clin. Med. 9, 220.

DOWDLE, E.B., MUSTARD, P. and EALES, L. (1967) S. Afr. med. J. 41, 1093.

DOWDLE, E.B., MUSTARD, P., SPONG, N. and EALES, L. (1968) Clin. Sci. 34, 233.

DRESEL, E.I.B. and FALK, J.E. (1953) Nature 172, 1185.

DRESEL, E.I.B. and FALK, J.E. (1954) Biochem. J. 56, 156.

DRESEL, E.I.B. and FALK, J.E. (1956) Biochem. J. 63, 388.

DRUYAN, R. and HAEGER-ARONSON, B. (1964) Scand. J. clin. Lab. Invest. 16, 498.

EALES, L. (1956) S. Afr. med. J. 30, 924.

EALES, L. (1959) S. Afr. med. J. 33, 822.

EALES, L. (1960) S. Afr. J. Lab. clin. Med. 6, 63.

EALES, L. (1961) Ann. Rev. Med. 12, 251.

EALES, L. (1963) S. Afr. J. Lab. clin. Med. 9, 151.

EALES, L. and CHAIT, J. (1939) Inyanga 1, No. 14, 39.

EALES, L. and DOWDLE, E.B. (1968) Brit. med. J. 1, 841.

EALES, L., DOWDLE, E.B., SAUNDERS, S.J. and SWEENEY, G.D. (1963) S. Afr. J. Lab. clin. Med. 9, 126.

EALES, L., LEVEY, M.J. and SWEENEY, G.D. (1966) S. Afr. med. J. 40, 63.

EDINGTON, G.M. (1954) cited by Uys, van der Walt, Potgieter and Golby 1960.

ELDER, T.D. and MENGEL, C.E. (1965) Clin. Res. 13, 43.

EPSTEIN, J. and REDEKER, A. (1965) Arch. Derm. 92, 286.

FALK, J.E., DRESEL, E.I.B. and RIMINGTON, C. (1953) Nature 172, 292.

FILIP, J. and BERMAN, J. (1957) cited by Saunders 1963.

FINDLAY, G.H. and BARNES, H.D. (1950) Lancet 2, 846.

FISCHER, H.F., CONN, E.E., VENNESLAND, B. and WESTHEIMER, F.H. (1953) J. biol. Chem. 202, 687.

FISCHER, H. and ORTH, H. (1937) cited by Gray 1964.

FORSANDER, O.A. (1966) Scan. J. clin. Lab. Invest. Suppl. 92, 143.

FOURIE, P.J.J. (1936) Onderstepoort J. vet. Res. 7, 535.

FRANKE, K. and FINKENTSCHER, R. (1935) Münch. med. Wschr. 82, 171.

FREEDMAN, A.D. and GRAFF, S. (1958) J. biol. Chem. 233, 292.

FREEDMAN, A.D. and KOHN, L. (1964) Science 145, 58.

FROOTKO, J. and PARNELL, J.L. (1957) see Barnes, Frootko and Parnell 1957.

GAJDOS, A. (1966) Acta med. Scand. Suppl. 445, 36.

GAJDOS, A. and GAJDOS-TÖRÖK, M. (1961) Lancet 2, 175.

GAJDOS, A., GAJDOS-TÖRÖK, M., PALMA-CARLOS, A. and PALMA-CARLOS, L. (1967) Nouv. Rev. franc. Hémat. 7, 15.

GARROD, A.E. (1923) Inborn Errors of Metabolism, second edition, London, H. Frowde.

GARROD, A.E. and HOPKINS, F.G. (1896) cited by Goldberg and Rimington 1962.

GELFAND, M. (1955) Trans. roy. Soc. trop. Med. Hyg. 49, 370.

GELFAND, M. (1957) The Sick African, third edition, Cape Town, Juta and Co.

GELFAND, M. and MITCHELL, J.D. (1957) Trans. roy. Soc. trop. Med. Hyg. 51, 62.

GIBSON, K.D. (1958) Biochim. biophys. Acta 28, 451.

GIBSON, K.D., LAVER, W.G. and NEUBERGER, A. (1958) Biochem. J. 70, 71.

GIBSON, K.D., NEUBERGER, A. and SCOTT, J.J. (1955) Biochem. J. 61, 618.

GILLMAN, J., MANDELSTAM, J. and GILLMAN, T. (1945) S. Afr. J. med. Sci. 10, 109.

GILLMAN, T., HATHORN, M. and CANHAM, P.A.S. (1959) Amer. J. Path. 35, 349.

GILLMAN, T., HATHORN, M. and LAMONT, N. McE. (1958) S. Afr. J. med. Sci. 23, 187.

GINSBURG, A.D. and DOWDLE, E.B. (1963) S. Afr. J. Lab. clin. Med. 9, 206.

GOLDBERG, A. (1953) cited by Goldberg and Rimington 1962.

GOLDBERG, A. (1968) Brit. med. J. 1, 509.

GOLDBERG, A., ASHENBRUCKER, H., CARTWRIGHT, G.E. and WINTROBE, M.M. (1956) Blood 11, 821.

GOLDBERG, A. and RIMINGTON, C. (1955) Proc. roy. Soc. B. 143, 257.

GOLDBERG, A. and RIMINGTON, C. (1962) Diseases of Porphyrin Metabolism, Springfield, Illinois, Charles C. Thomas.

GRANICK, S. (1954) Science 120, 1105.

GRANICK, S. (1958) J. biol. Chem. 232, 1101.

GRANICK, S. (1963) J. biol. Chem. 238, PC 2247.

GRANICK, S. (1966) J. biol. Chem. 241, 1359.

GRANICK, S. (1968) Fed. Proc. 27, 300.

GRANICK, S. and KAPPAS, A. (1967) J. biol. Chem. 242, 4587.

GRANICK, S. and MAUZERALL, D. (1958a) Ann. N.Y. Acad. Sci. 75, 115.

GRANICK, S. and MAUZERALL, D. (1958b) Fed. Proc. 17, 233.

GRANICK, S. and URATA, G. (1963) J. biol. Chem. 238, 821.

GRAY, C.H. (1964) in Biochemical Disorders in Human Disease, edited by R.H.S. Thompson and E.J. King, second edition, chapter 20, London, Churchill.

GRINSTEIN, M., KAMEN, M.D. and MOORE, C.V. (1948) J. biol. Chem. 174, 767.

GROSSER, Y., SWEENEY, G.D. and EALES, L. (1967) S. Afr. med. J. 41, 460.

GÜNTHER, H. (1911) cited by Goldberg and Rimington 1962.

GÜNTHER, H. (1922) cited by Goldberg and Rimington 1962.

HAEKEL, R. and HAEKEL, H. (1968) Biochemistry 7, 3803.

HAINING, R.G. and LABBE, R.F. (1967) Fed. Proc. 26, 828.

HAMNSTRÖM, B., HAEGER-ARONSEN, B., WALDENSTRÖM, J., HYSING, B. and MOLANDER, J. (1967) Brit. med. J. 4, 449.

HARLEY, V. (1890) cited by Goldberg and Rimington 1962.

HARPER, H.A. (1967) Review of Physiological Chemistry, 11th edition, Los Altos, California, Lange Medical Publications.

HEIKEL, T., LOCKWOOD, W.H., RIMINGTON, C. (1958) Nature 182, 313.

HICKMAN, R., SAUNDERS, S.J., DOWDLE, E.B. and EALES, L. (1967) Lancet 2, 656.

HICKMAN, R. SAUNDERS, S.M., DOWDLE, E.B. and EALES, L. (1968) Biochim. biophys. Acta 161, 197.

HICKMAN, R., SAUNDERS, S.J. and EALES, L. (1967) S. Afr. med. J. 41, 456.

HIGGINSON, J., GERRITSEN, T. and WALKER, A.R.P. (1953) Amer. J. Path. 29, 779.

HJELM, M. (1967) Clin. chim. Acta 15, 87.

HOAGLAND, M.B. (1966) in The Metabolic Basis of Inherited Disease, edited by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, second edition, Chapter 2, New York, McGraw-Hill.

HOARE, D.S. and HEATH, H. (1958) Nature 181, 1592.

HOARE, D.S. and HEATH, H. (1959) Biochem. J. 73, 679.

HOBERMAN, H.D. (1958a) J. biol. Chem. 232, 9.

HOBERMAN, H.D. (1958b) J. biol. Chem. 233, 1045.

HOBERMAN, H.D. and D'ADAMO, A.F. Jr. (1960) J. biol. Chem. 235, 519.

HOBERMAN, H.D. and PROSKY, L. (1967) Biochim. biophys. Acta 148, 392.

HOBERMAN, H.D., PROSKY, L., HEMPSTEAD, P.G. and ARFIN, H.W. (1964) Biochem. biophys. Res. Commun. 17, 490.

HOLTI, G., RIMINGTON, C., TATE, B.C. and THOMAS, G. (1958) Quart. J. Med. 27, 1.

HOPPE-SEYLER, F. (1871) cited by Goldberg and Rimington 1962.

HSIA, D., DOWBEN, R., SHAW, R. and GROSSMAN, A. (1960) Nature 187, 603.

HUFFNER, M. and HOLLOCHER, T.C. (1968) J. biol. Chem. 243, 3482.

IODICE, A.A., RICHERT, D.A. and SCHULMAN, M.P. (1958) Fed. Proc. 17, 248.

IPPEN, H. (1961) Dtsch. med. Wschr. 86, 127.

ISSELBACHER, K.J. and KRANE, S.M. (1961) J. biol. Chem. 236, 2394.

JOHNSON, W.J. and QUASTEL, J.H. (1953) Nature 171, 602.

JOUBERT, S.M. (1960) S. Afr. J. Lab. clin. Med. 6, 107.

JOUBERT, S.M., McKECHNIE, J.K. and DEPPE, W.M. (1963) S. Afr. J. Lab. clin. Med. 9, 227.

JOUBERT, S.M. and SHREEVE, W.W. (1968) Unpublished.

KAPPAS, A. and GRANICK, S. (1968) J. biol. Chem. 243, 346.

KAUFMAN, B.T. and KAPLAN, N.O. (1960) Biochim. biophys. Acta 39, 332.

KAUFMAN, S. (1955) in Methods in Enzymology, edited by S.P. Colowick and N.O. Kaplan, vol. 1, p.719, New York, Academic Press.

KEECH, D.B. and UTTER, M.F. (1963) J. biol. Chem. 238, 2609.

KEEN, G.A., SAUNDERS, S.J. and EALES, L. (1966) Lancet 1, 798.

KEELEY, K.J. (1962) S. Afr. med. J. 36, 602.

KEELEY, K.J., BOTHWELL, T. and KRAMER, S. (1960) Lancet 1, 601.

KELLIE, A.E. and WADE, A.P. (1957) Biochem. J. 66, 196.

KIKUCHI, G., KUMAR, A., TALMAGE, P. and SHEMIN, D. (1958) J. biol. Chem. 233, 1214.

KING, E.J. and COXON, R.V. (1950) J. clin. Path. 3, 248.

KING, E.J., HASLEWOOD, G.A.D., DELORY, G.E. and BEALL, D. (1942) Lancet, 1, 207.

KING, J. (1960) J. med. Lab. Technol. 17, 1.

KNUDSEN, K.B., SPARBERG, M. and LECOCQ, F. (1967) New Engl. J. Med. 277, 350.

KOOY, F.H. (1939) S. Afr. med. J. 13, 720.

KOTTRA, J. and KAPPAS, A. (1967) Ann. Rev. Med. 18, 325.

KRAMER, S. (1962) cited by Kramer 1963.

KRAMER, S. (1963) S. Afr. J. Lab. clin. Med. 9, 283.

KREBS, H. (1964) Proc. roy. Soc. B. 159, 545.

KREBS, H.A., GASCOYNE, T. and NOTTON, B.M. (1967) Biochem. J. 102, 275.

KURUMADA, T. and LABBE, R.F. (1966) Science 151, 1228.

LABBE, R.F. (1962) Lancet 1, 435.

LABBE, R.F. (1967) Lancet 1, 1361.

LABBE, R.F. (1968) Personal communication.

LABBE, R.F., KURUMADA, T. and ONISAWA, J. (1965) Biochim. biophys. Acta 111, 403.

LABBE, R.F. and NISHIDA, G. (1957) Biochim. Biophys. Acta 26, 437.

LABBE, R.F., TALMAN, E. L. and ALDRICH, R.A. (1954) Biochim. biophys. Acta 15, 590.

LAMONT, N. McE. (1963) S. Afr. J. Lab. clin. Med. 9, 303.

LAMONT, N. McE., HATHORN, M. and JOUBERT, S.M. (1961) Quart. J. Med. 30, 373.

LARDY, H.A., PAETKAU, V. and WALTER, P. (1965) Proc. nat. Acad. Sci. (Wash.) 53, 1410.

LASCELLES, J. (1957) Biochem. J. 66, 65.

LASCELLES, J. (1959) Biochem. J. 72, 508.

LASCELLES, J. (1960) J. gen. Microbiol. 23, 487.

LASCELLES, J. (1964) Tetrapyrrole Biosynthesis and its Regulation, New York and Amsterdam, W.A. Benjamin Inc.

LAVER, W.G., NEUBERGER, A. and UDENFRIEND, S. (1958) Biochem. J. 70, 4.

LEEVY, C.M. (1967) Fed. Proc. 26, 1474.

LEHNINGER, A.L. (1954) Harvey Lect. Series 49, 176.

LEVERE, R.D. (1966) Blood, 28, 569.

LEVERE, R.D. (1967) Biochem. Med. 1, 92.

LEVERE, R.D. and GRANICK, S. (1965) Proc. nat. Acad. Sci. (Wash.) 54, 134.

LEVERE, R.D. and GRANICK, S. (1967) J. biol. Chem. 242, 1903.

LIEBER, C.S. (1965) in Progress in Liver Diseases, edited by H. Popper and F. Schaffner, Vol. 2, p.134, New York, Grune and Stratton.

LIEBER, C.S. and RUBIN, E. (1968) Amer. J. Med. 44, 200.

LINDER, G.C. (1947) Lancet 2, 649.

LOCKHEAD, A.C., KRAMER, S. and GOLDBERG, A. (1963) Brit. J. Haemat. 9, 39.

LOCKWOOD, W.H. and RIMINGTON, C. (1957) Biochem. J. 67, 8P.

