OBSERVATIONS ON DRUG - INDUCED PORPHYRIA

With Especial Reference

to

The Role of Ribonucleic Acid

A Thesis

submitted to the University of Cape Town

for

the Degree of Doctor of Medicine

by

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This Thesis

is

dedicated

to

My Parents

and all others

who have made my University education

and this thesis possible.

"To know the nature of man
one must know the nature of all things. "

- Hippocrates.

Frontispiece:

Thin-layer chromatography plate photographed in ultra-violet light.

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    - RNA
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ABBREVIATIONS.

AIA	- Allyl-isopropyl-acetamide
ALA	- δaminolaevulinic acid
ALA-synthetase	- δaminolaevulinic acid-synthetase
AMP	- Adenosine monophosphate
ATP	- Adenosine triphosphate
CoA	- Coenzyme A
COPROgen	- Coproporphyrinogen
DNA	- Deoxyribonucleic acid
DDC	- Diethyl-2,4,6-trimethylpyridine-3,5-dicarboxylate
EDTA	- Ethylene-diamine tetra-acetic acid
FAD	- Flavin adenine dinucleotide
FMN	- Flavin mononucleotide
HCB	- Hexachlorobenzene
mRNA	- Messenger RNA
NAD	- Nicotine adenine dinucleotide
NADP	- Nicotine adenine dinucleotide phosphate
NADPH	- Reduced NADP
PBG	- Porphobilinogen
PROTOgen	- Protoporphyrinogen
RNA	- Ribonucleic acid

ABBREVIATIONS Contd.

RNase	- Ribonuclease
rRNA	- Ribosomal ribonucleic acid
r + mRNA	- Ribosomal + messenger ribonucleic acid
Rps. sphs.	- Rhodospseudomonas spheroides
tRNA	- Transfer ribonucleic acid
UROgen	- Uroporphyrinogen
SDS /SLS	- Sodium dodecyl (lauryl) sulphate

CHAPTER I: AN INTRODUCTION TO THE DISEASES OF PORPHYRIN METABOLISM.

> "Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path... for it has been found in almost all things, that what they contain of useful and applicable is hardly perceived unless we are deprived of them or they become deranged in some way." Harvey (1847)

The syndromes of disordered porphyrin metabolism have puzzled physicians and investigators alike since their first description in 1874 (1)(2). Although they are rare in most parts of the world, their catholic manifestations mimic diseases in all fields of medicine, but the presence of porphyrins in biological fluids is often rapidly detectable by their dramatic red fluorescence in ultra-violet light. However, although great advances have been made in porphyrin biochemistry, there are aspects of the biosynthetic pathway which remain to be elucidated.

HISTORICAL REVIEW.

First references to porphyrins were made in the 1840's by Scherer (3) and Mulder (4) who treated blood with concentrated sulphuric acid and revealed a purple-red iron-free fluid. This was named cruentine by Thudichum in a report to the Privy Council in Great Britain in 1867 (5). He demonstrated its fluorescence in a cone of sunlight collected by a system of quartz lenses, and could define its spectrum. Hoppe-Seyler (6) demonstrated two constituents in this fluid, of which

he named the major part haematoporphyrin. In 1874 (1)(2) the first case was described with symptoms and signs characteristic of congenital porphyria, although at the time it was entitled Pemphigus Leprosus. Investigation of the urine from this patient revealed two pigments designated urorubrohaematin and urofuschsohaematin. The former was later renamed urohaemotoporphyrin because of its resemblance to haemotoporphyrin. In 1911, Günther(7) collected all reports of syndromes and made the first classification of the disease. Garrod(8) included congenital porphyria as an inborn error of metabolism and credited Günther with its first recognition. In 1937, Waldenstrom(9) reviewed many of Günther's cases, and 103 patients with porphyria found in Sweden. He also reported on the chemical aspects of the disease which had been the especial concern of Fischer and Rimington. The name Porphyrias was proposed for the diseases, rather than Haematoporphyrias (as per Günther), because the naturally-occurring porphyrins had been found to differ structurally from the chemical artefact, haematoporphyrin. The porphyrins found in the urine and faeces were named uro- and coproporphyrin respectively, while that found as the naturally-occurring porphyrin of haem was known as protoporphyrin. Waldenstrom also noted the excretion of porphobilinogen by apparently healthy relatives of patients with acute porphyria and suggested a latent porphyria which he demonstrated in several generations.

Fig.I

A CLASSIFICATION OF PORPHYRIA.

- ERYTHROPOEITIC
    1: congenital erythropoeitic porphyria
    2: erythropoeitic protoporphyria
- HEPATIC
    Inherited
    1: swedish - acute intermittent
    2: s.a. genetic - variegate
    3: ? idiopathic coproporphyria

    Acquired
    1: porphyria cutanea tarda
    2: secondary to fungicide (HCB)
    3: secondary to hepatoma (1 case)
    4: secondary to other disease
- EXPERIMENTAL
    1: in vivo
    2: in vitro

A SUMMARY OF THE CLINICAL & BIOCHEMICAL FINDINGS IN VARIOUS OF THE SYNDROMES OF DISORDERED PORPHYRIN METABOLISM

TYPE	CLINICAL					BIOCHEMICAL					
						Urine				Stool	
	Heredity	Alcohol	Skin lesion	Acute attack		uro	copro	ALA	PBG	copro	proto
Congenital Erythropoeitic	+	-	+	-		I+	I+	n	n	n	n
Erythropoeitic Protoporphyria	+	-	+	-		n	n	n	n	n	+
Acute Intermittent	+	-	-	+	remission	+	n	2+	2+	n	n
					acute attack	3+	2+	3+	3+	n	n
S.A. Genetic	+	-	+	+	remission	+	+	n	n	2+	3+
					acute attack	3+	2+	2+	2+	3+	3+
Symptomatic	-	+	+	-		3+	+	n	n	+	+

Since that time, a number of classifications have been proposed but it is probable that a wholly satisfactory solution will only be achieved when all the enzymatic defects of the porphyrias have been elucidated.

CLASSIFICATION

A classification which is acceptable in the light of present knowledge may be compounded from those suggested by Watson(10) and Eales(11), and is shown in fig. I. A summary of the clinical and biochemical manifestations of some of these symptoms is presented in fig. II, and further description follows below.

A: ERYTHROPOEITIC

(i) Congenital Erythropoeitic porphyria - is a rare disease which is inherited as an autosomal recessive. The initial manifestation is of the excretion of red urine, often occurring in a young child. As exposure to sunlight increases, photo-sensitivity is revealed with the occurrence of a vesicular or bullous eruption hydroa aestivale, which in many cases proceeds to marked scarring and contracture(7) formation. Hypertrichosis(7)(12) and erythrodontia(7)(13) are other features. Splenomegaly is an almost constant accompaniment but the hypertension and neurological symptoms of other forms of porphyria are not found. Increased haemolytic activity is indicated by the finding of normochromic anaemia, with an increase in circulating normoblasts and reticulocytes, some of which fluoresce.

Faecal urobilinogen(14) is increased. This haemolytic component may only occur in certain phases of the disease. Urinary excretion of uroporphyrin is markedly increased(7)(15)(16) as to a lesser extent is coproporphyrin(7)(15)(16)(17)(18). Small amounts of porphyrins with seven, six, five and three carboxyl groups have been demonstrated(15)(16). The uro- and coproporphyrin occur predominantly in type I isomer form(19)(20) but type III isomers have been recovered (17)(18)(21). The excretion of porphyrin precursors is normal. Faecal coproporphyrin is increased, and uroporphyrin slightly, while protoporphyrin appears normal(16). Red cell porphyrins are increased(15) (16)(17)(22)(23). Because the primary defect appears to be the overproduction of isomer I, the most likely metabolic fault is in the mechanism converting porphobilinogen (PBG) to uroporphyrinogen (UROgen) (24)(25)(26). As will be detailed in chapter II, this involves the enzymes PBG deaminase or UROgen deaminase.

(ii) Erythropoeitic protoporphyria has only recently been described(27)(28). It does not exhibit any of the characteristics of congenital erythropoeitic porphyria, but photosensitivity manifests as solar urticaria(27)(29) with pruritus, erythema(27)(30) and oedema and little tendency to bullous formation. The spectrum of ultra violet light causing this has been shown to be precisely related to that of maximal absorption of porphyrins(27) (the Soret band). Scarring does not follow these cutaneous manifestations. In the

majority of patients the morphology of blood and bone marrow components is normal(27). The urinary excretion of porphyrin precursors and porphyrins(27)(29)(30) is normal, but protoporphyrin is markedly increased in circulating erythrocytes and in faeces(27). Occasionally, faecal coproporphyrin is increased. There is no increase of uroporphyrin in the faeces or tissues. The nature of the defect is suggested to be diminished activity of the ferrochelatase (haem synthetase), but iron utilisation(27) and haem synthesis by the bone marrow proceeds normally (31). The impression of various workers is that the defect is not limited to erythroid cells, but is present at least in the liver as well(32).

B: HEPATIC

Congenital

(i) Acute Intermittent Porphyria - has as its most prominent symptom abdominal pain of a colicky nature with minimal signs. The association with constipation and vomiting leads to misdiagnosis as a surgical abdominal emergency(33)(34). The manifestations are characteristically intermittent(35)(36)(37). Associated neurological manifestations may involve any aspect of neural function and are extremely variable. Tachycardia and hypertension occur frequently. The hyponatraemia accompanying acute attacks has been related to inappropriate secretion of anti-diuretic hormone(38)(39) but proof of this is lacking

in a situation where fluid balance is markedly disturbed. Acute attacks are often precipitated by exposure to drugs, especially the barbiturate and sulphonamide groups, griseofulvin or oestrogens(9)(36)(37)(40)(41)(42)(43)(44)(45). Chemically the characteristic finding is of markedly increased urinary PBG(9)(46)(47) and δ-amino-laevulinic-acid (ALA)(48)(49)50)(51)(52). Increased levels of porphyrins may occur only after the urine has been standing a while(53) and most occur as metal complexes, often with zinc(36)(54) although total zinc excretion is not increased(55). The concentrations of urinary amino acids(56) and indolic compounds(57) has been found to be increased. A slight to moderate increase in faecal porphyrin of all three types may occur. Only the liver has been shown to contain large amounts of PBG. Increased formation of ALA or a block in non-porphyrin metabolism after the formation of ALA are possible explanations for the emphasis on the excretion of porphyrin precursors(9)(22)(35)(36)(37). Both the phenomena of end-product inhibition(58)(59)(60) or defective genetic regulation of ALA-synthetase could account for part of the biochemical disorder, and this is suggested by the demonstration of elevated levels of ALA-synthetase in the livers of these patients(61)(62). Manifestation of this porphyria before puberty is rare(37) suggesting a relationship with hormonal activity. However, the administration of oestrogens to normal individuals has no effect on porphyrin metabolism(63). The condition is inherited as an autosomal dominant character.

(ii) S.A. Genetic Porphyria - also known as hereditary protoporphyria, is characterised by a family history of skin sensitivity to light and trauma known colloquially in South Africa as "sagte vel". Acute attacks occur occasionally with abdominal and neurological manifestations and are frequently precipitated by drugs(63)(64)(65) especially the barbiturates. Neuropathic involvement is often severe and persists after subsidence of the acute episode. During an acute attack, ALA and PBG are increased in the urine, with marked elevation of uro and coproporphyrin in the urine and coproporphyrin and protoporphyrin in the faeces. This elevation of porphyrin excretion in the stool persists during remission. The nature of the defect is not clear though suggestions have been made of a defect in the enzymes normally preventing oxidation of porphyrinogens to porphyrins(35), or a decrease in "cellular reducing potential"(66).

(iii) A separate form of coproporphyria(67) without symptoms has been described as hereditary or idiopathic depending on its occurrence in more than one member of a family.

Acquired

(i) Acquired porphyria in South Africa is usually related to alcohol ingestion(35)(68)(69). The associated skin lesions are similar to those occurring in S.A. genetic porphyria. Hyperpigmentation and scarring result(10)(11). Hepatomegaly is associated with laboratory and histological evidence of impaired liver function.

Acute attacks do not occur. Characteristically the excretion of urinary uroporphyrin is increased while faecal porphyrins are normal or only slightly elevated(41)(10)(11). Although it has not been demonstrated, an associated genetic defect is possible(10) because relatively few patients who chronically consume excessive amounts of alcohol, develop porphyria.

(ii) Another type of acquired porphyria occurred in Turkey in persons who had unwittingly ingested insecticide containing hexachlorobenzene (HCB)(72)(73)(74)(75).

(iii) A single case has been described of increased porphyrin excretion by a patient with a hepatoma(76). The symptoms and signs of disordered porphyrin metabolism disappeared following removal of the tumour.

(iv) Various metals and chemicals have been reported to increase porphyrin excretion. These include lead(77)(78)(79)(80)(81), aniline, arsenicals and methyl chloride, but since the increased porphyrin excretion occurs without associated symptoms and is predominantly of coproporphyrin, perhaps it is better to include these "chemically-induced" defects among the many other forms of liver disease in which coproporphyrin is excreted in excess(19)(35)(82).

C: EXPERIMENTAL PORPHYRIA

(i) In Animals

A number of compounds have been used to produce abnormal porphyrin metabolism in animals similar to human porphyria, but however

tempting it is to extrapolate such data to the manifestations in man, caution must be exercised. The drugs which have been especially used in animals are the barbiturates(83)(84) especially Sedormid(83)(85)(86) and its analogue AIA, DDC(87), Griseofulvin(88) and HCB(89). A comprehensive review up to 1964 is given by Ginsberg(90). Subsequent reports on animal experiments have confirmed the role of these particular drugs in the production of experimental porphyria, but a technique devised by Granick(91) using chick embryo liver cells in tissue culture has revealed that a much larger number of drugs will induce porphyrin fluorescence in such a system. This may provide a valuable means of screening drugs for potential toxicity, and is discussed in greater detail later, in conjunction with results reported in this thesis using the same technique.

(ii) In Man

Apart from the recognised exacerbation of acute intermittent and S.A. genetic types of porphyria by drugs, the concept of an entirely chemically-induced syndrome has fluctuated in popularity. As mentioned earlier, first reporters considered that the disease was induced only by drugs, but with Waldenstrom's(9) description of a latent form of porphyria precipitated by the use of these agents, interest moved away from a purely acquired toxic syndrome. The reports of experimental porphyria induced in animals(36)(85)(86) provided new support which was endorsed by the hexachlorobenzene "epidemic". This

appears to represent the first and only human counterpart described thus far of the experimental porphyria produced in animals(92)(93). The relationship between a patient with symptomatic porphyria and exacerbation of his disease by alcohol, may be regarded as similar to that between a patient with acute intermittent or S.A. genetic porphyria, and various drugs as yet, chloroquine remains the only drug to which a symptomatic porphyria has responded adversely(94), and the mechanism of this response is unexplained.