LONDON, I.M., BRUNS, G.P. and KARIBIAN, D. (1964) Medicine 43, 789.

LONDON, I.M., SHEMIN, D. and RITTENBERG, D. (1950) J. biol. Chem. 183, 749.

LOWENSTEIN, J.M. (1961) J. biol. Chem. 236, 1213.

LUNDÉN, R., WESTLAND, L. and FLORELL, C. (1966) Scand. J. clin. Lab. Invest. Suppl. 92, 114.

LUZIO, N.R. and HARTMAN, A.D. (1967) Fed. Proc. 26, 1436.

LWOFF, A. (1962) Biological Order, Cambridge, Mass., M.I.T. Press.

MACALPINE, I. and HUNTER, R. (1966) Brit. med. J. 1, 65.

MACALPINE, I., HUNTER, R. and RIMINGTON, C. (1968) Brit. med. J. 1, 7.

MACDONALD, R.A. (1963) Lancet 1, 727.

MAGNUS, I.A., JARRETT, A., PRANKHERD, T.A.J. and RIMINGTON, C. (1961) Lancet 2, 448.

MARVER, H.S. (1966) J. Lab. clin. Med. 6, 996.

MARVER, H.S., COLLINS, A., TSCHUDY, D.P. and RECHCIGL, M. (1966) J. biol. Chem. 241, 4323.

MARVER, H.S., TSCHUDY, D.P., PERLROTH, M.G. and COLLINS, A. (1966) J. biol. Chem. 241, 2803.

MARVER, H.S., TSCHUDY, D.P., PERLROTH, M.G., COLLINS, A. and HUNTER. G. (1966) Analyt. Biochem. 14, 53.

MAUZERALL, D. (1964) J. Pediat. 64, 5.

MAUZERALL, D. and GRANICK, S. (1956) J. biol. Chem. 219, 435.

MAUZERALL, D. and GRANICK, S. (1958) J. biol. Chem. 232, 1141.

MAXWELL, E.S. (1957) J. biol. Chem. 229, 139.

MEGYESI, C., SAMOLS, E. and MARKS, V. (1967) Lancet 2, 1051.

MONOD, J., JACOB, F. and GROS, F. (1962) cited by Tschudy 1965.

MUIR, H.M. and NEUBERGER, A. (1949) Biochem. J. 45, 163.

MUIR, H.M. and NEUBERGER, A. (1950) Biochem. J. 47, 97.

MULDER (1844) cited by Goldberg and Rimington 1962.

MUSTAFA, M.G., COWGER, M.L., LABBE, R.F. and KING, T.E. (1968) J. biol. Chem. 243, 1908.

NAKAO, K., WADA, O., KITAMURA, T., UONO, K. and URATA, G. (1966) Nature 210, 838.

NANDI, D.L., BAKER-COHEN, K.F. and SHEMIN, D. (1968) J. biol. Chem. 243, 1224.

NANDI, D.L. and SHEMIN, D. (1968) J. biol. Chem. 243, 1236.

NAUNYN, A. (1906) cited by Megyesi et al. 1967.

NEMETH, A.M., RUSSELL, C.S. and SHEMIN, D. (1957) J. biol. Chem. 229, 415.

NEUBERGER, A. and TURNER, J.M. (1963) Biochim. biophys. Acta 67, 342.

NEVÉ, R.A., LABBE, R.F. and ALDRICH, R.A. (1956) J. Amer. chem. Soc. 78, 691.

NIKKILÄ, E.A. and OJALA, K. (1963) Proc. Soc. exp. Biol. (N.Y.) 113, 814.

NYBERG, A., SCHUBERTH, J. and ÄNGGÄRD, L. (1953) Acta chem. Scand. 7, 1170.

OCHNER, R.K. and SCHMID, R. (1961) Nature 189, 499.

OCHOA, S. (1955) in Methods in Enzymology, edited by S.P. Colowick and N.O. Kaplan, Vol. 1, p.739, New York, Academic Press.

OCHOA, S., MEHLER, A.H. and KORNBERG, A. (1948) J. biol. Chem. 174, 979.

OCHOA, S., STERN, J.R. and SCHNEIDER, M.C. (1951) J. biol. Chem. 193, 691.

O'DONNEL, J.F., SCHIFF, L. and PILLER, M. (1962) J. Lab. clin. Med. 59, 963.

ORTEN, J.M., DOEHR, S.A., BOND, C., JOHNSON, H. and PAPPAS, A. (1963) Quart. J. Stud. Alcohol 24, 598.

OWEN, J.A., IGGO, B., SCANDRETT, F.J. and STEWART, C.P. (1954) Biochem. J. 58, 426.

OYAMA, H., SUGITA, Y., YONEYAMA, Y. and YOSHIKAYA, H. (1961) Biochim. biophys. Acta 47, 413.

PATTERSON, M.S. and GREENE, R.C. (1965) Analyt. Chem. 37, 854.

PERLROTH, M.G., MARVER, H.S. and TSCHUDY, D.P. (1965) J. Amer. med. Ass. 194, 1037.

PERLROTH, M.G., TSCHUDY, D.P., RATNER, A., SPAUR, W. and REDEKER, A. (1968) Metabolism 17, 571.

PETERS, J.P. and VAN SLYKE, D.D. (1932) Quantitative Clinical Chemistry, Vol. 2, p.602, Baltimore, Williams and Williams.

PETERS, T., GIOVANNIELLO, T.J., APT, L. and ROSS, J.F. (1956a) J. Lab. clin. Med. 48, 274.

PETERS, T., GIOVANNIELLO, T.J.. APT, L. and ROSS, J.F. (1956b) J. Lab. clin. Med. 48, 280.

PORRA, R.J. and FALK, J.E. (1964) Biochem. J. 90, 69.

PORRA, R.J. and JONES, O.T.G. (1963) Biochem. J. 87, 181.

PRIESTLEY, J. (1894) cited by Goldberg and Rimington 1962.

PURVIS, J.L. and LOWENSTEIN, J.M. (1961) J. biol. Chem. 236, 2794.

RADIN, N.S., RITTENBERG, D. and SHEMIN, D. (1950) J. biol. Chem. 184, 745.

REDEKER, A.G. (1963) S. Afr. J. Lab. clin. Med. 9, 302.

REINHOLD, J.G. (1953) Standard Methods in Clinical Chemistry, edited by M. Reiner, Vol. 1, New York, Academic Press.

RICHTER, G.W. (1957) J. exp. Med. 106, 203.

RIMINGTON, C. (1956) Brit. med. J. 2, 189.

RIMINGTON, C. (1966) Acta med. scand. Suppl. 445, 11.

RIMINGTON, C. (1968) Brit. med. J. 1, 509.

RIMINGTON, C. and De MATTEIS, F. (1965) Lancet 1, 270.

ROBINSON, E.A., KALCKAR, H.M. and TROEDSSON, H. (1963) Biochem. biophys. Res. Commun. 13, 313.

ROE, J.H. and COGWILL, G. R. (1935) Amer. J. Physiol. 111, 530.

RUBIN, E. and LIEBER, C.S. (1968) New Engl. J. Med. 278, 869.

SAIFER, A., GERSTENFELD, S. and VECSLER, F. (1961) Clin. Chem. 7, 626.

SALIMAN, P.M. (1964) Analyt. Chem. 36, 112.

SANDBERG, A.A. and SLAUNWHITE, W.R. (1956) J. clin. Invest. 35, 1331.

SANO, S. and GRANICK, S. (1961) J. biol. Chem. 236, 1173.

SANO, S., INOUE, S., TANABE, Y., SUMIYA, C. and KOIKE, S. (1959) Science 129, 275.

SAUNDERS, S.J. (1963) S. Afr. J. Lab. clin. Med. 9, 277.

SCHEFFÉ, H. (1959) The Analysis of Variance, New York and London, John Wiley and Sons inc.

SCHIFFMAN, E. and SHEMIN, D. (1955) cited by Shemin 1955.

SCHMID, R. (1963) S. Afr. J. Lab. clin. Med. 9, 212.

SCHMID, R. (1966) in The Metabolic Basis of Inherited Disease, edited by J.P. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, second edition, Chapter 36, New York, McGraw-Hill.

SCHMID, R., FIGEN, F. and SCHWARTZ, S. (1955) J. biol. Chem. 217, 263.

SCHMID, R. and SCHWARTZ, S. (1952) Proc. Soc. exp. Biol. (N.Y.) 81, 685.

SCHMID, R., SCHWARTZ, S. and WATSON, C.J. (1954) Arch. intern. Med. 93, 167.

SCHMID, R. and SHEMIN, D. (1955) J. Amer. chem Soc. 77, 506.

SCHULMAN, M.P., and RICHERT, D.A. (1957) J. biol. Chem. 226, 181.

SCHULTZ, J.H. (1874) cited by Goldberg and Rimington 1962.

SCHWARTZ, S. (1955) Fed. Proc. 14, 717.

SCOTT, J.J. (1955) in Ciba Foundation Symposium on Porphyrin Biosynthesis and Metabolism, edited by G.E.W. Wolstenholme and E.C.P. Millar, London, Churchill.

SCOTT, J.J. and GRAY, C.H. (1962) cited by Schmid (1966).

SEFTEL, H.C., ISAACSON, C. and BOTHWELL, T.H. (1960) S. Afr. J. med. Sci. 25, 89.

SEFTEL, H.C., KEELEY, K.J., ISAACSON, C. and BOTHWELL, T.H. (1961) J. Lab. clin. Med. 58, 837.

SHANLEY, B.C., ZAIL, S.S. and JOUBERT, S.M. (1968) Lancet 1, 70.

SHEMIN, D. (1955) Harvey Lect. Series 50, p.258.

SHEMIN, D., KIKUCHI, G. and ABRAMSKY, T. (1962) cited by Schmid 1966.

SHEMIN, D. and KUMIN, S. (1952) J. biol. Chem. 198, 827.

SHEMIN, D., LONDON, I.M. and RITTENBERG, D. (1948) J. biol. Chem. 173, 799.

SHEMIN, D. and RITTENBERG, D. (1945) J. biol. Chem. 159, 567.

SHEMIN, D. and RITTENBERG, D. (1946) J. biol. Chem. 166, 621, 627.

SHEMIN, D. and RUSSELL, C.S. (1953) J. Amer. chem. Soc. 75, 4873.

SHEMIN, D., RUSSELL, C.S. and ABRAMSKY, T. (1955) J. biol. Chem. 215, 613.

SHEMIN, D. and WITTENBERG, J. (1951) J. biol. Chem. 192, 315.

SHRAGO, E. and LARDY, H.A. (1966) J. biol. Chem. 241, 663.

SINEX, F.M., PLAZIN, J., CLAREUS, D., BERNSTEIN, W., VAN SLYKE, D.D. and CHASE, R. (1955) J. biol. Chem. 213, 673.

SLAUNWHITE, W.R. and SANDBERG, A.A. (1958) J. clin. Endocr. 18, 1056.

SMITH, M.E. and NEWMAN, H.W. (1959) J. biol. Chem. 234, 1544.

STENSTAM, T. (1946) Acta med. scand. Suppl. 177, 11.

STOKVIS, B.J. (1889) cited by Goldberg and Rimington 1962.

STOKVIS, B.J. (1895) cited by Rimington 1966.

STRACHAN, A.S. (1929) cited by Wainwright 1957.

SUTHERLAND, D.A. and WATSON, C.J. (1951) J. Lab. clin. Med. 37, 29.

SVEINSSON, S.L., RIMINGTON, C. and BARNES, H.D. (1949) Scand. J. clin. Lab. Invest. 1, 2.

SWEENEY, G.D. (1963) S. Afr. J. Lab. clin. Med. 9, 182.

SWEENEY, G.D., DOWDLE, E.B., SAUNDERS, S.J. and EALES, L. (1963) S. Afr. J. Lab. clin. Med. 9, 247.

SWEENEY, G.D., LEVEY, M., DOWDLE, E.B. and EALES, L. (1962) S. Afr. med. J. 36, 312.

TALMAN, E.L., CASE, J.D., NEVÉ, R.A., LABBE, R.F. and ALDRICH, R.A. (1955) J. biol. Chem. 212, 663.

TALMAN, E.L., LABBE, R.F., ALDRICH, R.A. and SEARS, D. (1959) Arch. Biochem. 80, 446.

TENGSTROM, B. (1966) Scand. J. clin. Lab. Invest. Suppl. 92, 132.

THERON, J.J., HAWTREY, A.O., LIEBENBERG, N. and SCHIRREN, V. (1963) Amer. J. Path. 43, 73.

THOMPSON, G.N. (1956) cited by Lieber 1965.

THORNE, C.J.R. (1960) Biochim. biophys. Acta 42, 175.

TIO, T.H. and LEIJNSE, B. (1958) Arch. Derm. 77, 568.

TIO, T.H., LEIJNSE, B., JARRETT, A. and RIMINGTON, C. (1957) Clin. Sci. 16, 517.

TOMKINS, G.M. (1957) J. biol. Chem. 225, 13.

TOMLINSON, G.A. and YAFFE, S.J. (1966) Biochem. J. 99, 507.

TOPPER, Y.J. (1957) J. biol. Chem. 225, 419.

TSCHUDY, D.P. (1965) J. Amer. med. Ass. 191, 718.

TSCHUDY, D.P., PERLROTH, M.G., MARVER, H.S., COLLINS, A., HUNTER, G. and RECHCIGL, M. (1965) Proc. nat. Acad. Sci. (Wash.) 53, 841.

TSCHUDY, D.P., ROSE, J., HELLMAN, E., COLLINS, A. and RECHCIGL, M. (1962) Metabolism 11, 1287.

TSCHUDY, D.P., WELLAND, F.H., COLLINS, A. and HUNTER, G. (1963) Lancet 2, 660.

TSCHUDY, D.P., WELLAND, F.H., COLLINS, A. and HUNTER, G. (1964) Metabolism 13, 396.

TYGSTRUP, N. and WINKLER, K. (1958) Clin. Sci. 17, 1.

URATA, G. and GRANICK, S. (1963) J. biol. Chem. 238, 811.

UTTER, M.F. and KEECH, D.B. (1963) J. biol. Chem. 238, 2603.

UTTER, M.F., KEECH, D.B. and SCRUTTON, M.C. (1964) cited by Krebs, Gascoyne and Notton 1967.

UYS, C.J. and EALES, L. (1963) S. Afr. J. Lab. clin. Med. 9, 190.

UYS, C.J., van der WALT, J.J., POTGIETER, G.M. and GOLBY, H.H. (1960) S. Afr. J. Lab. clin. Med. 6, 1.

VAN SLYKE, D.D., DILLON, R.T., MACFAYDEN, D.A. and HAMILTON, P.B. (1941) J. biol. Chem. 141, 627.

VAN SLYKE, D.D. and NEILL, J.M. (1924) J. biol. Chem. 61, 523.

VAN SLYKE, D.D., PLAZIN, J. and WEISIGER, J.R. (1951) J. biol. Chem. 191, 299.

VINE, S., SCHAFFER, H.M., PAULEY, G. and HARGOLIS, E.J. (1957) Ann. intern. Med. 47, 834.

WAINWRIGHT, J. (1957) S. Afr. J. Lab. clin. Med. 3, 1.

WALDENSTRÖM, J. (1937) Acta med. scand. Suppl. 82, 1.

WALDENSTRÖM, J. (1957) Amer. J. Med. 22, 758.

WALDENSTRÖM, J. (1967) see Hamnström, Haeger-Aronsen, Waldenström, Hysing and Molander 1967.

WALDENSTRÖM, J. and VAHLQUIST, B. (1944) Acta med. scand. 117, 1.

WATSON, C.J. (1954) Advanc. intern. Med. 6, 235.

WATSON, C.J. (1960) New Engl. J. Med. 263, 1205.

WATSON, C.J., LOWRY, P.T., SCHMID, R., HAWKINSON, V.E. and SCHWARTZ, S. (1951) Tr. Ass. Amer. Phycns. 64, 345.

WATSON, C.J., RUNGE, W. and BOSSENMAIER, I. (1962) Metabolism 11, 1129.

WELIKY, I. and SHEMIN, D. (1957) Fed. Proc. 16, 268.

WELLAND, F.H., HELLMAN, E.S., GADDIS, E.M., COLLINS, A., HUNTER, G.W. and TSCHUDY, D.P. (1964) Metabolism 13, 232.

WESTALL, R.G. (1952) Nature 170, 614.

WETTERBERG, L. (1964) Lancet 2, 1178.

WIELAND, O. and WEISS, L. (1963) Biochem. biophys. Res. Commun. 10, 333.

WILLIAMS, R.T. (1959) cited by Granick 1966.

WILLIAMSON, D.H., LUND, P. and KREBS, H.A. (1967) Biochem. J. 103, 514.

WILSON, M.L., IODICE, A.A., SCHULMAN, M.P. and RICHERT, D.A. (1959) Fed. Proc. 18, 352.

WILZBACH, K.E., KAPLAN, L. and BROWN, W.G. (1953) Science 118, 522.

WISE, E.M. Jr. and BALL, E. (1964) Proc. nat. Acad. Sci. (Wash.) 52, 1255.

WITH, T.K. (1955) Scand. J. clin. Lab. Invest. 7, 193.

WITTENBERG, J. and SHEMIN, D. (1949) J. biol. Chem. 178, 47.

WITTENBERG, J. and SHEMIN, D. (1950) J. biol. Chem. 185, 103.

WOODS, J.D. and BARNES, H.D. (1951) S. Afr. med. J. 25, 952.

WRISTON, J.C., LACK, L. and SHEMIN, D. (1955) J. biol. Chem. 215, 603.

YONEYAMA, Y., OYAMA, H., SUGITA, Y. and YOSHIKAWA, H. (1962) Biochim. biophys. Acta 62, 261.

YOUNG, J.W., SHRAGO, E. and LARDY, H.A. (1964) Biochemistry 3, 1687.

ZAIL, S.S. and JOUBERT, S.M. (1968) Brit. J. Haemat. 15, 123.

ZIEVE, L., HILL, E., SCHWARTZ, S. and WATSON, C.J. (1953) J. Lab. clin. Med. 41, 663.

ZIMMERMAN, T.S., McMILLIN, J.M. and WATSON, C.J. (1966) Arch. intern. Med. 118, 229.

ZUMOFF, B., BRADLOW, H.L., GALLAGHER, T.F. and HELLMAN, L. (1967) J. clin. Invest. 46, 1735.

ZUMOFF, B., BRADLOW, H.L. and HELLMAN, L. (1966) J. clin. Invest. 45, 1091.

APPENDIX A

METHODS

Reagents. Reagents employed were in all instances of a high grade of purity (usually Merck or Analar). Further purification was not carried out in the laboratory except where specifically stated. The sources of special reagents, e.g. enzymes, nucleotides, resins, etc., have been indicated in the appropriate sections.

A.1 Enzyme Assays

A.1.1 Liver ALA synthetase assays. Since ALA dehydratase is located in the cell cytoplasm while ALA synthetase is a mitochondrial enzyme, previous methods utilised isolated mitochondria for the assay of liver ALA synthetase (Granick and Urata 1963, Tschudy et al. 1964). Following the demonstration that high concentrations of ethylene-diaminetetra-acetic acid (EDTA) almost completely inhibit ALA dehydratase while enhancing ALA synthesis, it has become possible to determine ALA synthetase activity in liver homogenates (Marver, Tschudy, Perlroth and Collins 1966). This observation has simplified the assay considerably and in addition has made studies on needle biopsy material possible.

Accordingly, in the assay of this enzyme in both human liver needle biopsy specimens and rat liver, whole homogenates of liver containing TRIS and EDTA have been employed. The substrate for incubation comprised glycine either alone or in combination with sodium citrate. Contrary to the findings of Marver, Tschudy, Perlroth and Collins (1966), 0.05 M citrate was not found to inhibit ALA synthetase activity. Pyridoxal phosphate was omitted in studies on rats in accordance with Marver and co-workers (Marver, Tschudy, Perlroth and Collins, 1966), who found that the addition of this cofactor did not affect ALA production in porphyric rat liver homogenate; likewise this co-enzyme was not included in determinations on human liver since no significant effect was produced by its addition to the incubation medium.

Following the conversion of ALA and aminoacetone to pyrroles by condensation with acetylacetone, separation of the ALA pyrrole and aminoacetone pyrrole was achieved either by column chromatography on Dowex 1 acetate resin (Marver, Tschudy, Perlroth, Collins and Hunter, 1966), or by extraction of aminoacetone pyrrole from the mixture with equilibrated ether (Granick 1966).

ALA pyrrole thus isolated was subsequently determined by addition of modified Ehrlich's reagent and measurement of the optical density of the mixture as detailed below using a Zeiss PMQII or Beckman DB spectrophotometer.

The methods to be described were shown to give comparable results in the assay of ALA synthetase activity of rat and human liver (Section A.1.1.3).

A.1.1.1 Macro-methods for determinations in rat liver. Two methods were used for determination of ALA synthetase activity in rat liver. Initially (Chapter 6) column chromatography on Dowex 1 resin was employed for separation of the ALA and aminoacetone pyrroles - Method (i). Subsequently (Chapter 8) the simpler ether extraction procedure (Granick 1966) for separation of the pyrroles was preferred - Method (ii). Method (i). (Shanley et al. 1968). In a Potter-Elvehjem homogeniser, 1 in 3 homogenates of rat livers were made in ice-cold buffer (0.075 M tris, 0.01 M EDTA, 0.1 M glycine, pH 7.4). The homogenates for each group of 5 rats were pooled and 2.5 ml samples of the pooled homogenates were diluted with 7.5 ml of the same buffer and incubated at 37°C for one hour. The reaction was stopped with 2.5 ml of 25% trichloracetic acid and after 15 minutes the deproteinised solution was centrifuged in order to obtain a clear supernatant. For each incubation a similar control sample was treated immediately with 2.5 ml of 25% trichloracetic acid.

Following centifugation, ALA was determined in the supernatant fraction according to the method of Marver, Tschudy, Perlroth, Collins and Hunter (1966) as described below. Ten ml of this solution in a glass-stoppered boiling tube was adjusted to pH 4.5-6 by the addition of 2 ml of 2.5 M sodium acetate solution; 0.3 ml of acetylacetone was added and the mixture heated in a boiling water bath for 10 minutes.

After cooling, 10 ml of the solution containing the pyrroles were placed on a 1 x 7 cm column of Dowex 1 acetate. The resin was prepared from specially purified Dowex 1-X8 chloride (200-400 mesh) (Bio-Rad Labs., California) by washing a column with 20 bed volumes of 3 M sodium acetate solution, followed by batch washing with 50 bed volumes of distilled water. The aminoacetone pyrrole was eluted with 10 ml of n-butanol containing 0.01 M ammonium hydroxide after which the column was washed with 10 ml of 1 M acetic acid. Finally, the ALA pyrrole was eluted with 10 ml of a mixture of glacial acetic acid and methanol (1:2).

This final eluate was centrifuged for 10 minutes, if necessary, to remove any turbidity. An aliquot was mixed with an equal volume of modified Ehrlich's reagent containing perchloric acid (2M) (Mauzerall and Granick 1956). After 20-45 minutes, the optical density of the mixture at 556 mμ was determined in a cell of 1 cm optical length. The ALA content of the solutions was calculated using an E_m 556 of 6.6×10^4.

Method (ii). The second and more convenient method used for determination of ALA synthetase activity in rat liver was a macro-scale version of the micro-procedure described below for liver biopsy material (Section A.1.1.2).

1 in 10 homogenates of rat liver in ice-cold buffer (0.15 M tris, 0.02 M EDTA, pH 7.4) were made using a Potter-Elvehjem homogeniser. Two ml of homogenate was mixed with 2 ml of substrate

(0.1 M glycine, 0.1 M sodium citrate, pH 7.4) and the mixture incubated at 37°C for one hour. The reaction was stopped by the addition of 4 ml of 0.6 M trichloracetic acid. In a duplicate (control) sample enzyme activity was prevented by the immediate addition of 4 ml of 0.6 M trichloracetic acid. After fifteen minutes the deproteinised solutions were centrifuged to ensure clarity of the supernatant preparations.

Further procedures for determining ALA in the clear supernatant were identical with those described in Section A.1.1.2. except that the quantities of ingredients used were 10 times those stipulated.

A.1.1.2 Micro-method for needle biopsy specimens (Zail and Joubert 1968). Approximately 20-40 mg of liver was obtained by percutaneous liver biopsy performed under local anaesthesia with a Menghini needle. The tissue was immediately placed in ice-cold tris buffer (0.15 M, pH 7.4) containing 0.02 M EDTA for transport to the laboratory. Weighed samples of liver (10-20 mg) were placed in cold glass micro-homogenisers, cold tris buffer was added to a total volume of 0.2 ml and homogenisation was carried out in an ice bath using closely fitting ground glass pestles. After removal of 0.01 ml homogenate for phosphorus determination (Section A.2) the volume was reconstituted to 0.2 ml with tris buffer and then 0.2 ml of substrate (0.1 M glycine, 0.1 M sodium citrate, pH 7.4) was added. In each assay one homogenised sample was incubated in a water bath at 37°C for one hour whereafter the reaction was stopped by the addition of 0.4 ml of 0.6 M trichloracetic acid. In a duplicate (control) sample enzyme activity was prevented by the immediate addition of 0.4 ml of 0.6 M trichloracetic acid. After 15 minutes the deproteinised solutions were centrifuged for 5 minutes in a Beckman/Spinco Microfuge 152.

Further procedures for determining ALA in the clear supernatant fraction were a micro-scale modification of the method

described by Granick (1966). 0.5 ml of this supernatant solution was placed in a small glass-stoppered boiling tube and neutralised to pH 4.6 with 0.1 ml of 2.5 M sodium acetate solution; then 0.01 ml of acetylacetone was added and the mixture was heated in a boiling water bath for 10 minutes to convert ALA and aminoacetone to pyrrole derivatives.

After cooling, 0.2 ml of this solution was removed and mixed with 0.2 ml of Ehrlich-mercury reagent (Lwoff 1962). At 15 minutes after mixing the optical density (W) at 552 mμ was determined in a micro-cell of 1 cm optical length.

The remaining 0.4 ml after neutralisation to between pH 7 and 7.5 by addition of 0.01 ml of 0.5 M Na_2HPO_4 solution and 0.03 ml of 1.8 M NaOH solution was shaken with equilibrated ether for 1 minute. 0.2 ml of the aqueous phase which according to Granick (1966) contains 85% of the ALA pyrrole and 5% of the aminoacetone pyrrole was removed and mixed with 0.2 ml of Ehrlich-mercury reagent. The optical density (Z) of this mixture at 552 mμ was determined after 15 minutes as for (W).

Equilibrated ether was freshly prepared for each assay as follows. Peroxide-free ether was washed three times with an equal volume of 1 M NaOH to remove all traces of the antioxidant, propyl gallate; this was followed by several washings with distilled water to remove NaOH. Finally, the ether was shaken with an equal volume of an aqueous solution containing trichloracetic acid (0.227 M), sodium acetate (0.038 M), Na_2HPO_4 (0.011 M) and NaOH (0.012 M).

Under the conditions used to determine the optical densities (W) and (Z) the E_m 552 of the Ehrlich-colour salt of the ALA pyrrole is 5.8×10^4 and that of the aminoacetone pyrrole is 6.6×10^4 (Granick 1966).

ALA synthetase activity in mμ moles ALA formed per gram of liver (wet weight) per hour was calculated by subtracting the result given by the following equation for the control sample from that given for the incubated sample:

$$\text{mμ moles ALA/g liver} = \frac{47719\ (Z - 0.045\ W)}{\text{Weight of liver (mg)}}$$

This value was divided by the phosphorus content expressed as mg phosphorus/g liver to give ALA synthetase activity as mμ moles ALA formed per mg total liver phosphorus per hour.

Table A.1 Recovery of ALA pyrrole on Dowex 1 acetate.

Amount ALA added (mμ moles)	Percentage recovery	
25	74.9	Mean 77.0
50	76.5	
100	79.6	
200	76.8	

A.1.1.3 Comparison of methods used for liver ALA synthetase assay.

Marver, Tschudy, Perlroth, Collins and Hunter (1966) reported excellent separation of ALA pyrrole and aminoacetone pyrrole by means of chromatography on Dowex 1 acetate columns. They found the pyrroles in the respective eluates to be chromatographically pure; recovery of the pure synthetic pyrroles in aqueous solution from Dowex 1 acetate columns ranged from 89-99% and reproducibility was excellent.