THE ROLE OF DRUGS IN PORPHYRIA

The first report of a syndrome apparently precipitated by the newly-introduced hypnotics sulphonal, trional and tetronal and associated with haematoporphyrinuria, was made in 1889(82A). Numerous cases of exacerbation of the various syndromes of porphyria by drugs have been cited since then, the relationship being especially well-defined between acute intermittent porphyria and S.A.genetic porphyria and the barbiturates. In these last instances, fatalities have been reported(65).

Some drugs may be tested with extreme caution on patients with porphyria, especially if their use is essential to recovery, but for the most part, the possible deleterious effects preclude this(94)(95)(96)(97)(98)(99).

Although most reports have been of the deleterious effects of drugs in porphyria, some attempts to treat the disease with drugs

have been made. These are particularly difficult to evaluate in view of the occurrence of complete spontaneous remissions(100). Reports have been received from Tschudy's laboratory on the effect of dietary (101)(102) and hormonal regulation(103)(104) and from Gajdos concerning the beneficial effect of the administration of AMP(105)(106)(107).

The dietary theory of Tschudy relates to the inhibitory effect of glucose on many enzymes (which will be reported in greater detail elsewhere in this thesis). It was found that a patient(102) with acute intermittent porphyria in remission, excreted increased levels of porphyrin precursors in the urine when fed a low carbohydrate diet, or when isocaloric amounts of fat and protein were substituted for carbohydrate. The levels of these precursors returned to normal with resumption of a balanced diet.

The investigation of the effect of various hormones was motivated by the observation that in females with acute intermittent porphyria, an acute attack may occur at the time of menstruation(9). The effect of progesterone-derived oral contraceptive agents is thought to be the inhibition of gonadotrophin secretion with stabilisation of endogenous ovarian steroid production(108). The administration of stilboestrol (63)(109)(110) and other oestrogen derivatives may result in increased porphyrin production, while a case is reported whose symptoms were apparently alleviated by the administration of combined ethinyl oestradiol and norethynodrel (as Enovid)(103). Several other workers

advocate the use of oral contraceptive agents in the treatment of acute intermittent porphyria(104)(111)(112). The reports of progesterone-induced exacerbations are in the minority(113).

In bacteria, the administration of adenine nucleotides causes diminished ATP levels(114)(115)(116)(117)(118). This information and a postulate by Shemin(118) that in porphyria there was a deficiency of phosphorylated derivatives of adenosine prompted Gajdos to investigate the effect of adenine mononucleotides in experimental porphyria. He has reported several successes in experimental animals(106)(119) and in the treatment of acute attacks in acute intermittent porphyria(105)(106)(107). However, no effect was noted when AMP was used in a patient with congenital erythropoeitic porphyria(120), and the value of the therapy is not accepted by all investigators.

THE COMPOSITION OF THE THESIS

This thesis is concerned with two aspects of the role of drugs in porphyria:

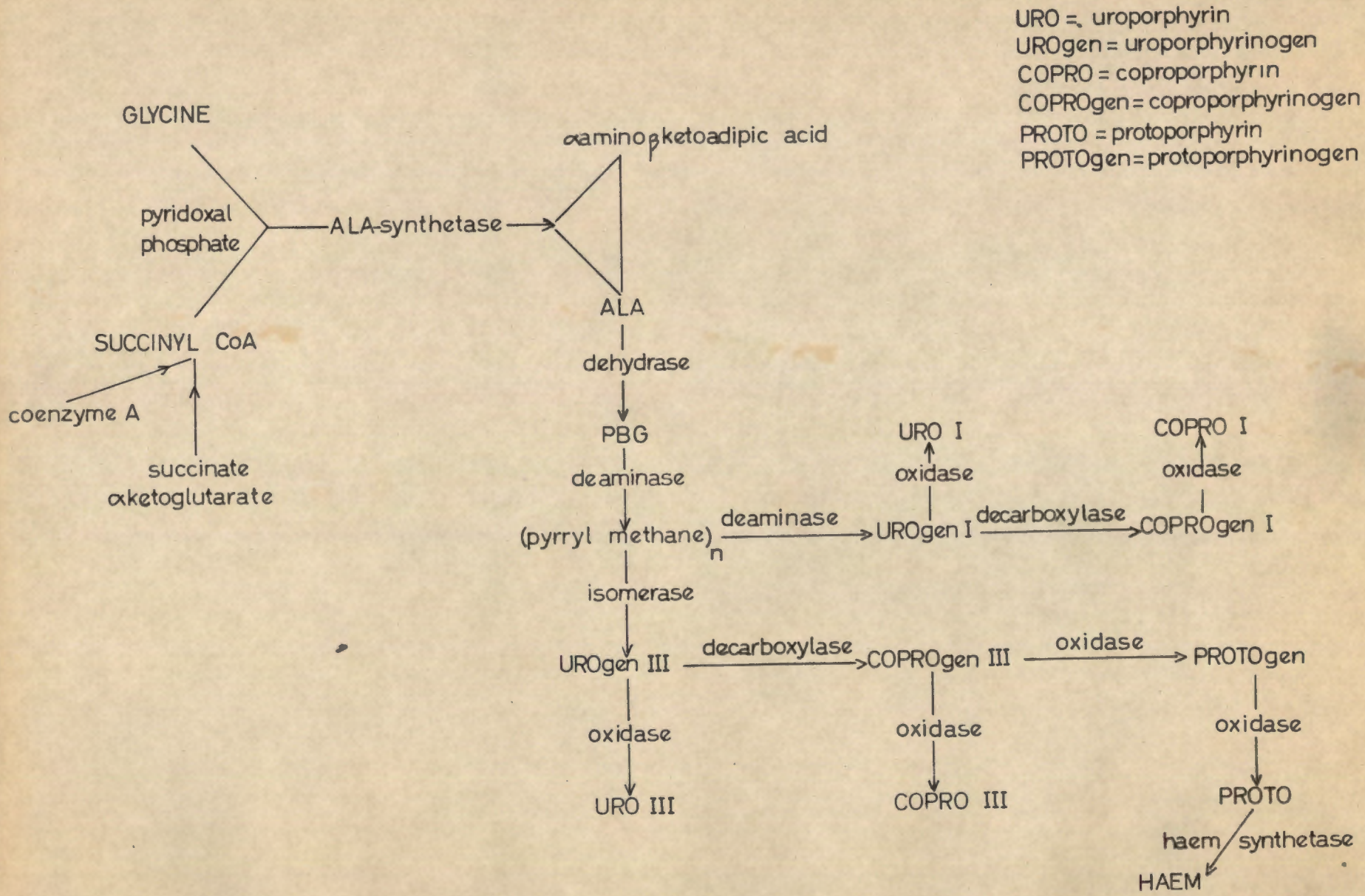
(i) The value of the tissue culture technique for detection of porphyrinogenic agents in vitro as a screening test for such substances which may exacerbate human porphyria, and

(ii) the testing of an hypothesis that drug-induced porphyria is mediated through an RNA mechanism.

Before details of the experimental design and results are given porphyrin biosynthesis and its control will be described in chapters II and IV, and modern concepts of protein biosynthesis will be briefly summarised in chapter III.

Fig: III

THE BIOSYNTHESIS OF PORPHYRIN.



CHAPTER II: THE BIOSYNTHESIS OF PORPHYRINS AND HAEM.

> "Porphyria is a genetically determined condition with a disturbance of pyrrole or porphyrin metabolism. It is a typical inborn error of metabolism."
> Waldenstrom - 1957.

A brief outline of the porphyrin biosynthetic pathway is presented as a background to the experiments reported in this thesis. (fig. III). Since the focal point of the discussion is the enzymic regulation of the synthesis of porphyrins, particular attention will be paid to the enzyme systems involved, especially to ALA-synthetase. Investigation has revealed that of all the enzymes in the biosynthesis tested thus far, only ALA-synthetase has been found to be rate-limiting.

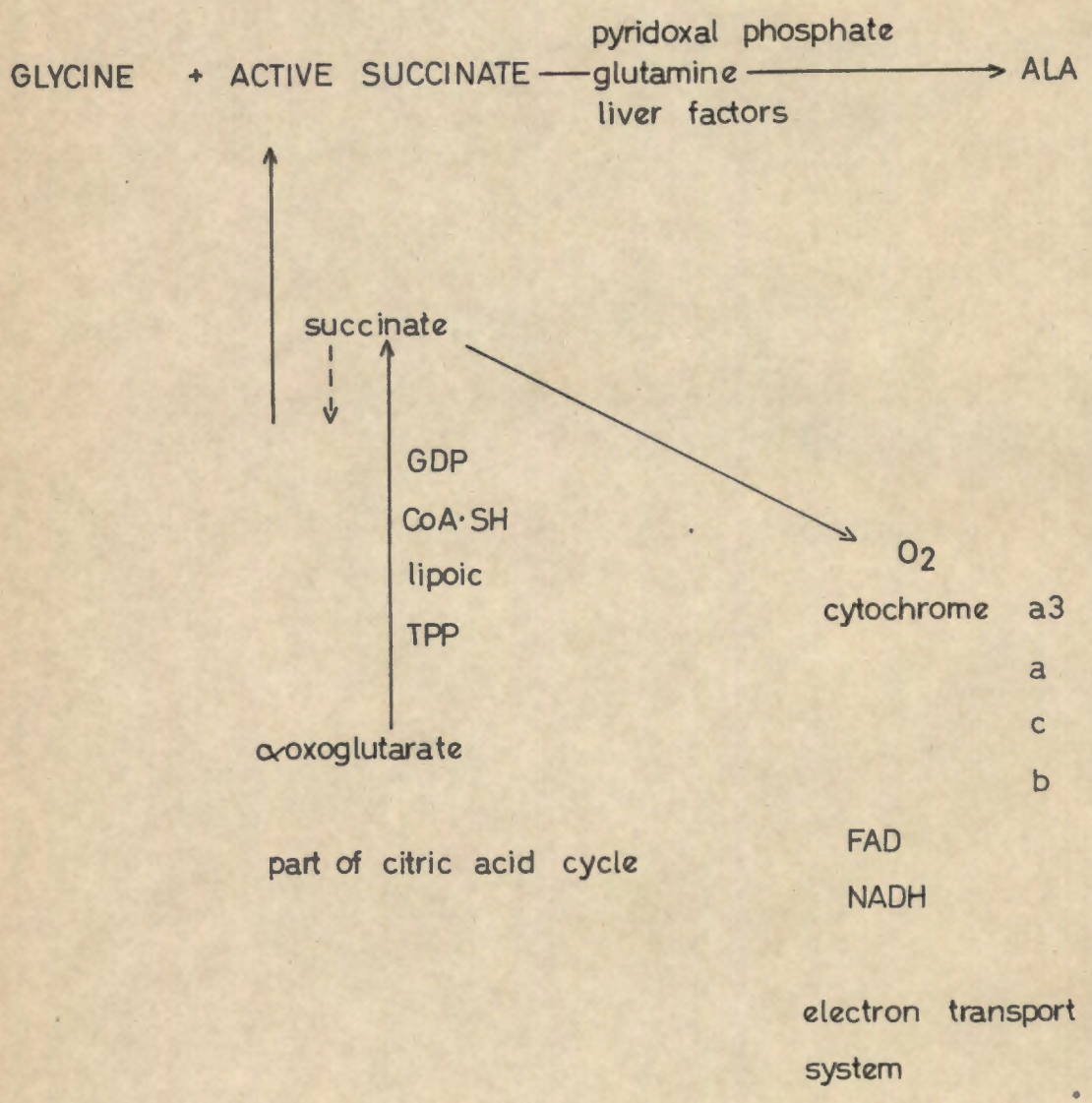
Numerous detailed reviews of the pathway have been published(121-129) which may be summarised as follows.

A RESUMÉ OF PORPHYRIN BIOSYNTHESIS.

Porphyrins, the precursors of haem, are derived from the enzymic condensation of 8 molecules of a non-essential amino-acid glycine, with succinate which has been activated by combination with acetyl co-enzyme-A (Co-A). Shemin, Rimington and Neuberger with various co-workers(130-138) have been major contributors to the understanding of this step with its resultant formation of αamino βketoadipic acid, and the elucidation of the succinate glycine cycle(118)(139).

Fig: IV

A SUMMARY OF THE PRINCIPAL FACTORS INVOLVED IN THE FIRST STAGE IN PORPHYRIN BIOSYNTHESIS.



ALA forms by spontaneous decarboxylation of the aminoketoadipic acid. The method of conversion of 2 molecules of ALA to PBG was elucidated after the determination of the structure of PBG, a pyrrole (53)(140). Through an ill-defined polypyrryl methane, 4 molecules of PBG undergo isomerisation to form UROgen III. The interaction of the 2 enzymes PBG deaminase (UROgen I synthetase)(141) and UROgen isomerase (UROgen III cosynthetase)(26) has not yet been fully elucidated. By consecutive decarboxylation of the acetic acid side chains, coproporphyrinogen (COPROgen) is formed, and final oxidation of the propionic acid groups to vinyl groups results in protoporphyrinogen (PROTOgen) Isomer IX of protoporphyrin incorporates iron to form haem.

THE ENZYME SYSTEMS INVOLVED.

The enzymes involved in the biosynthesis have been arbitrarily divided by Granick into 3 groups(142):

Group I: Enzymes of the citric acid cycle culminating in the condensation of glycine with succinyl CoA. (fig. IV)

Group II: The soluble enzymes converting ALA to COPROgen.

Group III: The enzymes concerned with the conversion of COPROgen to haem.

GROUP I:

ALA-synthetase is the catalyst of the reaction between succinyl CoA and glycine. The presence of this enzyme in animals was first

suggested by Laver(137) and by Shemin and Russell(118)(143). In vivo synthesis in man was proposed by Granick when ALA was recovered from the urine of a patient with acute intermittent porphyria(144). Direct measurement in animal livers has occurred following dicarbethoxy-dihydro-collidine (DDC) and allyl-isopropyl-acetamide (AIA) administration(145)(146)(147). Granick has devised a micro-method for the estimation of ALA-synthetase in the in the livers of porphyric chick embryos induced by AIA(91). Additional information has come from the work of Tschudy(61), and Dowdle(62), showing the presence in increased amounts of the enzyme in the livers of patients with various syndromes of porphyria.