In the present study recovery of ALA pyrrole made from pure synthetic ALA (supplied by Sigma Chemical Co., U.S.A.) was very consistent. There was only 5% variation with amounts of ALA ranging from 25-200 mμ moles (Table A.1). It will be noted however that the mean recovery of 77.0% was somewhat lower than that reported by Marver and co-workers (Marver, Tschudy, Perlroth, Collins and Hunter, 1966).

This discrepancy is not readily explicable but could possibly be related to the fact that in the present study it was found butanol-ammonium hydroxide invariably caused slight shrinkage of the resin leading to the development of a few small cracks in the column. This difficulty is not mentioned by Marver and his colleagues. On the other hand, these authors mention that during the condensation of pure ALA with acetylacetone a small amount (5-10%) of a compound behaving like the aminoacetone pyrrole is formed. This compound is quantitatively eluted by butanol-ammonium hydroxide. A second possibility, therefore, is that in the present study such a process may have led to greater losses of ALA than found by Marver's group (Marver, Tschudy, Perlroth, Collins and Hunter 1966).

The main reason for not using the chromatographic method in all the studies on rat liver ALA synthetase activity was that the solvent extraction procedure described by Granick (1966) proved to be far more convenient and useful in that as many as 10 individual assays could be carried out simultaneously. With the chromatographic

Table A.2 Comparison of micro-method and the chromatographic macro-method (i) for determination of liver ALA synthetase activity. Figures for the macro-method are corrected for a mean percentage recovery of 77.0% (Table A.1).

MATERIAL	ALA synthetase activity mμ moles ALA/g liver/hour	
	Micro-method	Macro-method (i)
Normal rat liver(fed)	8.8	7.9
Normal rat liver(fed)	6.0	6.1
* Porhyric rat liver	176.9	178.6
* Porhyric rat liver	283.4	306.6
Normal human liver	0.7	2.4
Normal human liver	1.5	5.5

* These rats were made porphyric by intraperitoneal administration of AIA (400 mg/kg) after a 24 hour fast. The animals were sacrificed 17 hours after administration of the drug.

technique this was quite impracticable, hence the pooling of samples for each group of animals.

To confirm the validity of the micro-method as a measure of hepatic ALA synthetase activity the results obtained with this technique were compared (Zail and Joubert 1968) with those obtained on the same specimens using the larger scale chromatographic procedure, macro-method (i). Table A.2 shows the figures obtained for normal and porphyric rat liver and normal human liver samples procured at laparotomy. It is obvious that the two methods yield comparable results. However, the micro-method was found to give higher values for intrinsic ALA content of unincubated samples of liver homogenate, suggesting that non-specific Ehrlich aldehyde-reacting chromogens are not removed by this technique but are separated from the aminoketones in the chromatographic method.

A.1.2 Coproporphyrinogen oxidase activity of rat liver mitochondria (Sano and Granick 1961). As indicated in Section 3.6 coproporphyrinogen oxidase (coproporphyrinogenase) is located intramitochondrially in mammalian liver. Sano and Granick (1961) have described an assay of this enzyme employing mitochondria solubilised with thioglycollate. On incubation with this mitochondrial preparation coproporphyrinogen yields protoporphyrinogen. The operation is carried out in the dark to prevent excessive autoxidation of the porphyrinogens which are highly unstable compounds. The presence of thioglycollate and diethylthiocarbamate in the incubation mixture is also designed to minimise unwanted autoxidation. At the end of the incubation the porphyrinogens are autoxidised under controlled conditions. Protoporhyrin is then extracted as described below and determined spectrophotometrically.

Preparation of mitochondria: Mitochondria were prepared from rat liver by differential centrifugation in 0.25 M sucrose as described for the assay of pyruvate carboxylase activity (Section A.1.3). The mitochrondrial pellet was stored at -20°C until required. In this form coproporphyrinogenase activity is stable for more than one year (Sano and Granick 1961).

At the time of assay the frozen mitochondria were suspended in cold 0.1 M tris buffer, pH 7.2, containing 0.05 M potassium thioglycollate and 0.001 M sodium diethyldithiocarbamate. Approximately 1/3 of the pellet obtained from a whole rat liver was suspended in 10 ml of this buffer.

The protein content of the mitochondrial suspension was determined using a biuret reaction (Reinhold 1953).

Preparation of coproporphyrinogen: Coproporphyrin III was prepared from its tetramethyl ester (supplied by Sigma Chemical Co., U.S.A.) by hydrolysis with 6 M HCl for 24 hours at room temperature in the

dark. Thereafter, the acid was evaporated in vacuo in the dark and the free porphyrin dissolved in 0.01 M KOH to make a stock solution of 5×10^{-4} M and stored at -20°C.

Coproporphyrinogen was freshly prepared before use by reduction of an aliquot of this stock solution using freshly ground 3% sodium amalgam (1 g/ml of solution) under nitrogen and in dim red light. Reduction was judged to be complete on disappearance of fluorescence in ultraviolet light. The solution was then filtered through a Millipore filter and titrated to pH 7.2 with 40% H_3PO_4 solution.

Assay procedure: 1.8 ml of the enzyme preparation in a 15 ml test tube was incubated for 2 hours with 0.2 ml of coproporphyrinogen solution aerobically at 38°C on a shaker and in the dark. At the end of this time the reaction was stopped by the addition of 10 ml of ethyl acetate-glacial acetic acid mixture (3:1) and the porphyrinogens were autoxidised to porphyrins by illumination with 50 foot-candles of light from a daylight fluorescent tube for 30 minutes at room temperature. Precipitated proteins were then removed by centrifugation. The supernatant solution containing the porphyrins was added to 30 ml of ether in a separating funnel while the protein precipitate was washed with a further 10 ml of ethyl acetate-acetic acid. After centrifugation, this second extract was also added to the separating funnel.

HCl was then used to remove the porphyrins from the ethyl acetate-acetic acid-ether solution. Extractions (usually 3 or 4) with 5 ml volumes of 2.5 N HCl were continued until there was no fluorescence in the upper layer. Prompt adjustment of the pH of the aqueous acid solution to 3.5 with saturated sodium acetate solution was carried out to avoid reaction of the vinyl groups of protoporphyrin with thioglycollate. The porphyrins were then extracted into 15 ml

of ether. This solution was washed 3 times with an equal volume of water. Three extractions each with 6 ml of 0.15 N HCl were performed to remove coproporphyrin and another porphyrin with 3 ionisable carboxyl groups. Protoporphyrin was finally extracted with 3 volumes of 3 ml 1.5 N HCl. This solution was then diluted to 10 ml with ethanol 96% and the optical density at 408 mμ was determined in cells of 1 cm optical length using a Zeiss PMQII spectrophotometer. Protoporphyrin concentration was calculated using an E_m 408 of 2.4×10^5. Coproporphyrinogen oxidase activity was expressed at mμ moles of protoporphyrin formed per mg mitochondrial protein.

Comment: Sano and Granick (1961) have used this method in determining the coproporphyrinogenase activity of a wide variety of tissues in different animals. In guinea pig liver mitochondria they found a specific activity (mμ moles protoporphyrin formed per mg protein) of 8.0 whereas in packed beef liver mitochondria purified 2.2 times the specific activity was 4.9. It would appear, therefore, that there is a species difference in the activity of this enzyme in liver. These authors also partially purified the enzyme from beef liver and were able to demonstrate an increase in specific activity up to 45 with increasing enzyme purity. No figures for rat liver are available, however.

In the present study, this assay was found to give highly consistent results. In the 18 individual assays carried out on rat liver in Chapter 8 the variation in specific activity was from 2.01 to 3.38. These results are comparable with those of Sano and Granick (1961) especially in view of the species difference mentioned above.

A.1.3 Pyruvate carboxylase activity of rat liver mitochondria (Utter and Keech 1963). As indicated in Chapter 9 pyruvate carboxylase catalyses the carboxylation of pyruvate to oxaloacetate in the presence of acetyl CoA, ATP and Mg^{++}. Utter and Keech (1963) have described a method for assaying this enzyme using $NaH^{14}CO_3$ whereby the activity could be determined as mμ moles $NaH^{14}CO_3$ fixed in unit time per mg mitochondrial protein.

Preparation of mitochondria: Rats were killed by decapitation and their livers immediately removed and chilled in ice-cold 0.25 M sucrose. In each case a 1 in 10 homogenate of liver in ice-cold 0.25 M sucrose was made using a Potter-Elvehjem homogeniser; mitochondria were isolated from this homogenate by differential centrifugation in an MSE High Speed 18 refrigerated centrifuge at 0-3°C. The nuclear sediment after centrifugation at 850 *g* for 10 minutes was discarded and the supernatant subjected to 7,000 *g* for 10 minutes. The resultant mitochondrial pellet was washed twice by resuspension in 0.25 M sucrose followed by centrifugation at 7,000 *g* for 10 minutes.

For determination of pyruvate carboxylase activity either fresh mitochondria or mitochondrial acetone powders were used. In the case of fresh mitochondria a suspension of the whole mitochondrial pellet obtained above was prepared in 1.6 ml of ice-cold 0.4 M tris buffer (pH 7.4) and used immediately in the assay. Mitochondrial acetone powders, on the other hand, were stored under vacuum at -20°C until required, when 20 mg of powder were suspended in 4 ml. of ice-cold tris buffer (0.4 M, pH 7.4).

The protein content of the mitochondrial suspensions used was determined by means of a biuret method (Reinhold 1953).

Assay procedure: The method employed was that of Utter and Keech (1963) with several minor modifications. Table A.3 shows the quantities of ingredients contained in 0.5 ml of incubation mixture.

Table A.3 Composition of incubation mixture in each tube for assay of pyruvate carboxylase activity.

Ingredients	Amount (μmoles)	Volume of solution (ml)
Mitochondrial suspension in 0.4 M tris buffer	80 (tris)	0.2
$NaH^{14}CO_3$ (14,650 c.p.m./μmole)	15	0.1
ATP (Sigma Chem.Co., U.S.A.)	1.25	0.05
Acetyl CoA (Sigma Chem.Co., U.S.A.)	0.35	0.1
Pyruvate	5.0	0.025
$MgSO_4$	4.0	0.025

Each tube contained slightly more tris (80 instead of 50 μmoles) than the mixture described by Utter and Keech (1963) and $MgSO_4$ was used instead of $MgCl_2$. These modifications did not affect the efficiency of the assay in any way. Control tubes from which pyruvate was omitted were used to permit correction for acetyl CoA carboxylase activity.

All tubes were pre-incubated for two minutes at 30°C and the reaction started by the addition of the mitochondrial suspension. After 5 minutes incubation at 30°C the reaction was stopped by freezing in a dry ice-acetone bath and the tubes were kept at -20°C until counting. The procedures employed were either proportional gas-phase or liquid scintillation counting (Section A.6). Before counting, excess unfixed $^{14}CO_2$ was removed by addition of acid and gassing of the solution with $^{14}CO_2$ for 15 minutes.

Pyruvate carboxylase activity was expressed as μmoles $NaH^{14}CO_3$ fixed in 5 minutes at 30°C per mg mitochondrial protein.

Comment: Utter and Keech (1963) showed that this assay is specific for pyruvate carboxylase and is not related to any of the CO_2-fixing reactions previously described. No activity could be demonstrated in the absence of any of the following reactants: pyruvate, ATP, acetyl CoA, and $NaHCO_3$. These authors (Utter and Keech 1963, Keech and Utter 1963) obtained comparable results using either this assay or a spectrophotometric method in the determination of pyruvate carboxylase activity in chicken liver fractions and in various rabbit tissues.

Freedman and Kohn (1964) used this isotope method to assay pyruvate carboxylase in various fractions of rat and mouse liver. They showed that fasting causes a marked increase in the activity of this enzyme. Mitochondria from starved animals exhibited up to 3 times the activity of those isolated from the livers of fed animals. This finding provides a ready means of testing the efficacy of the assay.

In the present study (Chapter 9) the results obtained with this method were consistently of the same order as those of the previously mentioned authors. In animals fasted for 40 hours the increase in enzyme activity was 2.7 times the relevant value for fed animals.

Table A.4 Composition of reaction mixture for the assay of malic enzyme activity.

Ingredients	Amount (μmoles)	Vol. in reaction mixture (ml)
Tris buffer (pH 7.4)	75	1.0
NADP (Sigma Chem.Co., U.S.A.)	135	0.2
Mn Cl_2	3	0.2
L-malate (pH 7.4)	1.5	0.1
Water	-	1.3
Enzyme solution	-	0.2

A.1.4 Assay of malic enzyme in rat liver (Ochoa 1955). At physiological pH malic enzyme which is NADP-dependent catalyses the oxidative decarboxylation of malate to pyruvate. The reaction may be followed by observing the change in optical density of the solution at 340 mμ (the absorption maximum for the reduced pyridine nucleotides) per unit time.

Preparation of enzyme solution: Rats were killed by decapitation and their livers removed and chilled in ice-cold 0.25 M sucrose. In each case a 1 in 10 homogenate of liver in ice-cold 0.25 M sucrose was made using a Potter-Elvehjem homogeniser. The nuclear and mitochondrial fractions were removed by centrifugation of homogenates at 15,000 *g* for 5 minutes in an MSE High Speed 18 refrigerated centrifuge at 0-3°C. The supernatant solution was centrifuged at 105,000 *g* for 30 minutes in a Beckman Model L 4 preparative ultracentrifuge at 0-3°C. This final supernatant preparation was used as the enzyme solution in the subsequent assay according to Ochoa (1955). The protein content of this solution was determined by means of a biuret method (Reinhold 1953).

Assay procedure: All the ingredients of the reaction mixture as listed in Table A.4 except the enzyme solution were added to a cell of 1 cm optical length. The reaction was then started by the addition of the enzyme solution and optical density measurements at 340 mμ were made in a Beckman DB spectrophotometer at 15 second intervals for one minute at room temperature. The change in optical density between 15 and 45 seconds after addition of the enzyme was calculated and from this was subtracted the change which occurred in the absence of added substrate (control). The value thus obtained was used together with the E_m 340 for $NADPH_2$ of 6.22×10^3 to calculate the enzyme activity which was expressed as mμ moles of malate decarboxylated per minute per mg protein.

Comment: This method is well established and in the present study was found to give satisfactory, reproducible results.

Table A.5 Composition of incubation mixture in each tube for assay of fumarate reductase activity.

Ingredient	Amount (μmoles)	Volume (ml)
ATP (Sigma Chem. Co., U.S.A.)	2	0.3
* Coenzyme A (" " ")	26 mμmoles	
Glutathione (" " ")	4	
$MgCl_2$	2	
Hydroxylamine (neutralised to pH 7)	192	
Tris (pH 7.4)	20	
$NADH_2$ (Sigma Chem.Co., U.S.A.)	4	
Mitochondrial suspension	-	0.1

* In Table 3 of Kurumada and Labbe (1966) the amount of acetyl CoA in 2 ml of incubation mixture is stated to be 13 mμmoles. This is an obvious typographical error. The quantity should be 130 mμmoles - the amount employed by Kaufman (1955) in the method upon which the measurement of fumarate reductase activity is based.

A.1.5 Fumarate Reductase Activity of Rat Liver Mitochondria. (Kurumada and Labbe 1966). Kurumada and Labbe (1966) have described an assay of fumarate reductase activity in liver mitochondria in which the $NADH_2$-linked reduction of fumarate is observed by conversion of the succinate so formed to succinyl CoA. The succinyl CoA generated reacts to form succinyl hydroxamate in the presence of hydroxylamine. The amount of hydroxamate present is then determined by spectrophotometric measurement of the colour produced upon addition of $FeCl_3$. (Kaufman 1955).

Preparation of mitochondria: Fresh mitochondria were prepared from rat or mouse liver by differential centrifugation in 0.25 M sucrose as described for the assay of pyruvate carboxylase activity (Section A.1.3); the mitochondrial pellet was resuspended in 0.25 M sucrose so that 1 ml of suspension would contain the mitochondria from about 1 g of liver. Mitochondrial acetone powders were also prepared in which case the powder obtained from 2 g of liver was resuspended in 1 ml of 0.25 M sucrose. The protein content of these suspensions was determined using a biuret method (Reinhold 1953).

Assay procedure: Table A.5 shows the amounts of ingredients in the incubation mixture contained in each tube. In order to conserve expensive ingredients the assay was conducted on a micro-scale. All quantities and volumes were 1/5 of those used by Kurumada and Labbe (1966).

The incubation was started by the addition of the mitochondrial suspension and was continued for 30 minutes at 37°C. The reaction was stopped by the addition of 0.6 ml of a solution containing equal volumes of 5% $FeCl_3$ (freshly made), 3 N HCl and 12% trichloracetic acid. Centrifugation was carried out immediately in a Beckman/Spinco Microfuge 152 for 1 minute and the optical density of the supernatant was read in a Beckman DB spectrophotometer at 540 mμ exactly 3 minutes after stopping the enzyme reaction. For each assay

a control sample was treated immediately with $FeCl_3$-HCl-trichloracetic acid mixture, centrifuged and the optical density of the supernatant determined.

A standard solution of malic hydroxamate was prepared from malic anhydride and neutralised hydroxylamine so that 0.4 ml of solution contained 0.8 µmoles of malic hydroxamate.

Fumarate reductase activity was expressed as mµmoles of hydroxamate formed per hour per mg mitochondrial protein.

Comment: Kurumada and Labbe (1966) used this method to demonstrate $NADH_2$-dependent fumarate reductase activity in liver mitochondria from both normal and AIA-treated mice. In porphyric animals they obtained values 2 to 3 times those found in normal mice. In the present study (Chapter 9), however, despite careful attention to detail and the use of different batches of ingredients in different experiments, it proved impossible to corroborate the findings of these workers. Although the standard preparations gave consistent readings, mitochondrial fumarate reductase activity could not be demonstrated with any consistency either in fresh mitochondria or mitochondrial acetone powders in normal or porphyric rats and mice. It must be concluded that, if fumarate reductase does exist in mammalian liver, this is not a reliable method for demonstrating its activity.

A.2 Total Liver Phosphorus (Saliman 1964). This method depends on the conversion of organic phosphorus to inorganic phosphate which is then determined by measuring the amount of colour produced after addition of ammonium molybdate and hydrazine.

Procedure: 0.01 ml of 1 in 10 liver homogenate plus 0.1 ml of 0.1 N H_2SO_4 was digested in a covered crucible with 2 ml of a mixture consisting of distilled HI (5% v/v), $Ca(OH)_2$ (0.06%), phenol (50%) and water (5% v/v) in glacial acetic acid for one hour on a steam bath. Thereafter iodine vapour was driven off by heating on a hot plate and when dry the crucible was heated in an oven at 700°C for 10 minutes. After cooling, 4 ml of a 0.25% ammonium molybdate solution and 1 ml of 0.06% hydrazine solution were added and the resultant colour read in a Zeiss PMQ11 or Beckman DB spectrophotometer at 830 mμ. A standard solution of 0.001 M KH_2PO_4 in 0.1 N H_2SO_4 was prepared. 0.1 ml of this solution (containing 3.1 μg P) plus 0.01 ml of the buffer solution used for making the liver homogenate was treated in the same way as the liver sample. Finally a blank solution consisting of 0.01 ml of buffer and 0.1 ml of 0.1 N H_2SO_4 was also treated similarly.

Total liver phosphorus was expressed as mg per g wet weight.

Comment: This method was found to be particularly suitable for small samples both from the point of view of sensitivity and reproducibility. Using 0.01 ml of 1 in 10 liver homogenate the optical density of the final solution was of the order of 0.5.

Table A.6 Composition of system for assay of acetyl CoA content of rat liver extract.

Ingredient	Amount	Volume (ml)
Tris-HCl (pH 7.0)	150 µmoles	)
Potassium malate	10 µmoles	) 0.15
NAD (Sigma Chem. Co.)	2 µmoles	)
Malate dehydrogenase (Sigma Chem. Co. Pig heart type 1)	2.4 µM units	0.1
Citrate synthetase (Sigma Chem. Co.)	25 µg	0.025
Liver extract pH 6.8	-	2.0

A.3 Determination of Acetyl CoA content of rat liver.

(Wieland and Weiss 1963). This method makes use of the assay system described by Ochoa et al. (1951) for the measurement of citrate synthetase activity and depends on the following reactions:

$$\text{Malate} + NAD^+ \xleftrightarrow[\text{dehydrogenase}]{\text{malate}} \text{oxaloacetate} + NADH + H^+ \quad (1)$$

$$\text{Acetyl CoA} + \text{oxaloacetate} + H_2O \xrightarrow[\text{synthetase}]{\text{citrate}} \text{citrate} + CoA \quad (2)$$

Hence

$$\text{Acetyl CoA} + \text{malate} + NAD^+ + H_2O \longrightarrow \text{citrate} + CoA + NADH + H^+ \quad (3)$$

Reaction (3) shows that 1 molecule of acetyl CoA gives rise to 1 molecule of NADH. By observing the change in optical density of the system at 340 mμ, the absorption maximum for NADH, a measure of the acetyl CoA content can be obtained.

Preparation of liver extract: Rats were killed by decapitation and the whole livers rapidly removed and dropped instantly into liquid nitrogen to halt all further metabolic activity. Each frozen liver was then quickly weighed and homogenised in 25 ml of 6% $HClO_4$ (ice-cold). The precipitated protein was removed by centrifugation at 0-3°C in an MSE High Speed 18 refrigerated centrifuge. The pH of the clear supernatant was adjusted to 6.8 with 2 N KOH solution. The mixture was allowed to stand in an ice bath for 15 - 30 minutes, the final volume was noted and then centrifugation was repeated to remove the precipitate of $KClO_4$. The acetyl CoA concentration of this supernatant was then determined as described below.

Determination of acetyl CoA: Table A.6 shows the composition of the assay system. All the ingredients except the 2 enzymes were added to a cell of 1 cm optical length in a Zeiss PMQ11 spectrophotometer. After the addition of malate dehydrogenase an increase in optical density at 340 mμ, indicating the formation of NADH, was observed until the reaction reached equilibrium. At this stage citrate synthetase was added and the further increase in optical density at 340 mμ was recorded. This latter increase represented further production of NADH

consequent upon conversion of acetyl CoA to citrate. Since the acetyl CoA present in the assay gave rise to an equimolar amount of NADH, the acetyl CoA content of the liver was calculated using the E_m 340 for NADH of 6.2×10^3 and was expressed as mμmoles acetyl CoA per g of liver.

Comment: Wieland and Weiss (1963) confirmed the linear relationship between NADH formation and acetyl CoA content when known amounts of the latter were added to the assay system. These workers also reported 95% recovery of acetyl CoA added to the acid liver homogenate. Using this assay they showed that in alloxan-diabetic rats, made ketotic by withdrawal of insulin and administration of cortisol, and in normal rats made ketotic by fat feeding there was a consistent 2.5 to 3-fold increase in the liver content of acetyl CoA. This is in keeping with the finding that pyruvate carboxylase activity is also increased in ketosis (Freedman and Kohn 1964).

In the present study this assay was found to yield very consistent results (Chapter 9). The values obtained for fed Wistar strain rats (mean 38.6 and 39.6 mμmoles acetyl CoA / g liver for normal and porphyric animals respectively) were, however, somewhat higher than those found by Wieland and Weiss (1963) in normal Sprague-Dawley rats (mean 19.9 mμmoles acetyl CoA / g liver).

A.4 Isolation of haemin from rat liver (Labbe and Nishida 1957). Liver haem is isolated as haemin using a mixture of glacial acetic acid (containing 2% strontium chloride) and acetone. In view of the small amount of haem in the liver a fixed amount of carrier in the form of washed erythrocytes is homogenised with the liver sample before isolation to facilitate the subsequent crystallisation of haemin.

Extraction and isolation procedure: Rats were killed by decapitation and their livers removed and immediately chilled in ice-cold saline. 1 g of liver was then homogenised with 4 ml of ice-cold saline and 5 ml of ice-cold washed red cells suspended in saline in a Potter-Elvehjem homogeniser surrounded by a jacket containing crushed ice. Within any one experiment the same amount of carrier was added to both the normal and porphyric homogenates; but from one experiment to another the quantity varied due to the use of different red cell suspensions containing between 10 and 15 g haemoglobin per 100 ml.

72 ml of a freshly prepared mixture of glacial acetic acid containing 2% strontium chloride (1 part) and acetone (3 parts) were then added to the homogenate in a 100 ml conical flask and extraction was allowed to proceed for 30 minutes. During this time the flask was brought momentarily to boiling point to aid extraction.

After cooling, the extract was filtered through acid-resistant filter paper and the filtrate was evaporated on a boiling water bath until all the acetone had been driven off. During this time the temperature was not allowed to exceed 90°C. Thereafter the hot concentrated haemin solution was poured into centrifuge tubes and allowed to stand overnight. The crystals of haemin were harvested by centrifugation and were subsequently washed twice with a mixture of glacial acetic acid and water (1:1), twice with a small quantity of ethanol 96% (v/v) and once with ether. After allowing the ether to evaporate the crystals were dried over fused $CaCl_2$ in a vacuum desiccator.

Comment: This is a well known method for isolation of haemin and in the present study was consistently found to produce a good yield. It will be noted that recrystallisation was not routinely carried out. Using the above-mentioned quantities, this procedure proved to be impracticable due to the poor recovery of crystals with each recrystallisation. With larger amounts of liver and carrier haem a sufficient quantity of crystals could be obtained after recrystallisation for counting purposes but recrystallisation was found to make no material difference to the specific activity of the isolated haemin in the present study and was therefore omitted.

A.5 Isolation and purification of Aspartic and Glutamic acids from rat liver. (Freedman and Graff 1958). These two dicarboxylic amino acids may readily be isolated from a 6 N HCl hydrolysate of homogenised whole liver. Humin is precipitated by phosphotungstic acid and the phosphotungstate-free supernatant is applied to Dowex 1-X10 resin which retains the dicarboxylic acids. After washing the resin free of other amino acids, glutamic and aspartic acids are differentially eluted with 0.5 M acetic acid. Further purification may be achieved by crystallisation of the hydrochlorides from the concentrated eluate by saturating the solution with HCl gas.

Procedure: Rats were killed by decapitation and their livers removed and homogenised in 1 N HCl. The HCl concentration of the homogenate was then raised to 6 N and the total volume adjusted to 20 times the original volume of tissue. Hydrolysis was carried out by refluxing for 18 hours and after cooling and dilution of the hydrolysate with 5 volumes of distilled water, humin was precipitated by addition of a slight excess of 30% phosphotungstic acid. The mixture was then heated briefly to approximately 90°C to effect partial solution of the phosphotungstates which were then allowed to crystallise overnight.

The supernatant was separated by filtration and concentrated to approximately 30 ml in vacuo. A further precipitate of phosphotungstates was removed again by filtration and the filtrate was washed 5 times with 20 ml portions of n-amyl alcohol to remove excess phosphotungstate and once with 20 ml of ether to remove amyl alcohol. Thereafter the aqueous solution was evaporated in vacuo to a brownish glass and placed in a vacuum desiccator over NaOH for several days to remove all traces of HCl.

The residue was dissolved in 100 ml of water, stirred with a small amount of charcoal for half an hour and filtered. The clear colourless filtrate was then placed on a 25 x 2 cm column of

Dowex 1-X10 resin (Bio-Rad Labs., California) in the acetate cycle. The column was washed with water until the washings were ninhydrin negative. Glutamic acid and aspartic acid were then eluted separately with 0.5 M acetic acid. The fractions containing these two amino acids were evaporated to dryness under reduced pressure and dried in a vacuum desiccator over fused $CaCl_2$. Carrier was not added.

Samples of dried aspartic and glutamic acids were then removed for

(a) the determination of percentage purity by means of the ninhydrin reaction (Van Slyke et al. 1941);

and (b) the determination of specific activity by means of liquid scintillation counting (Section A.6).

Comment: In the present study the percentage purity of the isolated amino acids as determined by the ninhydrin release of CO_2 was as follows:

glutamic acid 92.7%

aspartic acid 97.1%

While greater purity could have been achieved by the addition of carrier and crystallisation of the hydrochlorides with HCl gas, this procedure was omitted, since the specific activity of the final product would have been rendered too low for efficient counting. Moreover the percentage purity achieved for the free amino acids as shown above was considered entirely adequate for the purposes of the present study (Chapter 10).