The factors necessary for ALA-synthetase activity are:-

(a) glycine

(b) succinyl CoA

(c) cofactors

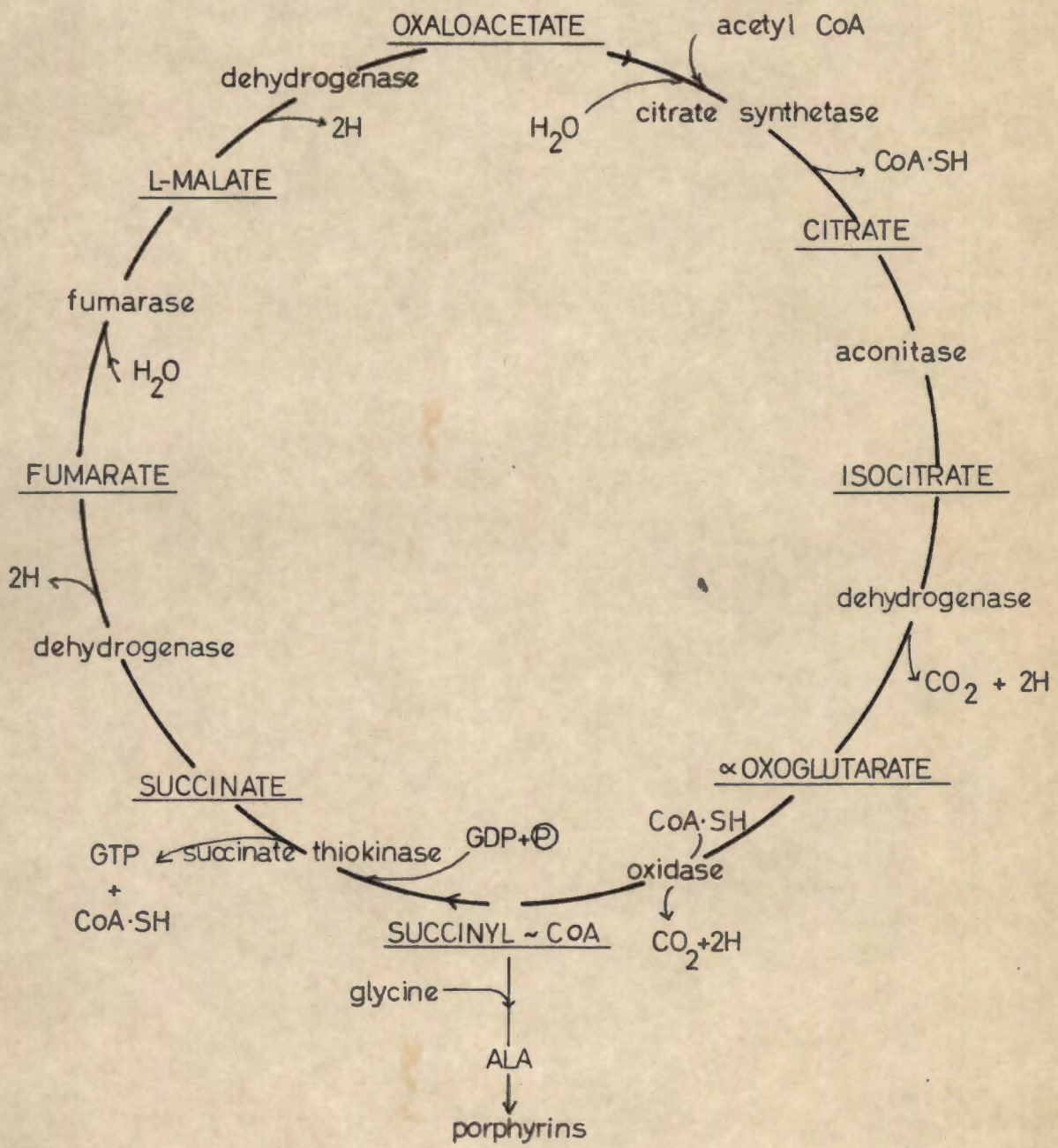
(d) other factors

(a) Glycine has been found essential for the formation of ALA. Various workers have found differing concentrations necessary(137)(145)(147)(148)(149)(150)(151), but no ALA synthesis occurs in its absence(90).

Radio-isotopic labelling of glycine has shown that all its nitrogen atoms(133), and 8 of the α(methyl) carbon atoms may be traced into the protoporphyrin molecule(53)(118)(130)(152). Labelled

Fig: V

THE CITRIC ACID CYCLE



carbon atoms appear also in the methyl groups of various compounds, the ureido group of purines, and the β carbon atom of serine, and into ketoglutaraldehyde(143). The remaining 26 carbon atoms of protoporphyrin are derived from acetic acid, especially from the methyl carbon(152)(153).

(b) Intermediates of the citric acid cycle (fig. V) exert variable influences depending on concentration. Citrate and d-isocitrate in particular give the highest yields of ALA and have been found to enhance protoporphyrin production(142). However, in high concentrations succinate, α ketoglutarate(137)(148) fumarate, oxaloacetate and malate inhibit ALA production by their action of suppressing oxidative decarboxylation of α ketoglutarate(145)(148).

While α ketoglutarate was initially thought to be the precursor of ALA, Shemin postulated that the succinyl-CoA complex, as an intermediate of the α ketoglutarate-succinate reaction, was the actual substrate. This concept was confirmed with the demonstration of the formation of succinyl CoA from α ketoglutarate in the presence of CoA and nicotine-adenine dinucleotide (NAD). It has also been shown that succinyl CoA may be formed directly from succinate, using differential labelling of succinate with inhibition by malonate. Greater ALA synthesis occurs if succinyl CoA is used as a substrate that if succinate, CoA or ATP alone are used(154).

In 1953 it was shown that ALA could replace succinate and glycine as a precursor of porphyrins(143). The formal condensation of glycine with succinic acid yielded ∝amino βketoadipic acid, which could undergo spontaneous decarboxylation to ALA(155). Thus the precursors of ALA appeared to be finally established.

(c) Cofactors

(i) The first and most important of these is pyridoxal phosphate, as shown by several lines of evidence. Blood from vitamin B deficient ducklings was found to incorporate glycine and succinate at a decreased rate(156). Incorporation was restored by the addition of pyridoxal phosphate, but other pyridoxal compounds were found ineffective. A direct influence has been noted on the porphyrin metabolism of chick haemolysates(138)(142)(148) and in mammalian systems(53)(156)(157)(158) diminished ALA synthesis resulted from decreased pyridoxal phosphate. In acute intermittent porphyria, the excretion of ALA and PBG decreased with the administration of a diet deficient in pyridoxal phosphate (157)(159).

(ii) Other B group vitamins have not shown this close association although in the bacterium Tetrahymena vorax diminished riboflavin concentration in the culture medium decreased porphyrin synthesis from glycine and ALA(160)(161), and deficient pantothenate, nicotinic acid, thiamine and thioctic acid decreased porphyrin synthesis from glycine. No effect was noted with altered folic acid concentration. The effect

of biotin in decreasing porphyrin synthesis(143)(168) is unrelated to ALA-synthetase activity(162), but possibly occurs due to diminished activity of pyridoxal phosphate in culture media.

Uroporphyrinogen is the first intermediate of the corrin ring of vitamin B12, but no relationship has been shown between this vitamin and porphyrin synthesis(163)(164)(165)(166). However, Rhodopseudomonas spheroides (Rps.sphs), a bacterium capable of porphyrin synthesis, has been found to be rich in vitamin B12(167).

(iii) The increased excretion of ascorbic acid in the urine of experimentally porphyric animals may be due to merely a non-specific effect of the vitamin on drug metabolising enzymes(167A).

(iv) In bacteria, adenosine-triphosphate (ATP) stimulates ALA synthesis, as it does in haemolysates of chick erythrocytes, using succinate as a substrate, but ATP inhibits ALA-synthesis when α keto-glutarate or isocitrate are substrates(148). With citrate as a substrate, addition of ATP to the medium results in increased ALA synthesis(90). However, in tissue culture of chick embryo liver cells, addition of ATP has been found to inhibit porphyrin production induced by AIA. Experiments performed in this connection will be described later (chapter VIII).

(v) Coenzyme A has been found to stimulate ALA synthesis in culture systems of chick erythrocytes(137)(145)(148)(154)(168).

(vi) NAD has only a small effect on ALA-synthetase(90)(138)(142)(149). However oxidative phosphorylation has been proved essential to

the enzyme by the inhibition of its action by dinitrophenol and the barbiturates. The effect of the latter will also be discussed later (chapter V).

(vii) The effect of magnesium is variable(58)(131)(138)(149)(154). Induction of succinyl CoA synthetase and accumulation of magnesium in mitochondria in experimental porphyria has recently been observed(169). However, magnesium appears to control the binding of "messenger" RNA (mRNA) to ribosomes and its absence results in disruption of this complex. This has only been shown so far in in vitro preparations, but is assumed to pertain in vivo also.

(viii) Ethylene-diamine-tetraacetic-acid (EDTA) has a pronounced stimulatory effect on ALA-synthetase, which is thought to be through the maintenance of structural integrity of the mitochondria(145). However, its use as a chelating agent has been advocated in the treatment of acute intermittent porphyria(170)(171).

(ix) Iron was initially thought to inhibit ALA-synthetase activity, thus providing a control mechanism for haem synthesis, by the formation of an iron-porphyrin complex(152)(172). Another mechanism has been suggested i.e. indirect enhancement by stabilisation of the Schiff base(66)(146) with a role in enzymatic transaminations which depend on pyridoxal phosphate as a cofactor(173).

(x) Ammonia enhances the formation of NAD which in frozen/thawed preparations at least, is essential for the conversion of α ketoglutarate to succinyl CoA(138)(160).

Other factors common to all enzyme regulation include:-

(i) permeability barriers which are thought to be the cause of diminished ALA production by preparations which have been frozen and thawed(138)(142)(145)(174).

(ii) oxygenation - although Rps.sphs needs anaerobic conditions in the light to produce porphyrins(175), in most experiments anaerobiosis is found to decrease protoporphyrin formation(150)(161).

(iii) buffers - of which the phosphate buffer is found best for experiments in vitro with ALA-synthetase; borate, barbitone or Tris have been found to diminish activity(137).

Chemically the reaction is described as the condensation of pyridoxal phosphate with glycine to form a Schiff base, resulting in a stabilised carbanion with the loss of a proton. Further condensation occurs with the acyl carbon atom of succinyl CoA acting as an electron acceptor. Decarboxylation occurs simultaneously or shortly afterwards. Enzyme activity is maximal at pH 6.9, and sulfhydryl groups have been found essential(176).

GROUP II:

ALA-dehydratase, the enzyme involved in the condensation of ALA to PBG requires sulfhydryl groups(177)(178)(179) and can be blocked by zinc, and other metals, and by EDTA.

Two steps are said to be involved in the conversion of PBG to UROgen. Deaminase condenses PBG to polypyrryl methane with the

elimination of NH_3, and an isomerase(26) acts through an unknown mechanism to form the isomer III sequence. Heat destroys this isomerase activity but PBGenase (deaminase)(141) function remains undiminished though the yield is of type I isomers.

Enzymatic conversion of UROgen to COPROgen requires sulfhydryl groups and either does not require a metal or binds it very tightly. The activity is influenced by the availability of the substrate, UROgen, which may be oxidised to uroporphyrin on which the enzyme has no effect. However, it does also decarboxylate isomer I. Oxidation of porphyrinogens to porphyrins is activated by light, and may be influenced by metals and glutathione.

GROUP III:

COPROgen decarboxylase converts COPROgen III to PROTOgen, but has no effect on COPROgen I, which may explain the specificity of isomer III conversion to protoporphyrin in nature. Finally, with the incorporation of ferrous iron by ferrochelatase (haem synthetase), haem is formed.

CHAPTER III. THE ORGANIZATION FOR PROTEIN BIOSYNTHESIS

> "Protein synthesis is the cardinal manifestation of life, and yet the manner in which it is achieved is still the great problem of biochemistry and of biology" - Felix Haurowitz in Chemistry and Biology of Proteins, 1950.

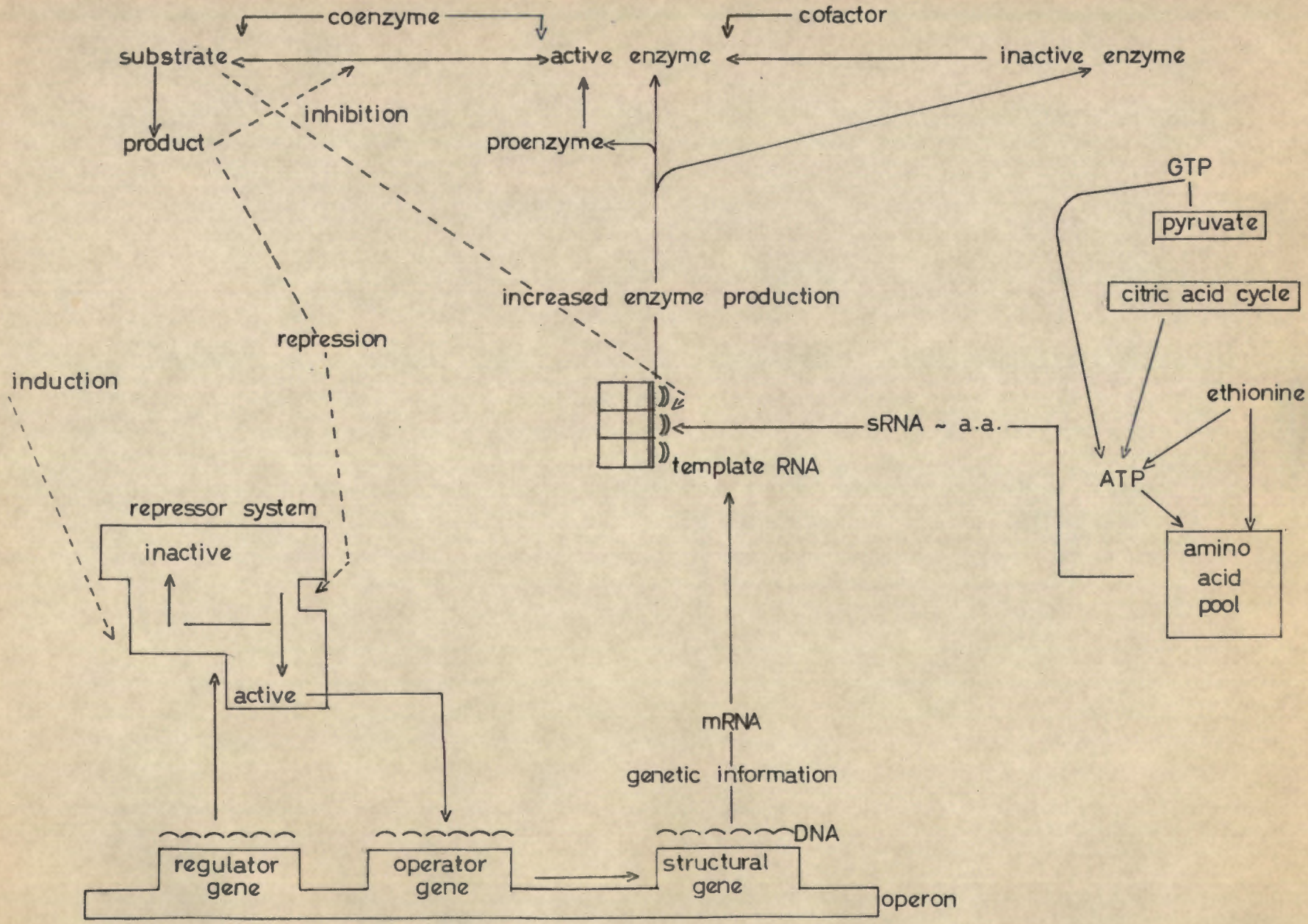
Inasmuch as porphyrin production results directly from the activity of the enzyme, ALA-synthetase, a brief description is given of the current concepts of protein, and therefore, enzyme synthesis.