A.6 Determination of Radioactivity.

A.6.1 Proportional Gas-Phase Counting. This method was used for the determination of radioactivity of haemin labelled with ^{3}H or ^{14}C, the tritiated compounds used in Chapter 10, and the reaction mixtures containing fixed $^{14}CO_2$ from pyruvate carboxylase assays.

A.6.1.1 Combustion of Samples.

a. ^{3}H-labelled haemin. The dried haemin crystals were weighed in zinc boats and placed, together with a mixture of granulated zinc, nickel oxide (Baker's Analysed grey-black powder) and water, as described by Wilzbach et al. (1953), in combustion tubes of special (Supramax) glass able to withstand 670°C without becoming deformed and impermeable to methane and hydrogen at this temperature. These tubes which were provided with break-tips were then sealed under vacuum, shaken to mix the contents thoroughly, and placed horizontally in a furnace at 670°C for 3 hours.

Under these conditions tritium in organic compounds is quantitatively converted to a mixture of methane and hydrogen (Wilzbach et al. 1953).

b. Standardisation of ^{3}H-labelled precursors. Aqueous solutions of the tritium-labelled precursors (Chapter 10) were accurately diluted to a concentration of approximately 1 mμc/mg and in each case 3-5 mg of the diluted preparation was weighed in a quartz weighing bottle and sealed. The bottle was then placed in a combustion tube charged with granulated zinc and nickel oxide as described previously. In this case no water was added. After sealing of the combustion tube under vacuum, the enclosed weighing bottle was broken by vigorous shaking and the contents thoroughly mixed before combustion at 670°C as described above.

c. ^{14}C-labelled haemin. Samples of dried haemin were accurately weighed and placed in the combustion attachment to a Van Slyke-Neill manometric apparatus (Van Slyke and Neill 1924). Combustion was then carried out according to Van Slyke et al. (1951) using a mixture of fuming sulphuric and phosphoric acids together with potassium iodate and potassium dichromate. The evolved carbon dioxide was measured manometrically before quantitative transfer to the counting tube.

d. Reaction mixtures - pyruvate carboxylase assay. After removal of excess unfixed $^{14}CO_2$ as described in Section A.1.3 known volumes of reaction mixture following incubation were combusted in a Van Slyke-Neill manometric apparatus (Van Slyke and Neill 1924) as described in Section A.6.1.1 (c). The evolved CO_2 was measured manometrically before quantitative transfer to the counting tube.

A.6.1.2 Transfer of gases.

a. Tritium. Following combustion the reaction tubes were cooled to room temperature before transfer of the gases to counting tubes by means of the apparatus shown in Fig. A.1. This consisted of a closed system in which a high vacuum was produced by means of a rotary oil pump and a mercury diffusion pump connected in series in order to eliminate tritium "memory" in the counting tubes.

Once a suitable vacuum was achieved (10^{-5} mm Hg) the break tube (1) was isolated and the break-tip of the combustion tube broken. The released gas was then transferred by a Toepler pump (2) to the evacuated Bernstein-Ballentine tube (3) (Bernstein and Ballentine 1950). Under these conditions only 1.5% of the tritium is retained

Fig. A.1 Apparatus used for the quantitative transfer of tritium from combustion tubes to Bernstein-Ballentine counters.

1 = break-tube

2 = Toepler pump

3 = Bernstein-Ballentine counter

2
3

within the combustion tube (Wilzbach et al. 1953). Before counting the Bernstein-Ballentine counter was filled with "P-10" quenching gas (10% methane in argon) at atmospheric pressure.

b. Carbon Dioxide ($^{14}CO_2$). $^{14}CO_2$ liberated in a Van Slyke-Neill manometric apparatus as described in Section A.6.1.1 was transferred quantitatively to a Bernstein-Ballentine counter and then "P-10" gas was added at atmospheric pressure.

A.6.1.3 Counting procedure. The electronic instrumentation used was as described by Sinex, Van Slyke et al. (1955) and consisted of a high voltage supply, amplifier, discriminator and scaling circuit. The Bernstein-Ballentine counter was placed within a two-inch thick lead shield, connected to the high voltage power supply and amplifier, and counted at plateau voltage (Sinex, Van Slyke et al. 1955). The plateau was established on each occasion for each counter and the background count was checked to ensure elimination of ^{3}H "memory".

The efficiency of this method of counting is of the order of 98% (Sinex, Van Slyke et al. 1955). Due to the design of the Bernstein-Ballentine counter, however, each tube contains some dead space - on the average about 15%. Thus the overall counting efficiency is of the order of 83%. The dead space of each counting tube is accurately known and allowed for in calculations.

A.6.2 Liquid Scintillation Counting. This method was used for determination of radioactivity of samples containing:

a. $HO^{3}H$.
b. Tritiated aspartic and glutamic acids
c. Aetiocholanolone-1,2-^{3}H
d. $^{14}CO_2$ fixed in pyruvate carboxylase assay reaction mixtures.

A.6.2.1 Scintillants and solvents.

Primary scintillant - "PPO" ... 2,5-diphenyloxazole

Secondary scintillant - "POPOP" ... p-bis-[2-(5-phenyl-oxazolyl)]-benzene

Solvents - Toluene

- Toluene plus Triton-X 100

A.6.2.2 Solutions for counting.

Aetiocholanolone-1,2-^{3}H was dissolved in toluene containing 0.4% PPO and 0.01% POPOP. In the case of aqueous solutions, as for tritiated water, solutions of tritiated aspartic and glutamic acids, urine collected after administration of aetiocholanolone-1,2-^{3}H and pyruvate carboxylase assay reaction mixtures containing fixed $^{14}CO_2$, this scintillant solution was mixed with the detergent preparation, "Triton-X 100" (Patterson and Greene 1965) in the proportions : 2 parts toluene to 1 part Triton-X 100.

A.6.2.3 Counting procedure.

The radioactive solutions were placed in 20 ml glass counting vials (Beckman Instruments Inc.) and counted in a Nuclear-Chicago Mark 1 Liquid Scintillation Computer Model 6860, using the channels ratio method.

A.7 Enzymatic determination of galactose in blood (Hjelm 1967).

Under the influence of galactose oxidase, D-galactose is oxidised to D-galacto-hexodialdose with the formation of hydrogen peroxide :

$$\text{D-galactose} + O_2 \xrightarrow[\text{oxidase}]{\text{galactose}} \text{D-galacto-hexodialdose} + H_2O_2$$

Thereafter in the presence of the enzyme peroxidase, hydrogen peroxide reacts with the colourless reduced form of the dye, o-dianisidine, to yield the coloured oxidised form :

$$\underset{\text{(reduced)}}{\text{o-dianisidine}} + H_2O_2 \xrightarrow{\text{peroxidase}} \underset{\text{(oxidised)}}{\text{o-dianisidine}} + H_2O$$

The amount of colour formed is proportional to the galactose content of the sample and is measured spectrophotometrically.

Assay procedure : The galactose oxidase reagent (Galax^R) and galactose standard solutions used were supplied by Kabi Pharmaceuticals, Stockholm. The assay procedure employed was one recommended by the manufacturer and was essentially identical with the method of Hjelm (1967).

Venous blood was collected in tubes containing EDTA as anticoagulant and 0.1 ml of each sample was immediately transferred to a tube containing 2 ml of 0.025 N NaOH. After allowing 1 minute for complete haemolysis to occur, 0.1 ml of a solution of $ZnSO_4$ (of such a concentration as to exactly neutralise 2 ml of 0.025 N NaOH) was added. The contents of the tubes were thoroughly mixed and centrifugation was carried out to obtain a clear supernatant. 0.2 ml of this supernatant was then mixed with 0.5 ml of galactose oxidase reagent (Galax^R). The latter was prepared by adding 21 ml of distilled water to a sealed bottle containing freeze-dried galactose oxidase, peroxidase, o-dianisidine and phosphate buffer. The use of smaller volumes of supernatant and enzyme solution than were recommended, while not affecting the accuracy of the assay, made it possible to conserve the costly galactose oxidase reagent.

One hour later the fully developed colour was measured in a micro-cell of 1 cm optical length at 450 mμ in a Beckman DB spectrophotometer.

Standard solutions of galactose containing 25, 50 and 100 mg / 100 ml were prepared from a solution containing 200 mg / 100 ml. 0.1 ml volumes of these solutions and 0.1 ml of water as a blank were treated in the same manner as the blood samples. The optical densities of the standard solutions were used to construct a standard curve from which the galactose content of the blood samples in mg / 100 ml was read.

Comment : Hjelm (1967) has made a thorough study of the enzymatic determination of galactose in whole blood, plasma and erythrocytes and has concluded that "blood galactose can be determined by a very simple procedure and with a high degree of sensitivity and reliability by using galactose oxidase and an indication system employing peroxidase and o-dianisidine".

It must be stressed, however, that specially purified reagents such as those supplied by Kabi are required. Commercially available galactose is often contaminated with varying amounts of other carbohydrates and not all preparations of galactose oxidase are satisfactory (Lundén et al. 1966).

The accuracy of the method was demonstrated by Hjelm (1967) in the following way. Erythrocytes were suspended in saline containing various concentrations of galactose and then in each case the quotient

$$\frac{\text{galactose in erythrocytes}}{\text{galactose in saline}}$$

was determined using both the enzymatic method and ^{14}C-galactose. The two methods yielded the same result for galactose concentrations up to 100 mg / 100 ml.

Various substances such as uric acid and ascorbic acid by interfering with the oxidation of the chromogen by H_2O_2 may give falsely high or low values for blood galactose determined by the enzymatic method. However, at physiological blood levels the effect of these substances is negligible (Hjelm 1967).

In the present study this method proved to be entirely satisfactory.

A.8 Determination of free aetiocholanolone-1,2^{3}H in plasma

(Slaunwhite and Sandberg 1958, Sandberg and Slaunwhite 1956). Free, unconjugated aetiocholanolone is soluble in chloroform whereas the conjugated form is not, requiring prior hydrolysis before extraction (Slaunwhite and Sandberg 1958).

Procedure : Blood samples of 30 - 40 ml were collected in flasks containing heparin and were immediately centrifuged to separate plasma and cells. Fifteen ml of plasma in each case was then extracted three times with equal volumes of freshly redistilled chloroform (Sandberg and Slaunwhite 1956). Centrifugation was necessary to obtain clear separation of the phases.

Water was removed from the pooled chloroform extracts by addition of anhydrous sodium sulphate. This was followed by filtration, the residue on the filter paper being washed twice with approximately 50 ml chloroform. The filtrate and washings were mixed and evaporated almost to dryness at 37°C in a rotary evaporator under reduced pressure. The last traces of chloroform were removed by passing a stream of air through the flask.

The material remaining in the flask was dissolved by addition of 16 ml of toluene containing 0.4% PPO and 0.01% POPOP. Finally 15 ml of this solution were pipetted into a 20 ml glass vial for determination of radioactivity as described under Section A.6.

A.9 Quantitation of porphyrins and porphyrin precursors in urine and stool.

A.9.1 Total aminoketones and porphobilinogen in urine

(Mauzerall and Granick 1956). Urine at pH 5 - 7 was passed through a column of Dowex 2-X8 resin (Baker Chem. Co., U.S.A.) in the acetate form. PBG was held on the column while urea and aminoketones were washed from the Dowex 2. PBG was then eluted from the column with acetic acid and determined spectrophotometrically after mixing with modified Ehrlich's reagent.

The washings from the first column were passed through a column of Dowex 50-X8 resin in the acid form. Urea was washed from the column and the aminoketones were then eluted with sodium acetate and allowed to react with acetylacetone at pH 4.6. The resulting pyrroles were determined spectrophotometrically with Ehrlich's reagent.

Comment : This method has obvious limitations since it does not achieve separation of the aminoketone pyrroles and because the Ehrlich aldehyde-reacting chromogens are not identified. It has nevertheless been in use for some 12 years and abundant evidence of its usefulness in the quantitation of porphyrin precursor excretion is to be found in the literature.

A.9.2 Porphyrins in urine and stool.

a. Urinary uroporphyrin (Sveinsson, Rimington and Barnes 1949).

Porphyrins are adsorbed on to a precipitate of calcium phosphate formed by addition of $CaCl_2$ and NaOH to an aliquot of urine. After washing, this precipitate is dissolved in HCl and the uroporphyrin concentration of the acid solution is determined spectrophotometrically.

Procedure : To 2 ml of urine in a 10 ml graduated centrifuge tube

were added 3 ml of 3% $CaCl_2$ solution followed by 5 ml of 1 N NaOH. After mixing, 10 minutes was allowed for complete adsorption to occur. The tube was then centrifuged and the supernatant discarded. The precipitate bearing the adsorbed porphyrins was washed once with 10 ml of 0.1 N NaOH and once with 10 ml of water. Finally it was dissolved in 5 ml of 0.1 N HCl and the volume of the solution made up to 10 ml with water. The optical density of the solution was determined spectrophotometrically at 430, 380 and 405 mμ, the peak of the Soret band for uroporphyrin, using a cuvette of 1 cm optical length in a Zeiss PMQ11 spectrophotometer. (Readings were taken at 406, 405, 404 and 403 mμ to ensure selection of the maximum).

Uroporphyrin concentration was calculated by application of the correction formula

$$D_{405}^{corr} = \frac{2D_{405} - (D_{430} + D_{380})}{1.844}$$

and the value of $E_{1\ cm}^{1\%}$ for uroporphyrin in 0.5 N HCl of 6535 (With 1955).

b. Urinary coproporphyrin (Holti, Rimington et al. 1958).

Urine is extracted with successive volumes of ether. The ether-soluble porphyrins, after washing with dilute iodine to convert the porphyrinogens to porphyrins, are transferred to 5% HCl and the coproporphyrin concentration determined spectrophotometrically.

Procedure : The quantities used were one-fifth of those employed by Holti, Rimington et al. (1958). 10 ml of urine were mixed with 1 ml of glacial acetic acid and 20 ml of ether and shaken. After separation, the aqueous layer was again shaken with 20 ml of ether and the combined ether extract was washed twice with 3% sodium acetate solution (0.3 vol.), once with freshly prepared 0.005% iodine solution (0.1 vol.) and once with water. Porphyrin was transferred to HCl by shaking with successive small volumes of 5% HCl until the ether layer no longer fluoresced in ultraviolet light. The HCl extracts were

combined, the volumes noted and the optical density at 380, 430 and 401 mμ, the peak of the Soret band for coproporphyrin in this solvent, read in a cuvette of 1 cm optical length using a Zeiss PMQ11 spectrophotometer.