The primary polypeptide structure of proteins first proposed by Emil Fischer(180) has been confirmed by Sanger(181) with the establishment of the amino acid sequence of insulin, and by du Vigneaud(182) with the synthesis of the hormones of the posterior pituitary. The studies of Pauling and Corey(183) led to the concept of a secondary structure of protein molecules in the form of a helix. A similar structure has been shown for the nucleic acids, leading Watson and Crick(184) to propose the double-stranded DNA molecule, and Kornberg(185) to its enzymatic synthesis. The concept of the transcription of the message encoded in a deoxyribonucleic acid (DNA) molecule to ribonucleic acid (RNA) and its further translation into an amino acid sequence with a resultant protein

Consequent upon this knowledge has come the postulate for control of enzyme induction which may be applied to all systems (186-193).

Fig: VI

FACTORS INVOLVED IN THE REGULATION OF ENZYME ACTIVITY AND SYNTHESIS (194)



THE FACTORS KNOWN TO BE INVOLVED IN THE REGULATION OF ENZYME ACTIVITY AND SYNTHESIS

Currently, the factors known to be involved in regulation of enzyme activity and synthesis(194) are as shown in fig.VI. These will be discussed in succession.

(i) Controllers of enzyme regulation

Chemical analysis has resulted in the evolution of long-established controllers of enzyme regulation - these being substrate and enzyme concentration, which determine the rate of the reaction, and pH, temperature and the presence of inhibitors which combine to mediate activity.

(ii) Factors influencing the size of the enzyme population

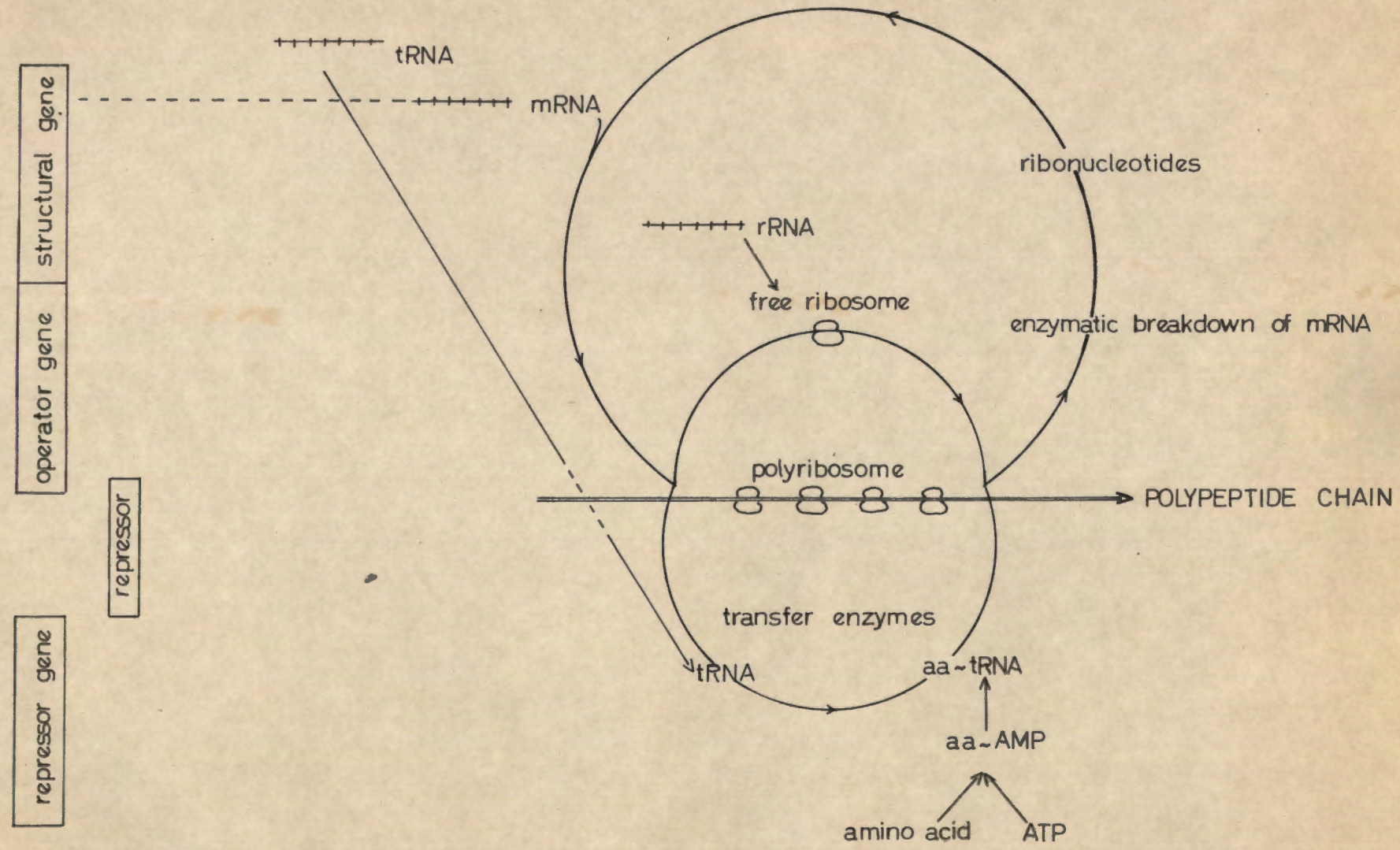
The use of the term "enzyme population" focuses attention on the dynamic aspects of the maintenance of a number of enzyme molecules by a continuous process of renewal and destruction. It emphasizes the heterogeneity in the stages of synthesis and breakdown, and underlines the possibility that certain enzyme molecules may be deficient, malformed or inactive(195). It also implies the concept of isozymes(196).

The synthesis and maintenance of an enzyme population depends on - (i) the presence of an intact enzyme forming system comprising genetic directives from DNA to messenger RNA,

(ii) the existence of a functioning template RNA on available ribosomal sites,

Fig: VII

A MODEL FOR PROTEIN SYNTHESIS



(iii) the presence of amino acid complexes with soluble RNA,

(iv) a balanced amino acid pool and

(v) the availability of energy from glycolysis and/or respiration.

(iii) The activity of enzymes

The activity of enzymes(197) is closely controlled by the presence of co-enzymes and the pyridine nucleotides NAD and NADP; flavin derivatives FAD and FMN; quinones, cytochromes, thiamine pyrrophosphate, lipoic acid, biotin, tetrahydrofolic acid, and, of particular note to this discussion, pyridoxal phosphate, CoA and the nucleotide phosphates.

PROTEIN PRODUCTION VIA RNA FROM DNA

The dexcription of protein production via RNA from DNA has become the subject of numerous reviews and investigations, not all of which have been confirmed. The hypotheses which guide current experiments on protein synthesis are the Watson-Crick model of DNA(184) the discovery of the enzymatic mechanism for DNA replication(185) and the elucidation of the DNA-dependent enzymatic synthesis of RNA(189)(191).

Protein biosynthesis commences from two points which combine after various reactions, and thereafter interreact(198)(204)(fig.VII).

(i) The first point of origin is the DNA helix, one strand of which is thought to form the template for RNA, and the other the template for renewed DNA synthesis. The concept of base-pairing as first shown by Chargaff(205) with the consistent pairing of the bases adenine with

uracil (or thymine in RNA) or guanine with cytosine, suggested the mechanism for RNA replication.

The existence of a fraction with the properties of a template RNA is suggested by many observations, at first only in bacteria(198), but now also in mammalian systems(206)(210). As yet not all conclusions are reliable. However at present, current theory embraces the existence of such a molecule, known as "messenger" RNA (mRNA), which is thought to traverse the cytoplasm from nucleus to ribosome bearing the encoded message for amino acid sequence(210A).

(ii) The activity of this mRNA in the ribosome is dependent on the arrival from the second point of origin, of activated amino acids. These are originally free amino acids, activated by specific enzymes to combine with ATP(211) and yield enzyme bound amino acid-adenylates, which become attached to a low molecular weight soluble or transfer RNA (tRNA) also known as the acceptor or adaptor(212)(213)(214). Binding occurs by an ester bond involving the 2'3'-hydroxyl group of the terminal adenosine moeity with the AAC-ending of all transfer RNA's(197)(200)(215). The nucleotide sequence of the adaptor is specific for a particular amino acid(215)(216), thus recognition of the specific amino acid activating enzyme occurs and of the site characteristic of the amino acid on the RNA template. The kinetic relationship between the ribosome and the messenger RNA is uncertain(200) - that is, whether the polysomes move along the RNA or whether messenger

RNA moves through the polysome(217). However, it is known that polysomes, when extracted in a medium deficient in magnesium, break up into constituent ribosomes. The connecting material is thought to be mRNA which requires the stabilising presence of magnesium(218)(219)(220).

The code for amino acid sequence is read in the ribosome and the correct amino acid selected and positioned. As the peptide bonds form, the transfer RNA and activating enzymes are released to further amino acid conscription, and on completion of the translation of the message, the assembled protein is shed, and mRNA returns to the circulation for further coding(200). The re-utilisation of mRNA is suggested by the minute proportion (about 2%) of this fraction relative to others(200).

THE GENETIC CODE

The genetic code is believed to be comma-less, and is read from start to a fixed stop(215)(217). The degeneracy of the code(215)(221), that is, the fact that more than one triplet sequence codes for each amino acid, has provoked great interest in the language, and recently each of the 64 possible triplets (if combinations of nucleotide bases are made), has been assigned a purpose(222)(223)(224)(225). Most code for amino acids, but as yet the reason for several codes for certain amino acids has not been determined. Two of the triplets (UAA (amber) and UAG (ochre)) are believed to have no significance with

regard to determining a sequence, but may provide the "punctuation" and prevent further reading of the code(223)(226). Interference with triplet sequences results in the formation of nonsense triplets which prevent adequate protein synthesis. Such interference is the property of many antibiotics(227)(228)(229).

INHIBITORS

Much of the information concerning all fractions of RNA has been derived from experiments in the use of inhibitors(230). The mechanisms involved are of competitive and non-competitive, uncompetitive and mixed inhibition(231). The number of recognised inhibitors of nucleic acids is increasing. The majority of studies of the effects of inhibitors on DNA or RNA involve either the effect of antibiotics on bacteria, or various anti-cancer agents on tumour cells in tissue culture. Inhibitors may be classified according to their site of action in the sequence of protein synthesis.

(i) Actinomycin D, although known to have anti-metabolic activity is under suspicion at present as regards its precise site of action. The original suggestion was that of formation of a complex with DNA, preventing transcription to RNA(232-236). The property involves absolute specificity for guanine and especially for its amino group(237).

(ii) The mitomycin group of antibiotics, prepared from Streptomycetes, is thought to cause depolymerisation by forming covalent cross-linkages of the strands of DNA, attaching to guanine or cytosine

residues or both(238)(239). Phleomycine, the structure of which is unknown, is also thought to inhibit transcription by preferential binding to the adenine or thymine bases.

(iii) Puromycin and acetoxycycloheximide inhibit at the ribosomal level where the polypeptide chain is assembled(215)(240)(241)(242)(243). The former is thought to act by replacing the entrant amino acid at the carboxyl end of the peptide chain(241). The antibiotics chloramphenicol(215)(242) and chlor-(228) and oxytetracycline(215) also inhibit the incorporation of amino acids into protein, although the former has not been found to have this effect in mammalian systems.

(iv) Competitive inhibition is the basis of action of homocitrullyladenosine(245) and of 5-fluorouracil(215)(246).

(v) Ethionine, although neither an antibiotic nor anti-tumour agent, has especial relevance to this discussion because of its triple properties of diminution of hepatic ATP and blood glucose, and of its induction in rats, of increased porphyrin synthesis. Its effect on protein synthesis is not clear, although it is known to form the compound s-adenosyl-ethionine in place of s-adenosyl methionine(247) and may thereby interfere with trans-methylation reactions. Also, s-adenosyl-ethionine is known to obstruct oxidative phosphorylation in mitochondrial preparations(248). It appears to block the long-lived protein secreting cells while DNA synthesis continues in the

shorter-lived proliferating cells which do not secrete protein(249)(250). This may be associated with differences in the effect of ethionine on RNA synthesis in the various cell types, or to differences in cellular ability to convert ethionine to s-adenosyl-ethionine. More recently it has been found that the inhibition of RNA synthesis, in female rats fed ethionine follows in time, the decreased ATP concentration, and precedes the inhibition in protein synthesis(247).

(vi) The nuclear protein histone, is reported to inhibit both RNA synthesis and nuclear function(251)(252). This action of hormones is thought to be that of combination with histone, resulting in de-repression of the operon and uncontrolled mRNA production.

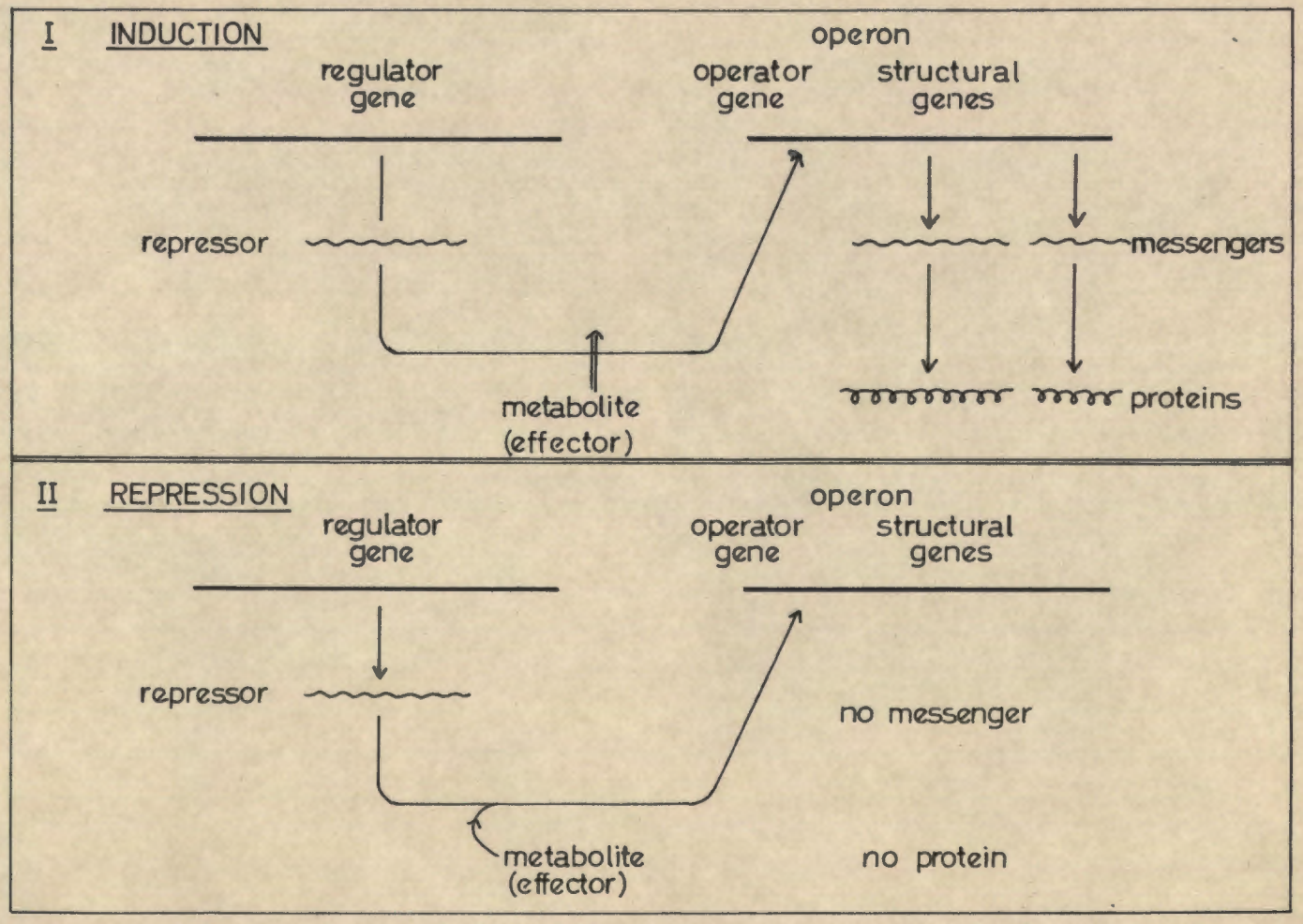
STIMULATORS

The stimulatory effect of many hormones on nucleic acid synthesis(253) forms a separate category. Protein synthesis is diminished in hypophysectomized rats(254) and growth hormone(255) is found to increase the incorporation of amino acids both in these rats and in controls, and can restore the amino acid incorporating activities in animals injected with Actinomycin D.

The mechanism of stimulation by cortisol may be through increased DNA(256) or by action as an effector(257). Thyroxine accelerates the transfer of amino acids from amino acid-RNA to rat liver microsomal and ribosomal preparations(258)(259). The stimulatory effect of oestrogen on protein synthesis in rat uterus preparations has recently

Fig. VIII

MODELS OF THE REGULATION OF PROTEIN SYNTHESIS(198)



been studied(260)(261)(262). The mechanism may be interaction with a component of the cell with the ability to control protein synthesis, or less likely, may directly activate protein synthesizing systems(261).

THE REGULATION OF GENETIC EXPRESSION

A model for the regulation of genetic expression was proposed in the Nobel Prize-winning studies of Jacob and Monod(198)(fig.VIII). This envisages various different types of gene, endowed with specific activities in the control mechanism. The model consists of four fundamental characters(176)(263).

(i) The structural gene(264) is thought to be the DNA segment of the chromosome which gives rise to messenger RNA.

(ii) The operon is a segment of DNA consisting of an operator gene, and one or more adjacent regulator genes and permease genes, and is responsible for the initiation of messenger RNA synthesis.

(iii) The regulator gene is the DNA segment responsible for the production of repressor to block a specific operator.

(iv) The effector combines reversibly with repressor, to prevent inhibition of the operator gene. Effector may also however, combine with aporepressor to form repressor. Recent conclusive evidence of the existence of a repressor has been presented(265).

In this model, the postulated final production of mRNA is the result of a succession of stimuli. The incentive for induction is received by the regulator gene which prevents any active repressor

from entering the repressor system. The condition of the inactive repressor is noted by the operator gene, which, by its close proximity to the structural gene, initiates the transcription to mRNA. In the case of repression, an active repressor is formed and no mRNA results.

This modern concept of protein, and,therefore,enzyme synthesis, forms the basis of many of the experiments reported in this thesis.

CHAPTER IV THE CONTROL OF PORPHYRIN BIOSYNTHESIS

> "...the disturbance in porphyrin metabolism in this disease cannot be located in a given organ or a given cellular system, but must be explained by modification of enzyme systems which are very widely distributed in the whole organism."
>
> Gajdos - 1955.

> "The mechanism of control of RNA synthesis and function are a basic part of the overall control of the mammalian cell."
>
> Penman - 1967.

The problem of the regulation of porphyrin biosynthesis(25)(59)(266-270) is of especial interest in the elucidation of the various syndromes of porphyria. A hypothesis has been developed by Granick(91) for the control of porphyrin biosynthesis, and the discussion in this chapter is based on the data reviewed in chapters II and II, and upon this hypothesis.

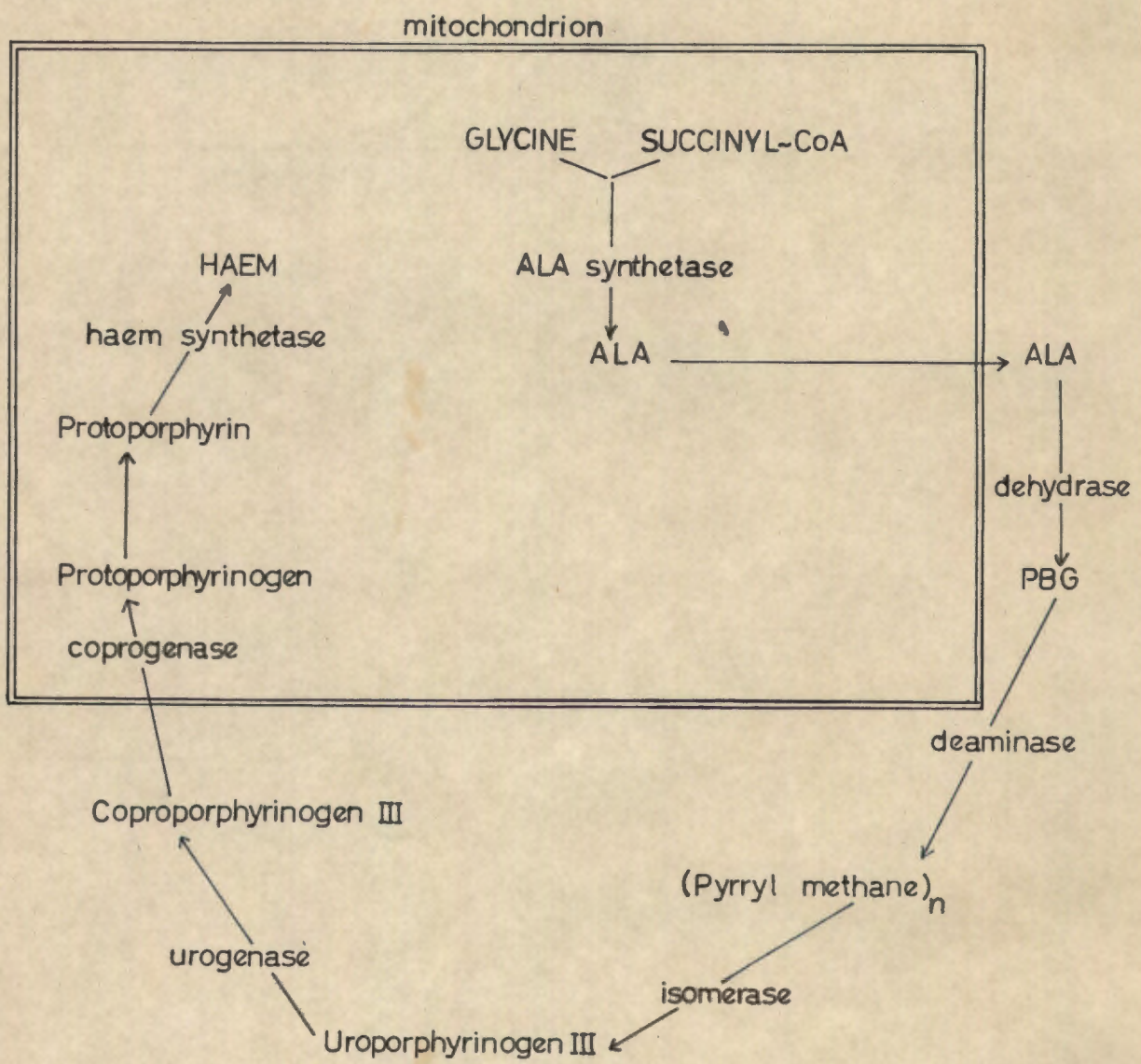
THE REGULATION OF ALA SYNTHETASE ACTIVITY

(i) Negative feedback inhibition

The concept of negative feedback inhibition of the action of enzymes of the pathway of porphyrin biosynthesis has been elaborated mainly in experiments with Rps. sphs(127)(172)(271). It has been found that haem, either as haem-protein or haem-protoporphyrin-metal complexes inhibit ALA formation(127)(172)(266)(270)(272). The inhibition of ALA dehydrase(162) by protoporphyrin has also been reported, and Kikuchi(58) in the same system has described the presence but not the nature of an inhibitor of ALA synthetase.

Fig: IX

THE INTRACELLULAR LOCATION OF PORPHYRIN BIOSYNTHESIS

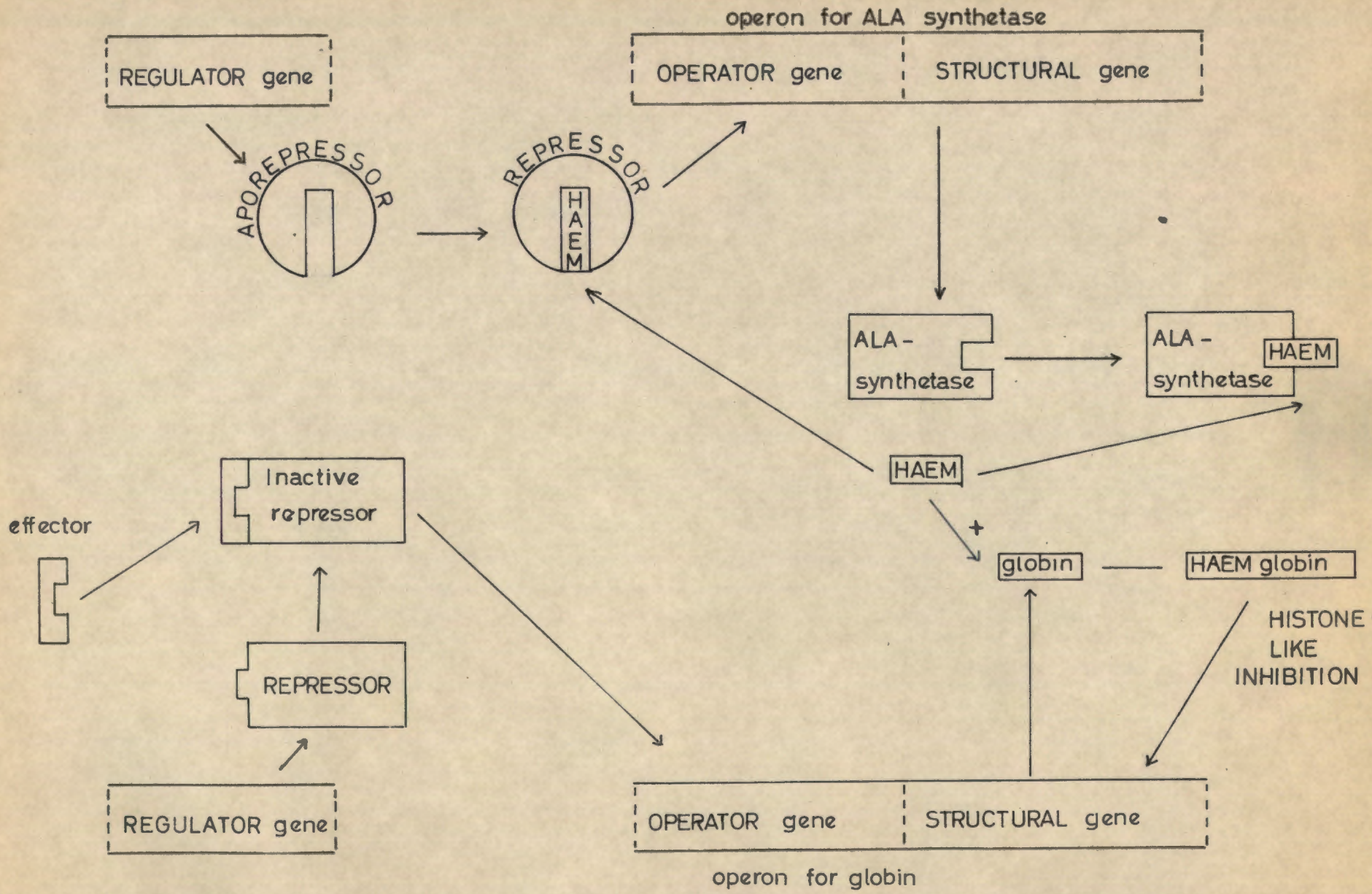


(ii) Repression

The postulate that instead of inhibition, the action of haem is one of repression of enzyme induction has received attention in recent years. Both this theory and that of end-product inhibition gained importance when the intracellular location of the enzymes of the pathway was determined(273)(fig.IX). Granick(145) correlated the occurrence in mitochondria both of electron transport and oxidative phosphorylating systems, with the citric acid cycle enzymes necessary for ALA synthesis. The siting of ALA synthetase in the mitochondrion was proved in guinea pigs fed DDC(145) and in rats fed AIA(61), HCB(274) and DDC. The position of both haem and ALA-synthetase in the mitochondrion provided support for the role of haem in inhibition or repression of ALA-synthetase. In addition, haem alters mitochondria - both they and the cristae enlarge(175). It is not known whether this change in the mitochondrion is due to increased ALA-synthetase, and it would be of interest if the synthetase was found on the cristae. If so, this would account for the difficulty with which ALA-synthetase can be washed from mitochondrial preparations into the surrounding medium(175). The proposal of this localisation of ALA-synthetase on the cristae would require that succinyl CoA be generated by ketoglutarate oxidase on the cristae, and close juxtaposition of succinyl CoA synthetase and ALA synthetase on the cristae is likely in view of the active deacylases which have been found in mitochondrial fractions(175).