Coproporphyrin concentration was calculated by application of the formula (Holti, Rimington et al. 1958) :

$$\text{Coproporphyrin conc. of solution } \mu g / ml = \frac{2D_{401} - (D_{430} + D_{380}) \times 1.7}{1.833}$$

c. Stool coproporphyrin and protoporphyrin (Holti, Rimington et al. 1958). Stool was extracted repeatedly with a mixture of glacial acetic acid and ether until the extract no longer fluoresced under ultraviolet light. The combined extracts were washed first with sodium acetate solution, then with iodine solution and finally with water as described in Section A.9.2 b.

Coproporphyrin was extracted from the acetic acid-ether solution with successive volumes of 0.1 N HCl. Thereafter protoporphyrin was extracted with successive volumes of 5% HCl. In each case the volume of the combined extracts was measured and the optical density of the final solution measured spectrophotometrically at 380 and 430 mμ and at the Soret maximum (401 mμ for coproporphyrin and 407 mμ for protoporphyrin), using cuvettes of 1 cm optical length in a Zeiss PMQ11 spectrophotometer.

Coproporphyrin and protoporphyrin concentrations were calculated using the formulae proposed by With (1955) and were expressed as μg / g dry weight.

Comment on methods used for quantitation of porphyrins in urine and stool : The methods described in this section for the quantitation of porphyrins in urine and stool are representative of the quantitative methods that are routinely used in many laboratories for diagnostic purposes. In the present study they have likewise been employed in establishing the diagnosis of symptomatic porphyria and also in

monitoring the 24-hour output of urinary uroporphyrin and coproporphyrin both in human subjects (Chapter 7) and in rats on various experimental regimes (Chapter 8).

Physical separation of porphyrins by these methods is at best only crude. It can be demonstrated by simple chromatographic techniques that fractions labelled e.g. "uroporphyrin" or "coproporphyrin" are in fact mixtures of porphyrins with various numbers of carboxyl groups. Spectrophotometric determination of any particular porphyrin in such a mixture is therefore liable to inaccuracies.

On the other hand chromatographic techniques, including thin layer chromatography, in general achieve excellent separation of porphyrins either as free acids or methylesters (Grosser et al. 1967). At the present time, however, none of these techniques is readily applicable to the quantitation of porphyrins in biological samples. Thus in the absence of any more suitable methods those employed in the present study were considered to be adequate for the purposes mentioned above.

A.10 Urinary creatinine (Peters and Van Slyke 1932).

This method makes use of the Jaffe reaction, the production of a red colour with an alkaline picrate solution. The Jaffe reaction is not specific for creatinine and there are other substances in blood, particularly in the erythrocytes, which also give this colour. However, in urine only up to 5% of the chromogens may be non-creatinine substances (Owen et al. 1954).

A.11 Liver function tests.

Determination	Method	Normal Range (Serum)
Serum bilirubin	King and Coxon (1950)	0.1-0.8 mg/100ml
Serum alkaline phosphatase	King et al. (1942)	3 - 13 K.A. units/100ml
Total protein	Reinhold (1953)	6 - 8 g/100ml
Total globulins	Saifer et al. (1961)	1.8-3.6 g/100ml
Albumin	Total protein - Total globulins	3.5-5.3 g/100ml
Serum glutamic oxalacetic transaminase (S.G.O.T.)	King (1960)	9 - 40 Karmen units/ml
Serum glutamic pyruvic transaminase (S.G.P.T.)	King (1960)	5 - 35 Karmen units/ml

A.12 Serum iron and total iron binding capacity were determined according to Peters et al. (1956).

Normal values ..... serum iron ..... 50-180 µg/100 ml

TIBC ..... 250-420 µg/100 ml

TABLE B.1 Type and frequency of presenting symptoms among patients with symptomatic porphyria.

Presenting symptom	No. of cases
Swelling of abdomen	10
Abdominal pain	6
No history - mentally confused	6
Diarrhoea and vomiting	4
Skin changes	4
Cough, dyspnoea	3
Passage of "red" urine	2
Paraesthesiae	2
Burning on micturition	1
Back pain	1
Swollen forearm	1
Ulcers on feet	1
Tumour on leg	1

APPENDIX B

CLINICAL, HISTOLOGICAL AND BIOCHEMICAL DATA ON ALL PATIENTS STUDIED.

The number of patients who were studied in the course of the investigations described in this thesis is such as to render it unpractical to include individual case reports. Consequently, the relevant data have been summarised in the ensuing sections with the aid of a number of tables.

B.1 Patients with symptomatic porphyria.

B.1.1 Symptoms. Of the 35 patients studied only 6 complained of symptoms referable to symptomatic porphyria. In 4 cases, this involved either darkening of the skin of the face or the appearance of blisters, chiefly on the hands. Two patients had noticed the passage of 'red' urine.

In most instances, the presenting symptom (Table B.1) was not related to symptomatic porphyria but to some other condition. Swelling of the abdomen was the most common complaint, followed by abdominal pain, either generalised or localised to the right upper quadrant. A proportion of patients were mentally confused and could give no account of themselves on admission.

B.1.2 Consumption of ethanol. Without exception, all 35 patients freely admitted to regular consumption of alcoholic beverages over a number of years. These included Zulu beer (brewed either domestically or by various local authorities), illicit concoctions such a 'shimiyane' and 'gaveen' (Lamont et al. 1961), and more recently, since the removal of restrictive legislation, spirits such as brandy and cane spirit. While some patients claimed to drink only Zulu beer in moderation, others admitted they drank heavily and were not particular as to the type of liquor.

B.1.3 Signs of porphyria. Hyperpigmentation of the face was

present in all cases and was usually of the characteristic blotchy nature described by Lamont et al. (1961). All but 2 patients had typical scars in the areas exposed to sunlight but chiefly on the dorsa of the hands. Only 3 subjects had evidence of vesicles. This accords with the finding that few complained of skin lesions and is probably due to the fact that the subjects studied were of necessity in-patients, admitted to hospital for other complaints. Those patients presenting at the Dermatology Clinic with acute skin lesions would normally be treated as out-patients and not be admitted. Hypertrichosis of the forehead was present in 57% of cases (80% of the women and 48% of the men).

B.1.4 Other diseases. As shown in Tables B.2, B.4 and B.6, these patients were suffering from a variety of complaints apart from symptomatic porphyria. The commonest diagnosis was cirrhosis, with or without ascites, hence the frequency of the complaint of swelling of the abdomen.

B.1.5 Histopathology of the liver. As mentioned in Chapters 7 and 12, portions of liver tissue obtained by percutaneous biopsy were examined histologically only in those cases where information was required for diagnostic reasons unconnected with symptomatic porphyria.

The findings in the 16 cases examined microscopically which appear in Tables B.2, B.4 and B.6 correspond with the pattern described in Section 2.2.3.1. In 2 cases (J. Mthembu and S. Ngubane), however, hepatocellular carcinoma was diagnosed. It may be argued that these patients should not have been included. Nevertheless, since they presented with the clinical and biochemical features of symptomatic porphyria and their response in the various studies was no different from that of the other porphyric subjects, it was decided to

include the findings in these two patients.

B.1.6 Biochemical findings. The results of analysis of casual urine and stool specimens for porphyrins and porphyrin precursors appear in Tables B.3, B.5 and B.7. It will be noted that the pattern is typical of symptomatic porphyria (Section 2.2.3.2).

In addition, a number of investigations were carried out on each patient in order to provide a broader 'biochemical profile'. These included a battery of 'liver function' tests and determination of SGOT, SGPT, prothrombin index, serum iron and haemoglobin. These data are also recorded in Tables B.3, B.5 and B.7. Once again the similarity between these results and those reported by other authors for symptomatic porphyria (Section 2.2.3.2 is immediately evident.

Several points of difference between the present findings and those of Lamont et al. (1961) are the absence of any haemoglobin values greater than 17.0 g per 100 ml and the lower incidence of hypersideraemia (35% > 170 µg/100 ml) in the present patients.

B.2 Non-porphyric subjects with hepatocellular disease. In Chapter 12 the rate of clearance of aetiocholanolone from the plasma was compared in 10 patients with symptomatic porphyria and 7 non-porphyric subjects with various types of hepatocellular pathology. The clinical, histological and biochemical details of these cases appear in Tables B.8, B.9. All of these non-porphyric patients lacked both clinical and biochemical evidence of symptomatic porphyria but 3 of them exhibited slightly raised urinary uroporphyrin excretion.

Table B.2 Clinical and histological data on porphyric patients included in study on liver ALA synthetase activity (Chapters 7, 11).

Name	Age (Yrs)	Sex	Clinical Diagnosis*	Histological findings (liver biopsy)
Mkize, F.	40	M	Cirrhosis	"Active cirrhosis", moderate siderosis
Ngubane, N.	65	M	Idiopathic epilepsy	--------
Buthelezi, I.	42	F	Mental confusion (associated with alcohol and head injury)	Cirrhosis and marked siderosis.
Thabede, R.	40	M	Peripheral neuritis (? alcoholic)	Liver cell damage, portal fibrosis, moderate siderosis
Ndaba, E.	49	F	Gastro-enteritis	--------
Mkize, S.	49	M	Idiopathic epilepsy	Portal fibrosis, marked siderosis.
Miya, C.	69	F	Cirrhosis	Cirrhosis and marked siderosis.
Pondo, V.	68	F	Pyelonephritis	--------
Sokhela, W.	42	M	Symptomatic porphyria only	--------
Mthembu, M.	60	M	Cirrhosis with ascites	--------
Ndimande, M.	40	M	Tuberculous peritonitis	Moderate siderosis, minimal fatty change.
Ngubane, J.	30	M	Pellagra	Septal fibrosis, marked siderosis.
Hlongwana, N.	30	M	Cirrhosis	Cirrhosis, moderate siderosis.
Majola, Z.	50	M	Unexplained hepatomegaly	Septal fibrosis, moderate siderosis.
Mthembu, D.	46	F	Alcoholic hypoglycaemia	Moderate siderosis, minimal fatty change.

* i.e. clinical diagnosis apart from symptomatic porphyria.

Table B.3 Biochemical data on porphyric patients included in study on liver ALA synthetase activity (Chapters 7, 11.)

NAME	Hb (g/100ml)	P.I. (%)	SERUM							URINE			STOOL (dry wt.)	
			Bili-rubin (mg/100ml)	Alk. Phos. (KA units)	Alb. (g/100 ml)	Glob. (g/100 ml)	GOT (Karmen units)	GPT (Karmen units)	Fe (ug/100ml)	ALA (ug/ml)	PBG (ug/ml)	URO (ug/100ml)	COPRO (ug/g)	PROTO (ug/g)
Mkize, F.	14.6	80	1.7	9	3.7	4.3	77	61	260	2.0	<1	740	38	17
Ngubane, N.	11.1	99	0.7	17	1.5	5.9	86	63	112	3.6	<1	573	79	10
Buthelezi, I.	10.8	86	1.2	4	2.5	5.6	56	28	95	5.2	<1	1131	172	62
Thabede, R.	11.5	-	0.5	15	2.0	7.0	54	14	31	1.6	<1	91	14	78
Ndaba, E.	14.2	94	0.9	9	3.2	5.2	137	168	-	-	-	+	138	63
Mkize, S.	13.8	100	0.6	15	3.3	5.0	68	51	224	8.3	<1	710	112	52
Miya, C.	9.9	100	3.4	16	1.9	6.0	47	54	240	-	-	+	-	-
Pondo, V.	10.5	100	0.7	14	2.9	5.7	-	-	108	<1.0	0	143	300	146
Sokhela, W.	16.0	91	0.9	10	3.2	4.4	68	121	360	3.5	<1	409	250	32
Mthembu, M.	10.3	71	0.9	7	2.4	5.0	65	27	93	1.2	<1	123	30	39
Ndimande, M.	10.3	79	0.9	17	1.9	7.5	65	34	64	11.5	1.2	1948	172	95
Ngubane, J.	13.8	92	-	13	3.6	4.5	56	48	190	1.8	<1	141	18	9
Hlongwana, N.	16.0	85	1.7	31	2.9	6.2	104	74	92	1.0	<1	252	18	2
Majola, Z.	8.9	82	0.8	7	2.5	4.1	48	33	65	1.4	<1	82	27	7
Mthembu, D.	12.0	92	0.8	10	3.7	4.3	52	33	106	1.0	<1	73	67	85

+ = screen test positive

Table B.4 Clinical and histological data on porphyric patients included in study of daily urinary porphyrin excretion (Chapter 7).

Name	Age (Yrs)	Sex	Clinical Diagnosis*	Histological findings (liver biopsy)
Mkize, F.	40	M	Cirrhosis	"Active cirrhosis", moderate siderosis
Gumede, H.	63	F	Fibrosarcoma of right leg	--------
Mazibuko, E.	70	F	Cirrhosis with ascites	--------
Nkosi, N.	60	M	Peripheral neuropathy	--------
Mhlongo, Z.	38	M	Mental confusion (? alcoholic)	--------
Msomi, R.	37	M	Cellulitis (R) forearm	--------
Sokhela, W.	42	M	Symptomatic porphyria only	--------
Hlongwa, S.	34	M	Right-sided pneumothorax	--------
Mthembu, J.	44	M	Carcinoma of liver	Hepatocellular carcinoma
Mthembu, E.	50	M	Mental confusion (? alcoholic)	--------
Mbotho, M.	40	M	Lung abscess	--------
Ndaba, T.	46	F	Cirrhosis with ascites	--------

* i.e. clinical diagnosis apart from symptomatic porphyria

Table B.5 Biochemical data on porphyric patients included in study of daily urinary porphyrin excretion (Chapter 7).