Fig: X

THE CONTROL OF HAEM BIOSYNTHESIS



(iii) The control of ALA-synthetase and haem synthesis

A current theory proposed by Granick(91) and others(275) for the control of ALA-synthetase and haem synthesis is shown diagrammatically in fig. X. The importance of ALA-synthetase in porphyrin synthesis depends on the fact that it has been shown to be both rate-limiting(58)(61)(137)(138)(142)(145)(172)(276) and inducible(145)(146)(274)(277), characteristics which have not, as yet, been applied to any other enzyme in the biosynthesis of porphyrins.

The scheme as described makes several assumptions which are as yet merely hypotheses(176). These are:-

(i) that globin synthesis may be "turned-on" independent of haem synthesis, but

(ii) because of globin synthesis, the haem synthesis rate increases. However

(iii) no globin polypeptide is completed in the absence of haem.

These assumptions attempt to account for the 1:1 correspondence between haem and globin in a haemoglobin monomer.

With these hypotheses in mind, the control of haemoglobin synthesis may be described as follows:

Prior to the initiation of haemoglobin synthesis, the operon responsible for globin synthesis is activated but repressed by the regulator gene. This also applies to the operon for ALA-synthetase. It assumes that the aporepressor produced by the regulator gene

requires haem for effectively repressing the operon for ALA-synthetase. The entry of the effector substance renders inactive the repressor of the globin operon and globin synthesis begins. Erythropoeitin or metabolites from neighbouring cells may act as globin effectors, the former having been shown to stimulate haem synthesis in the culture of bone marrow cells. Globin utilizes free haem, and deprives the repressor of ALA-synthetase of its effect, thus allowing the synthesis of ALA-synthetase. The selection of free haem as the aporepressor for the ALA-synthetase operon obviates the possibility of over-production of haem, although even if ALA-synthetase formation ceases, haem continues to form from pre-formed ALA. An additional regulatory mechanism is provided by the histone-like activity upon DNA of haemoglobin in normoblast nuclei.

Using the above hypothesis, Granick(91) has suggested the presence of 3 operons for haem regulation - one situated in each of liver and erythroid cells, and one in other cells. He postulates defects in one or all of these to explain the various syndromes of porphyria.

THE REGULATION OF THE SUBSTRATES OF ALA-SYNTHETASE

(i) Glycine

Some amino acids other than glycine have been shown to decrease the synthesis of porphyrin from glycine(142) while a diminished concentration of such amino acids has resulted in increased ALA-synthesis(174). The activity of these amino acids is L-cysteine > L-serine > L-arginine>

L-proline > L-alanine. Addition to chick haemolysates decreases the production of ALA with glycine as substrate but has no effect upon PBG production with ALA as substrate.

It has also been noted that old cultures of Rps. sphs. produce more porphyrin, possibly consequent on the diminished amino-acid concentration in the culture medium(160).

(i-a) Pyridoxal phosphate

Pyridoxal phosphate, being a ubiquitous co-enzyme, is often involved in competitive inhibition. The substances L-penicillamine (137)(138)(145), cysteine(142) cyanide and isonicotinic hydrazide (145)(162) react with the aldehyde group of pyridoxal phosphate and prevent the formation of a Schiff's base.

(ii) Citric acid cycle components

The role of citric acid components has been the interest of Labbe et al.(278)(279) and others who envisage this to be the point of control of haem biosynthesis(280). The inhibitory action of pyruvate may be competition with succinate to form aminoacetone, or with ∝ketoglutarate for coenzymes(142). Normal liver preferentially converts succinyl CoA and glycine to amino-acetone(145)(281) and the concept developed that the rate of condensation of glycine with acetyl CoA could regulate the amount of succinyl CoA available for haem synthesis(282). This was not supported however, when it was shown that elevated levels of ALA and aminoacetone could occur simultaneously (142) and that patients excreting large amounts of ALA and PBG also excreted aminoacetone(283).

Succinyl CoA may be formed in a number of ways(284):

(i) as mentioned, via the citric acid cycle from ∝ketoglutarate;

(ii) directly from succinate and CoA catalyzed by a nucleoside triphosphate;

(iii) from acetoacetyl CoA by the action of a CoA transferase, and

(iv) especially in animals, from propionyl CoA in combination with CO2 and a carboxylating, biotin-containing enzyme, resulting in methylmalonyl CoA which is converted to succinyl CoA in the presence of a vitamin B12 containing enzyme.

The first-mentioned route is that most likely to be affected by events of intermediary metabolism, and has been closely studied by Rimington(284). He showed the involvement of the lipothiamide system which also implicates availability of oxygen. This has been confirmed by the depressant effects on porphyrin production of elevated oxygen tension although in vitro effects do not necessarily mirror the intracellular state.

Further proof of the importance of oxygen tension on the biosynthesis has come from the observation of the effects of many drugs - firstly those applied directly for their suppressive effect on oxidative phosphorylation, namely 2,4,dinitrophenol(145)(149)(150) iodoacetamide(137)(138) and p-chloromercuribenzoate which inhibit porphyrin synthesis in vitro; and secondly the sedative group of drugs(285-288) whose action is through the mechanism of uncoupling

oxidative phosphorylation(98)(289) but which in vivo and in vitro increase porphyrin synthesis. The presence of an additional effect by the drugs is suggested. Seconal has been found to inhibit NADH2-oxidase and the incorporation of succinate into haem. Acetoacetate acts as an oxidant of NADH2 as catalysed by β OH-butyrate dehydrogenase, and reverses the inhibition by Seconal. It may also reverse the incorporation of succinate inhibited by malonate. This may be the reason for the inhibition by Seconal of porphyrin and haem synthesis in homogenates, but for the increase of synthesis in vivo, a different behaviour of the compartmented cell is suggested.

All the enzymes beyond ALA require reduced sulfhydryl groups for their action, and are inhibited by heavy metals. Reducing compounds are necessary to prevent the irreversible oxidation of porphyrinogens to porphyrins. Modification of the cellular "reduction potential" may control whether porphyrinogen is converted to haem or to porphyrin.

The simultaneous increase of haem and fatty acid biosynthesis in experimental porphyria has been explained by the block by malonyl CoA of the conversion of succinyl CoA to succinate. Succinyl CoA is directed to the haem pathway. This possible controlling role of malonate has been confirmed by demonstration of the inhibition of the incorporation of labelled haem by malonate(278).

Fumarate reductase and succinyl CO synthetase have recently been postulated to be inducible. An increase in succinyl CoA was

noted in animals fed AIA with associated increase in the reduction of fumarate to succinate; de novo formation of succinyl-Co-synthetase and accumulation of magnesium. All these observations precede a chemically detectable increase in tetrapyrrole formation(290).

ANOTHER PATHWAY OF ALA METABOLISM

The further breakdown of ALA to purines has been proposed as a controlling mechanism of porphyrin synthesis(291). Labbe et al (292)(293)(294)(295) have noted decreased uric acid concentration in the allantoin of chick embryos in which porphyria has been induced with Sedormid, but no interference with purine oxidation or excretion. They postulate inhibition of purine synthesis with a resulting accumulation of ALA leading to increased porphyrin production. However, it was found later by de Matteis et al(296) that there was no evidence of impaired purine metabolism in porphyric rats, nor was there any significant change in the levels of nucleic acid or total adenine purine nucleotide concentrations.

CHAPTER V. THE INDUCTION OF PORPHYRIN SYNTHESIS IN TISSUE CULTURE USING DRUGS AND CHEMICALS

"It was generally believed that for every ill that flesh is heir to, nature has designated some plant as the appropriate cure".
L.C. Miall - The Early Naturalists.

CONFIRMATION OF TISSUE CULTURE METHOD

(i) Materials

Experiments were conducted on a tissue culture system prepared from chick embryo liver cells.

(a) Eggs from white Leghorn fowls were obtained from the Silversands Poultry Farm, and kept in the incubator of the Department of Bacteriology, U.C.T. Medical School until their approximate age was 16-18 days. They were then transferred to the laboratories where the tissue cultures were set up under sterile conditions. Taking care to avoid incising the gall bladder, the livers were removed and washed in a balanced salt solution (Hanks). They were dried of excess fluid, weighed, and then transferred to Petri dishes in which they were finely diced to a size of about 0.5 mm. using two scalpels. After the addition of the culture medium (Eagle's), the cultures were placed in an incubator at 37°C under constant humidity and in an atmosphere of 5% CO2 in air. The incubation period after addition of drugs to the medium was 60 hours, and after addition of RNA was 16 hours.

(b) Hanks solution was prepared according to Paul(297) by the addition to distilled water of:-

NaCl.-8G/L; KCl.-400mg/L; CaCl2.-140mg/L; MgSO4.7H2O.-100mg/L; MgCl2.6H2O.-100mg/L; Na2HPO4.2H2O.-60mg/L; KH2PO4.-60mg/L.

The solution was either passed through a scintered glass filter, or sterilized in the autoclave of the Department of Bacteriology at a pressure of 10 lb./sq. inch for 10 minutes. Lactalbumin hydrolysate (DIFCO) 5% in distilled water, glucose-2G/L and phenol red-20mg/L were added and the pH adjusted to 7.2 with 1 ml. 5% sodium bicarbonate.

(c) Eagle's tissue culture medium was also prepared according to Paul but the recommended amounts of amino acids and vitamins were quadrupled(298).

All amino acids were in the L-form if available and were obtained with the vitamins from British Drug Houses. The concentrations in mg/1000 ml. before quadrupling were:-

Arginine-105;cystine-24;histidine-31;isoleucine-52;leucine-52; lysine-58;methionine-15;phenylalanine-32;threonine-48;valine-46; glutamine-292;tryptophan-10;tyrosine-36.

Choline-1;nicotinic acid-1;pantothenic acid-1;pyridoxine-1; riboflavine-0.1;thiamine-1;i-inositol-2;folic acid-1.

Stock solution 2 was prepared by addition of arginine, histidine, lysine, leucine, isoleucine, methionine, phenylalanine, threonine and valine to 100 ml. Hanks and heating to 80°C.

Stock solution 3 consisted of tyrosine and cystine dissolved in approximately 50 ml. 0.1 N HCl, but complete solution is never obtained.

Stock solution 4 consisted of all the vitamins with the exception of folic acid and glutamine dissolved in 200 ml. Hanks.

The solution of folic acid (stock 5) in 100 ml. Hanks was achieved by the addition of a few drops of 0.5N NaOH until the pH is neutral.

Stock 6 was prepared by the addition of 200,000U penicillin and 0.5G streptomycin dissolved in 100 ml. distilled water.

Stock 7 consists of glutamine dissolved in 100 ml. Hanks and kept frozen.

Preparation of 100 ml. of the final medium consisted of 84 ml. Hanks, 1 ml. each of stock solutions 2, 3, 5 and 7, and 0.5 ml. stock 4. After filtering through a scintered glass filter, 10 ml. calf serum was added. This was prepared at the Department of Bacteriology from blood obtained from the Municipal abbatoirs, by Miss S. von Olm, and was kept frozen in 15 ml. aliquots.

Both tissue culture medium and Hanks solution were kept in the refrigerator.

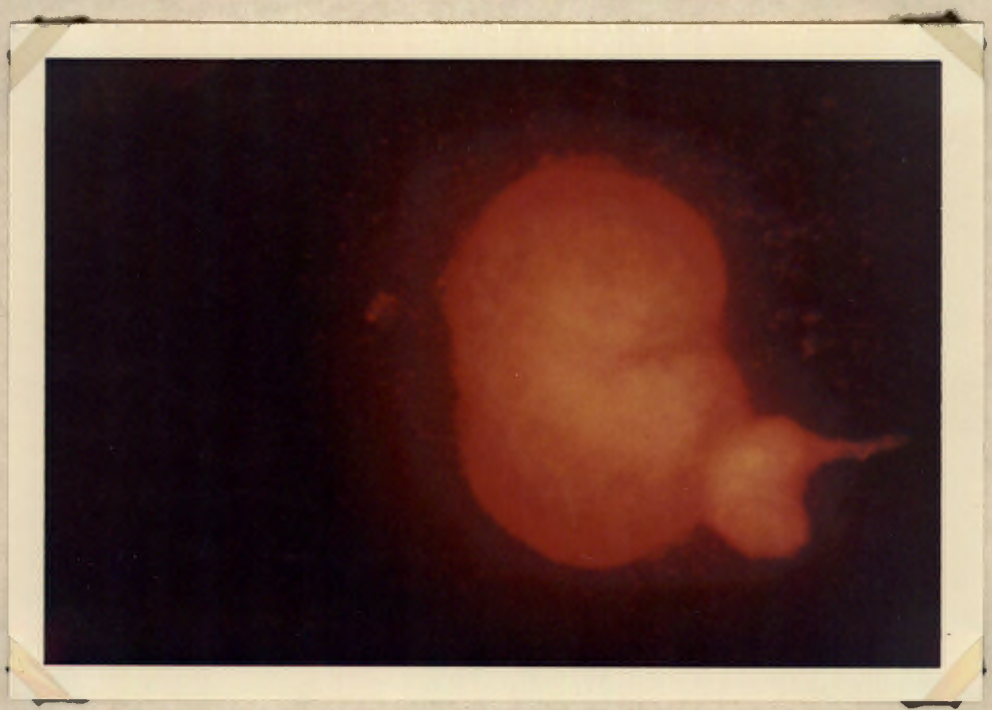
(ii) Method

Initially the method and integrity of tissue culture was confirmed using known inducers. The method was adapted from that

Fig. XI

A normal explant photographed in ultra-violet light.

Fig XII



A porphyric explant photographed in ultra-violet light.

described by Granick(91), although trypsinization of cells was not performed. Approximately 20 explants were placed in a small Petri dish using a Pasteur pipette, and after allowing a few minutes for adherence, were covered with 1 ml. of medium. The diameter of a maximum of 0.5 mm. was chosen since the viability and oxygenation of such explants is reported to be 70%. Increased concentrations of the constituents of Eagle's medium were used to enhance this.