NAME	Hb (g/100ml)	P.I. (%)	SERUM							URINE			STOOL (dry wt.)	
			Bili-rubin (mg/100ml)	Alk. Phos. (K.A. units)	Alb. (g/100 ml)	Glob. (g/100 ml)	GOT (Karmen units)	GPT (Karmen units)	Fe (ug/100ml)	ALA (ug/ml)	PBG (ug/ml)	URO (ug/100ml)	COPRO (ug/g)	PROTO (ug/g)
Mkize, F.	14.6	80	1.7	9	3.7	4.3	77	61	260	2.0	<1	740	38	17
Gumede, H.	10.2	91	0.5	5	2.3	6.0	76	32	108	1.0	1.0	212	70	37
Mazibuko, E.	10.5	80	0.7	12	3.0	4.3	66	29	86	0.3	1.2	219	166	82
Nkosi, N.	11.3	87	0.6	10	3.9	3.7	58	36	99	Nil	<1	44	48	22
Mhlongo, Z.	10.8	86	0.2	6	3.1	3.6	35	31	104	2.4	<1	32	5	13
Msomi, R.	11.1	-	0.7	9	1.1	5.4	78	43	190	5.4	<1	615	128	60
Sokhela, W.	16.0	91	0.9	10	3.2	4.4	68	121	360	3.5	<1	409	250	39
Hlongwa, S.	11.5	77	0.6	10	3.2	6.0	47	35	220	2.1	Nil	97	12	74
Mthembu, J.	13.8	70	10.2	41	2.5	6.1	98	79	141	1.0	<1	208	13	51
Mthembu, E.	14.2	-	1.1	13	1.6	5.7	70	59	240	1.3	Nil	92	122	21
Mbotho, M.	12.3	96	1.7	10	1.5	8.9	81	32	175	3.2	Nil	179	17	32
Ndaba, T.	10.5	72	0.7	10	2.7	6.7	59	47	81	1.0	<1	34	27	39

Table B.6 Clinical and histological data on patients with symptomatic porphyria included in study of aetiocholanolone $T_{\frac{1}{2}}$ (Chapter 12).

Name	Age (Yrs)	Sex	Clinical Diagnosis*	Histological findings (liver biopsy)
Chonco, A.	45	M	Cervical spondylosis	--------
Mkize, H.	57	M	Pulmonary tuberculosis	Marked siderosis, septal fibrosis, metastatic tubercles.
Mokhantso, J.	50	M	Cirrhosis with generalised oedema	--------
Kuzwayo, C.	60	F	Cirrhosis with ascites	Cirrhosis with siderosis
Ndlovu, F.	37	F	Cirrhosis with ascites	--------
Mbele, E.	58	M	Pellagra	--------
Ngubane, S.	29	M	Cirrhosis with ascites	Hepatocellular carcinoma
Mcetshwa, G.	54	M	Cirrhosis with ascites	Severe siderosis, septal fibrosis
Sibiya, N.	40	M	Cirrhosis with ascites	--------
Khumalo, D.	38	M	Emphysema and pneumonitis	Cirrhosis with mild siderosis

* i.e. clinical diagnosis apart from symptomatic porphyria

Table B.7 Biochemical data on patients with symptomatic porphyria included in study of aetiocholanolone $T\frac{1}{2}$ (Chapter 12).

NAME	Hb (g/100ml)	P.I. (%)	SERUM							URINE			STOOL (dry wt.)	
			Bili-rubin (mg/100ml)	Alk. Phos. (K.A. units	Alb. (g/100 ml)	Glob. (g/100 ml)	GOT (Karmen units)	GPT (Karmen units)	Fe (ug/100ml)	ALA (ug/ml)	PBG (ug/ml)	URO (ug/100ml)	COPRO (ug/g)	PROTO (ug/g)
Chonco, A.	10.2	62	3.8	11	1.8	5.8	65	32	132	7.4	<1	186	5	4
Mkize, H.	14.6	85	0.6	26	1.9	5.8	61	32	89	5.2	0	314	20	87
Mokhantso, J.	8.7	72	0.8	6	2.2	7.8	58	36	147	0.7	<1	46	16	236
Kuzwayo, C.	12.3	82	0.6	7	2.6	4.4	58	36	94	5.4	1.2	39	3	5
Ndlovu, F.	12.7	86	0.6	29	2.4	6.7	68	86	85	2.7	<1	313	29	163
Mbele, E.	9.9	-	0.7	10	2.2	4.7	25	14	72	4.5	<1	551	94	160
Ngubane, S.	10.8	73	1.8	15	2.5	3.8	149	177	100	6.9	<1	3522	20	2
Mcetshwa, G.	15.4	86	0.5	16	2.7	6.1	>300	69	204	1.5	<1	68	22	25
Sibiya, N.	14.2	57	1.2	18	2.7	4.4	45	22	210	1.2	<1	65	11	31
Khumalo, D.	12.7	80	0.6	19	3.4	5.7	44	-	160	2.3	<1	107	11	6

Table B.8 Clinical and histological details of non-porphyric patients included in study of aetiocholanolone $T\frac{1}{2}$ (Chapter 12).

Name	Age (Yrs)	Sex	Clinical Diagnosis	Histological findings (liver biopsy)
Mbambo, P.	45	M	Cirrhosis with ascites	--------
Khubisa, M.	49	M	Lung abscess, cirrhosis	Cirrhosis with severe siderosis
Tusi, E.	31	M	Cirrhosis with ascites	--------
Mtetwa, P.	47	M	Cirrhosis	Severe siderosis, minimal fibrosis
Ngcobo, M.	57	F	Tuberculous spondylitis	Severe siderosis and septal fibrosis, mild fatty change
Shusha, A.	25	M	Unexplained hypoglycaemia	Hepatocellular carcinoma
Mabaso, M.	57	M	Amoebic liver abscess	Low grade hepatitis

Table B.9 Biochemical data of non-porphyric patients included in study of aetiocholanolone $T_{\frac{1}{2}}$ (Chapter 12).

NAME	Hb (g/100ml)	P.I. (%)	SERUM							URINE			STOOL (dry wt.)	
			Bili-rubin (mg/100ml)	Alk. Phos. (K.A. units)	Alb. (g/100 ml)	Glob. (g/100 ml)	GOT (Karmen units)	GPT (Karmen units)	Fe (ug/100ml)	ALA (ug/ml)	PBG (ug/ml)	URO (ug/100ml)	COPRO (ug/g)	PROTO (ug/g)
Mbambo, P.	12.7	87	1.3	25	2.6	5.5	67	67	194	1.0	0	12	10	14
Khubisa, M.	12.0	73	0.8	16	3.3	5.7	28	28	130	4.2	1	0	3	55
Tusi, E.	13.4	95	0.5	9	3.2	4.8	46	17	54	2.8	1.6	19	12	24
Mtetwa, P.	12.7	97	0.4	13	1.4	5.0	47	40	93	5.5	1.1	3	11	16
Ngcobo, M.	12.7	93	0.8	10	3.0	4.3	25	-	84	1.1	0	23	31	55
Shusha, A.	11.4	73	1.1	12	3.5	4.5	161	48	29	2.8	1	3	13	17
Mabaso, M.	11.1	93	0.3	23	1.3	7.2	34	61	32	6.2	1	3	5	2

Table C.1 Mean urinary uroporphyrin excretion over the last 5 weeks of the experiment in the 6 groups of rats studied in Chapter 8 (5 observations in each case ; values in µg uroporphyrin / rat / day).

Diets	Plus water	Plus alcohol	Mean
Normal	1.83	1.51	1.67
Maize	1.77	1.99	1.88
Maize + iron	3.13	5.29	4.21
Mean	2.24	2.93	-

Table C.2 Analysis of variance for Table C.1 according to Scheffé (1959) for equal numbers of observations.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	F table value 5% points
Diets (adjusted for alcohol)	2	39.6655	19.8327	* 32.8737	3.40
Alcohol (adjusted for diets)	1	3.5639	3.5639	* 5.9103	4.26
Interaction between diets and alcohol	2	8.4451	4.2225	* 6.9990	3.40
Error	24	14.4801	0.6033	-	-
Totals	29	66.1546	-	-	-

* denotes significant values

APPENDIX C

STATISTICAL ANALYSES

C.1 Analysis of results in Chapter 8.

C.1.1 Urinary porphyrin excretion. Urinary porphyrin excretion over the last 5 weeks of the experiment was compared in the 6 groups of rats. Separate analyses of variance of the values for uroporphyrin (Table C.1) and coproporphyrin (Table C.3) were carried out as shown in Tables C.2 and C.4 respectively. The procedures employed were as described in Chapters 3 and 4 of Scheffé (1959) for cases involving equal numbers of observations.

From the F ratios shown in Table C.2 it appears that both diet alone and alcohol alone significantly affected urinary uroporphyrin excretion. In addition interaction between diet and alcohol occurred.

Scheffé's S method of multiple comparisons (p.67 of Scheffé 1959) was then used to test individual differences. This revealed that the maize and iron diet produced an effect significantly different from both the normal and the maize diets. In addition alcohol interacted significantly with the maize and iron diet.

Similarly, from Table C.4 it can be seen that diet alone significantly affected urinary coproporphyrin excretion while alcohol alone did not. Again there was interaction between diet and alcohol. Application of Scheffé's S method of multiple comparisons to test individual differences revealed exactly the same pattern as found for uroporphyrin.

Table C.3 Mean urinary coproporphyrin excretion over last 5 weeks of the experiment in the 6 groups of rats studied in Chapter 8 (5 observations in each case ; values in µg coproporphyrin/ rat / day).

Diets	Plus water	Plus alcohol	Mean
Normal	0.99	0.92	0.96
Maize	1.14	1.14	1.14
Maize + iron	2.51	3.99	3.25
Mean	1.55	2.02	-

Table C.4 Analysis of variance for Table C.3 according to Scheffé (1959) for equal numbers of observations.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	F table value 5% points
Diets (adjusted for alcohol)	2	32.5756	16.2878	* 29.8038	3.40
Alcohol (adjusted for diets)	1	1.6924	1.6924	3.0968	4.26
Interaction between diets and alcohol	2	3.8425	1.9212	* 3.5155	3.40
Error	24	13.1158	0.5465	-	-
Totals	29	51.2263	-	-	-

* denotes significant values

Table C.5 Mean liver mitochondrial coproporphyrinogen oxidase activity at 48 weeks in 6 groups of rats studied in Chapter 8 (3 observations in each case ; values in mμmoles protoporphyrin formed / mg mitochondrial protein).

Diets	Plus water	Plus alcohol	Mean
Normal	2.27	3.05	2.66
Maize	2.61	2.03	2.32
Maize + iron	2.76	2.98	2.87
Mean	2.55	2.69	-

Table C.6 Analysis of variance for Table C.5 according to Scheffé (1959) for equal numbers of observations.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	F table value 5% points
Diets (adjusted for alcohol)	2	0.9109	0.4554	3.3658	3.89
Alcohol (adjusted for diets)	1	0.0882	0.0882	0.6519	4.75
Interaction between diets and alcohol	2	1.4016	0.7008	* 5.1796	3.89
Error	12	1.6230	0.1353	-	-
Totals	17	4.0237	-	-	-

* denotes significant values

C.1.2 Liver mitochondrial coproporphyrinogen oxidase activity.

Table C.5 shows the values obtained for liver mitochondrial coproporphyrinogen oxidase activity in the 6 groups of rats at the end of the study. Analysis of variance was carried out as described in Chapters 3 and 4 of Scheffé (1959) for cases involving equal numbers of observations.

From Table C.6 it appears that neither alcohol alone nor diet alone significantly influenced coproporphyrinogenase activity. On the other hand significant interactions occurred.

Individual differences were tested for by Scheffé's S method of multiple comparisons (p.67 of Scheffé 1959). This revealed that alcohol plus a normal diet produced a significantly greater effect than a normal diet alone. On the other hand, the effect of alcohol plus a maize diet was less than the maize diet alone. Lastly alcohol in combination with either a normal diet or a maize plus iron diet produced a significantly greater effect than alcohol plus a maize diet.

C.1.3 Liver ALA synthetase activity (48 weeks). The values obtained for liver ALA synthetase activity in the 6 groups of rats at the end of the study are shown in Table C.7. In this case the numbers of observations were different for the various groups, necessitating a different approach to the analysis of variance. The following procedures were employed :

1) Interactions were tested for according to Scheffé (1959), pages 112 - 115;
2) with significant interactions, main effects were tested for according to the procedures set out in Scheffé (1959), pages 116 - 119.

The results of these analyses appear in Table C.8. It appears that besides significant interactions, alcohol alone had a significant effect, whereas diet alone did not.

Application of Scheffé's S method of multiple comparisons

Table C.7 Liver ALA synthetase activity at 48 weeks in 6 groups of rats studied in Chapter 8 (values in μmoles of ALA formed / g liver / hour).

Diets	Plus water		Plus alcohol		Mean
	No. of observ.	Mean	No. of observ.	Mean	
Normal	5	33.3	1	59.5	37.7
Maize	4	32.8	4	69.5	51.2
Maize + iron	4	39.5	3	76.8	55.5
Totals	13	35.1	8	70.9	-

Table C.8 Analysis of variance for data in Table C.7 according to Scheffé (1959) for unequal numbers of observations. The following figures have been extracted from several analyses of variance (Section C.1.3).

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F ratio	F table value 5% points
Diets (adjusted for alcohol)	2	9.5260	4.7630	0.0304	3.68
Alcohol (adjusted for diets)	1	4883.2532	4883.2532	*31.1934	4.54
Interaction between diets and alcohol	2	2426.4862	1213.2431	*7.7500	3.68
Error	15	2348.2117	156.5475	-	-

* denotes significant values

(Scheffé 1959, p.67) to test individual differences revealed that the only significant interaction was between the maize diet and alcohol.

C.2 Analysis of results in Chapter 11. In order to ascertain whether any correlation existed between liver ALA synthetase activity and galactose $T\frac{1}{2}$ the correlation coefficient r was calculated from the values shown in Table 11.1. The procedures used were those set out in Chapter 9 of Bailey (1959).

When all the values in Table 11.1 were included r was calculated to be 0.4663. When the values pertaining to those patients who received ethanol prior to the study (Table 11.1) were excluded from the calculation r was found to be 0.6642.

In both cases the calculated value for r was less than expected (0.602 and 0.707 respectively) for significance at the 5% level, i.e. no significant correlation was found at the 5% level between liver ALA synthetase activity and galactose $T\frac{1}{2}$.

C.3 Analysis of results in Chapter 12. In order to ascertain whether the mean values for aetiocholanolone $T\frac{1}{2}$ shown in Table 12.1 for porphyric and non-porphyric subjects differed significantly Student's t-test was applied. No significant difference at the 5% level was found between the means for the two groups.