(iii) Necessity for control cultures

Because of the variability of porphyrin production by individual livers, a control culture was prepared from a common pool for every experiment. The stimulation of fluorescence by the addition of ALA was used as confirmation that enzyme systems were still intact, if it was found that fluorescence was not induced by a particular drug.

(iv) Estimation of porphyrin fluorescence

The porphyrin production could be estimated subjectively by scanning the explants under a fluorescence microscope. A low power of 25x magnification is adequate, and fluorescence is exhibited best by using an exciting filter of wavelength 3500-4500 Å, and a barrier filter transmitting above 5200 Å. In this case, using a Zeiss Ultraphot fluorescence microscope, the exciting filter was BG 12, and the barrier filter Zeiss "50". Although the red fluorescence is a distinct occurrence (figs XI,XII) it was not felt that great significance could be attached to the allocation of pluses to indicate

degrees of intensity of fluorescence in explants, as used by Granick with hepatocyte monolayers. Instead it was preferred to reserve this technique for the screening of a number of drugs or to determine the time required for visible fluorescence to occur, as in the case of experiments to be described later, using RNA.

(v) Quantitation

(a) Porphyrin extraction.

For quantitation, the more accurate technique of extraction of porphyrins and quantitation using scanning of thin layer chromatographs was preferred. For this method, more livers were used and were divided amongst the culture dishes (usually 4). Each dish contained between 0.5 and 1.0 g. liver in 10 ml. medium. After incubation, explants and medium were transferred to wide-mouthed tubes and were separated by centrifugation (2,000 rpm.)

Porphyrins were extracted from the explants after repeated homogenisations in a Potter Elvehjem-type homogeniser with a glass pestle. The homogenising solution consisted of a total of 2.5 ml. acetone and 0.015 ml. conc. HCl. After 3 centrifugations, the combined supernates were adjusted to pH 3.0-3.5 with saturated sodium acetate. The end-point was determined by the appearance of a pale blue-grey colour when the solution was tested on Congo red paper. To prevent reduction of porphyrinogens to porphyrins, 0.05 ml. iodine was added after adjustment of the pH, and the porphyrins extracted

three times into butanol:ethyl acetate 1:1. A half volume of petroleum ether was added to the pooled extracts and the porphyrins taken into 2.5 ml. of 1.5N HCl.

The medium was adjusted to pH 3.0-3.5 using approximately 2.0 ml. glacial acetic acid, iodine was added and the porphyrins taken into butanol:ethyl acetate. Final extraction with 1.5N HCl was performed until the acid layer was no longer pink in UV-light. The combined extracts from explants and medium were taken to dryness on a Rotary evaporator and each esterified with approximately 5 ml. methanol:sulphuric acid (5%) in the dark at room temperature for 24 hours. The mixture was then adjusted to pH 3.0-3.5 with saturated sodium acetate, and the porphyrin methyl esters extracted into 7 ml. redistilled chloroform which was then repeatedly washed with distilled water. The chloroform was dried in a water-bath not exceeding 60°C under a stream of nitrogen, and the dried extract dissolved in 100 µl. redistilled chloroform.

(b) Thin-layer chromatography.

Using a Hamilton micro-syringe, 10 µl. of the solution of porphyrins in chloroform were spotted on a thin-layer chromatography plate coated with a layer of Kieselgel G nach Stahl (Merck) of thickness 0.2 mm. Ten microlitres of a marker consisting of a mixture of proto-, copro- and uroporphyrin methyl esters, and 5 µl. of a coproporphyrin standard were spotted alongside the test samples,

and the plate was developed in a solvent of chloroform:kerosene:n-propanol (60:34:2) at 37°C for 45 minutes. After drying, the plate was scanned. The scanning device was constructed by Dr G. Sweeney since no commercial instruments were available in Cape Town. The construction is as follows: exciting light is provided by a Zeiss ST41 high pressure mercury arc and incorporates a reflecting mirror, condensing lens and Hg365 filter, and is powered with regulated direct current. This light falls on a 2mm. slit immediately below the plate being analysed. This plate is held on a perspex carriage moved in the direction of the long axis of the plate by a small synchronous motor coupled also to a potentiometer providing a variable voltage to the X axis of a Moseley model I X-Y recorder. Immediately above the layer of adsorbent on the upper surface of the plate is a second slit 1 mm. across and a Wratten no.15 filter. Light passing through a second (1 mm. slit) and this filter is focussed on to the photocathode of an IP 21 photomultiplier tube. After suitable amplification (photomultiplier photometer, American Instrument Co., Silver Springs MD) the output of the tube is fed on to the Y axis of the recorder.

(c) Calculation

The areas under the peaks which recorded each band of porphyrin were measured by means of an OTT hand planimeter, and the amount of porphyrin calculated by comparison of these areas with that obtained for the standard. Use was made of conversion factors to compensate

for the difference in intensities of fluorescence between the coproporphyrin and other methyl esters. These factors are 4.77 for protoporphyrin and 2.63 for uroporphyrin, and are valid at least over the range 0.01 - 1.0 µg of porphyrin in a given fraction, while the accurate detection of porphyrin varies between 0.005 µg and 10 µg. This method was derived by Dr G.D. Sweeney from standard porphyrin extraction techniques.

(d) Materials

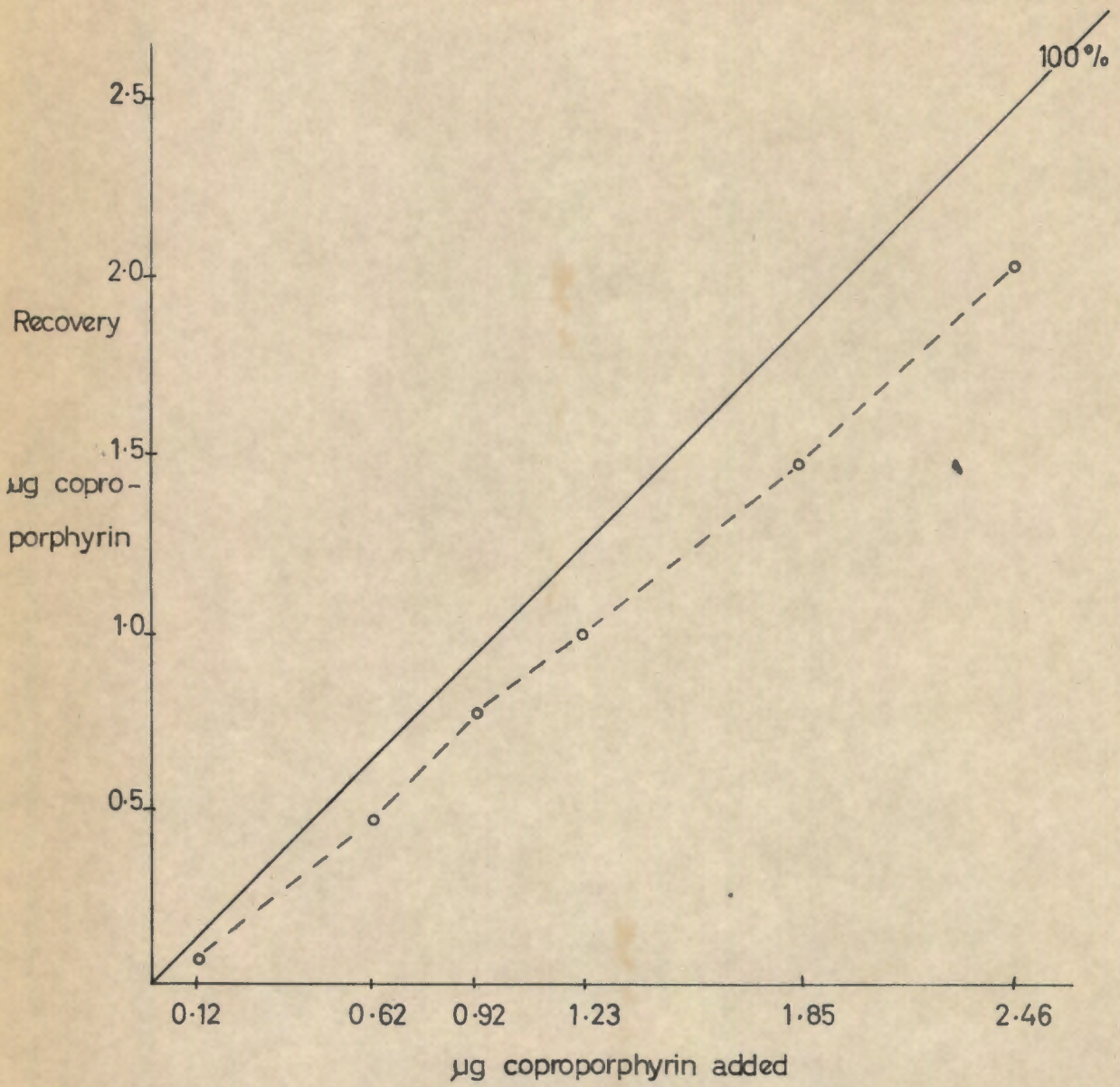
All reagents were of "ANALAR" grade and were re-distilled.

Thin-layer plates were prepared on standard apparatus (Shandon Scientific Co. Ltd.), and were dried for 45 minutes in a thermostatically controlled over at 120°C. They were kept in a dessicator until required.

The marker solution was prepared from the methyl esters of porphyrins extracted from faeces of patients currently being analysed in the laboratory, which were used in arbitrary amounts.

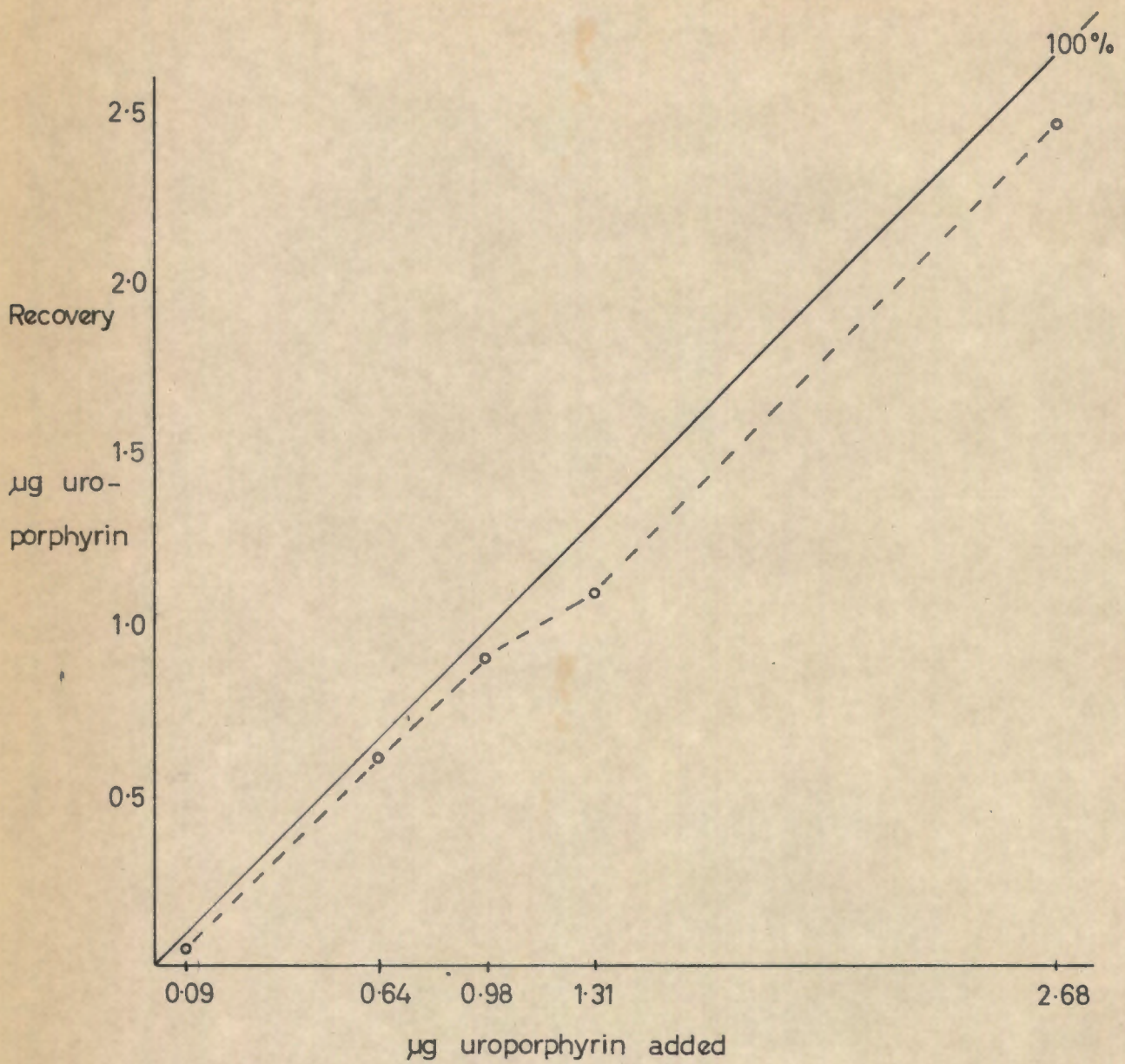
Coproporphyrin for preparation of the standard was recrystallised from faeces, and an aliquot of the crystals hydrolysed in a small amount of 7.5N HCl kept in the dark at room temperature for at least 48 hours. The pH of the solution was then corrected to 3.5 with saturated sodium acetate, and the porphyrins extracted into ether. The ether was repeatedly washed with distilled water to remove any hydrophilic porphyrins, and was then dried on a vacuum pump.

Fig: XIII Recovery of free Coproporphyrin* from tissue culture.



* The coproporphyrin was dissolved in 1·5N HCl and added to tissue culture. The porphyrin was extracted immediately. The final yields are expressed as the amounts of coproporphyrin found in the experimental cultures minus amounts found in control cultures.

Fig: XIV Recovery of free Uroporphyrin* from tissue culture.



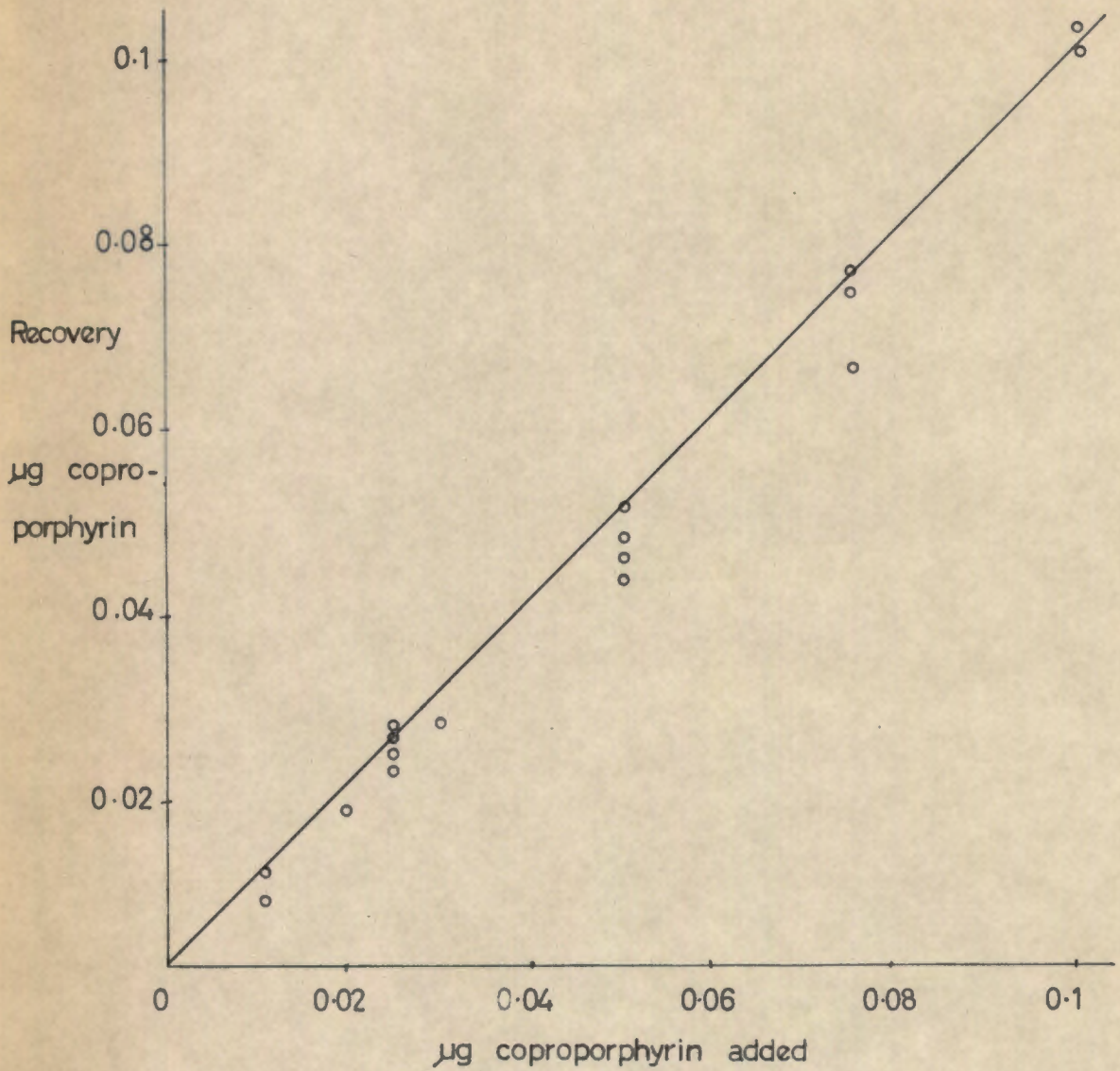
* The uroporphyrin was dissolved in 1·5N HCl and added to tissue culture. The porphyrin was extracted immediately. The final yields are expressed as the amounts of uro-porphyrin found in the experimental cultures minus amounts found in control cultures.

Esterification was achieved with 25-30 ml. methanol:sulphuric acid (5%) in the dark at room temperature for 24 hours. The pH of the solution was re-corrected to 3.5 with saturated sodium acetate, and the porphyrins extracted into chloroform which was washed and finally dried on a Rotary evaporator. The dried extract was dissolved in 5-10 ml. chloroform and the solution carefully layered over a column of activated alumina which had been washed with chloroform. The porphyrin was eluted off by the addition of more chloroform and the adjustment of flow rate to 120 dpm. The first fraction obtained contained coproporphyrin which could be repurified by drying and repetition of the column purification. The final solution was tested by thin-layer chromatography to detect impurities. It was then dried and dissolved in a known amount of chloroform. A dilution was made and the concentration of porphyrin determined in a Zeiss spectrophotometer. One microgram amounts were dried on the Rotary evaporator and stored in stoppered tubes at $-4^{\circ}C$ until required. For use, 100 µl. redistilled chloroform was added to dissolve the porphyrin and 5 µl. was used. At a photomultiplier sensitivity of 0.1, this amount of porphyrin yielded an area of approximately 100.

(vi) The accuracy and reproducibility of these results has been tested.

(a) Known concentrations of free coproporphyrin and uroporphyrin were dissolved in 1.5N HCl and were added in various dilutions to the

Fig: XV Recovery of Coporphyrin methyl ester* from thin layer chromatographic plates.



*coproporphyrin methyl ester was dissolved in redistilled chloroform and spotted on thin layer plates. After chromatography, the amount of coproporphyrin was quantitated as described in the text, and the actual recovery is shown graphically. Each point indicates a single experiment, and the line drawn represents 100% recovery.

to the culture medium. The porphyrins were extracted immediately and the results quantitated. The amounts of the respective porphyrins produced after subtraction of the amount formed by the control cultures, was expressed as a percentage of the amount added.

The results are shown graphically in figs. XIII and XIV. It may be seen that the recovery of coproporphyrin averaged 80% and of uroporphyrin 90%. This confirms previous studies done in the laboratory when the extracted porphyrin was estimated in a spectrophotometer.

(b) An additional check was made on the accuracy of the chromomatography method alone. Various samples of the coproporphyrin standards used routinely, were diluted and different concentrations chromatographed against a usual standard of 5 µl. The results of 20 such estimations showed an average recovery of 94% with the range 86 to 110% (fig. XV).

In all experiments, incomplete esterification of porphyrins was revealed by failure of the chloroform solution to move off the baseline when chromatographed, and such results were discarded and the estimations were repeated.

EXPERIMENTS USING ALA AND AIA

(i) ALA.

With the accuracy of the method thus established, the effect of addition of ALA was tested. ALA was dissolved in Hanks solution of 0.5N saline, in a final concentration of 1 µmole/ml. A bright red

Experiments to show the increase in porphyrin production by tissue culture after the addition of δ-amino-laevulinic acid.

Expt. No.	CONTROL			ALA 1µg/ml		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
I	2	0·52	0·94	2	5·94	13·10
	4	0·14		4, 5	1·49	
	8	0·28		7, 8	5·65	
II	4	0·28	0·52	2	4·77	52·72
				4, 5	3·25	
	8	0·24		7, 8	44·70	
III	4	0·26	0·94	2, 3	2·00	66·04
				4, 5	0·79	
	8	0·68		7, 8	63·25	
IV	2	0·29	0·61	2	17·48	33·30
				4	12·40	
	4	0·32		8	3·42	
V	2	0·31	1·19	2	26·04	50·55
	4	0·81		4	13·61	
	8	0·07		8	10·90	

MEAN INCREASE ALA - CONTROL : 42·3 ± 20·4

($p < 0·001$)

Table I

Experiments to show the increase in porphyrin production by tissue culture after the addition of allyl-isopropyl-acetamide

Expt. No.	CONTROL			AIA 3mg/ml		
	COOH	µg porphyrin	Total	COOH	µg porphyrin	Total
I	2 4 8	0·52 0·14 0·28	0·94	2 4 8	2·63 0·90 2·27	5·80
II	4 8	0·28 0·24	0·52	2 4 8	0·39 0·28 4·48	5·15
III	2 4 8	0·13 0·25 0·24	0·62	2 4 8	0·04 0·17 5·04	5·25
IV	8	1·63	1·63	2 8	0·36 6·08	6·44
V	2 4 8	0·93 0·23 0·32	1·48	2 4 8	0·86 0·78 3·32	4·96

MEAN INCREASE AIA – CONTROL: 4·3 ± 0·6
($p < 0.001$)

Table II

fluorescence could be detected within 2-3 hours, and porphyrin production reached a maximum at 4-5 hours. Dependent upon the viability of the explants and the integrity of enzyme systems, the response to ALA was always marked. Table I shows the result of 5 experiments using ALA. The range of increase varies from 14 to 100-fold and is highly significant ($p < 0.001$).

An intact enzyme system is essential to the manifestation of fluorescence after the addition of ALA and it is for this reason that ALA is used to prove the integrity of enzyme systems.

It may also be seen from the Table that ALA stimulates the formation of porphyrine of all carboxyl groups.

(ii) The drug AIA has been used by other investigators to induce porphyrin synthesis both in animals and in tissue culture. The drug in this case was dissolved in Hanks solution or in 0.5N saline to a final concentration of 3 mg/ml. Fluorescence was first noticeable 24 hours after addition of the drug to cultures, and porphyrin production reached a maximum within 36 to 48 hours. However, at no time was the fluorescence as bright or the porphyrin production as great as that occurring after the addition of ALA.

The results of 5 experiments using AIA are presented in Table II. There is a significant ($p < 0.001$) increase in porphyrin production from three to ten-times control levels. It may be seen however, that the induction of intermediate carboxyl groups occurred less often than was the case with ALA addition.

A higher concentration of AIA was used than that reported by Granick(91); 3 µg/ml failed to induce porphyrin fluorescence in explants as it had in trypsinized cells, possibly due to permeability barriers.

Further discussion of the method of action of AIA will be discussed after the presentation of experiments conducted with other drugs.

EXPERIMENTS WITH DRUGS AND CHEMICALS

(i) Materials

The drugs added to tissue culture were the standard pharmaceutical preparations, obtained from Groote Schuur Hospital by courtesy of the Chief Pharmacist, Mr. Shapiro. The concentrations used of many of these were as described by Granick(91), and those not detailed by him were prepared in similar concentration. Those not supplied in liquid form were dissolved in Hanks solution as required. Other constituents of tablets, such as starch and lactose were tested on the cultures for effect prior to commencement of the study. Occasionally, if good dissolution of the tablet could not be obtained, a little Pulv. Tragacanth was added. This had been similarly tested prior to use.

Solutions of the drugs were added directly to the tissue culture medium before incubation.

TABLE III.

Drugs which produce fluorescence when added to tissue culture.

Dosage is that found to produce optimal fluorescence when added to 1 ml culture medium.

Trade Name.	Generic Name.	Chemical Name.
SEDATIVES:		
Phenobarbitone Sodium. 4mg	Phenobarbitone	Phenobarbitone
Pentobarbitone Sodium. 2mg	Pentobarbitone	Pentobarbitone
Noludar. 1 mg	Methyprylon	2,4,dioxo-3,3-diethyl 5methylpiperidine
Doriden. 5mg	Glutethimide	Ethyl-2-phenyl-2-glutarimide
Amytal. 1 mg	Amobarbital	Ethyl,methyl,butyl, barbituric acid
Seconal. 1 mg	Secobarbital	Sodium-allyl(methyl-1-butyl)5,barbituric acid
Sulfonal. 50µg		Sulphonmethane
TRANQUILLIZERS AND STIMULANTS.		
Caffeine. 100µg		1,3,7-trimethylxanthine
Allergron. 2.5mg	Nortriptylene HCl.	
Amphetamine. 750µg	Amphetamine	β-phenyl-isopropylamine
Miltown. 8mg	Meprobromate	2-methyl-2n-propyl-1,3-propanediol,dicarbamate

Trade Name.	Generic Name.	Chemical Name.
TRANQUILLIZERS AND SEDATIVES cont.		
Surmontil. 500µg	Trimipramine	Phenothiazine type
Tofranil. 500µg	Imipramine	N(γ-dimethyl-amino-propyl)-imino-dibenzy-lium HCl.
Tryptanol. 500µg	Amitriptylene HCl	
ANALGESICS AND ANTIPYRETICS:		
Zactirin. 2.5mg+ 6.5mg		Ethoheptazine (1methyl-1-carbethoxy) + acetyl salicylic acid.
Butazolidine. 2mg	Phenylbutazone	3,5-dioxo-1,2,-diphenyl-4n-butyl,pyrazolidine
Zyloprim. 2mg	Allopurinol	4-hydroxy,pyrazolo, pyrimidine HCl
Indocid. 500µg	Indomethacin	
Benemid. 10mg	Probenecid	N-propyl-p-sulphanyl, benzoic acid.
Berazolene. 4mg	Aminopyrine	Pyrazolone
Phenazone. 1 mg	Antipyrine	Phenyl-pyrazolone derivative.
Sodium salicylate. 1 mg		Sodium salicylic acid
Phenacetin. 1 mg	Acetophenetidin	Para-amino phenol derivative
ANTIBIOTICS AND ANTIHELMINTHICS:		
Ambilhar. 2mg	Nitrothiazolyl-imidazolidinone	1,5-nitro-2-thiazolyl-2-imidazolidinone
Antiphen. 10mg	Dichlorophen	2,2'-dihydroxy-5,5'-dichloro,diphenyl,methane

Trade Name.	Generic Name.	Chemical Name.
ANTIBIOTICS AND		
ANTIHELMINTHICS Contd.		
Grisovin, F.P. 2.5mg	Griseofulvin	Griseofulvin
Sulphadiazine Sodium. 5µg		Sulphadiazine
Albamycin. 50ug	Novobiocin	Carbenoy(oxy)-4-tetrahydro,hydroxy,-3-methoxy,dimethyl-6-6-dipyran-2-yloxyl-7-hydroxy-4-hydroxy-4-methyl,butaryl-2,3-benzamido-3-methyl-8-coumarine.
HORMONES:		
Syntometrine. 12.5mg + 0.125 i.u.		Ergometrine tartrate+ Syntocinon
Norinyl. 20µg + 1 µg		Norethisterone + Mestranol
Stilboestrol. 100µg	Diethyl-stilboestrol	Trans,bis(hydroxy-4-phenyl)-3,4-hexane
Ergometrine. 10µg		Ergometrine maleate
ANAESTHETIC AGENTS:		
Pentothal Sodium. 5mg	Thiopentone	
Fabantol. 25mg	Propanidid	Propyl-4-n,n,diethyl, carbamoyl,methoxy-3-methoxy,phenyl,acetate.
Tubarine. 500µg	Curare	Tubocurarine Cl
Flaxedil. 10mg	Gallamine	V-phenyl,tris(oxyethylene)tris,triethyl,ammonium iodide

Trade Name.	Generic Name.	Chemical Name.
ANAESTHETIC AGENTS:		
Contd.		
Leostesin. 4µg	Lidocaine	Diethyl,amino,dimethyl-2-6,acetanilide HCl
Viadril. 1 mg	Hydroxydione Sodium	T21-hydroxy-pregnane-3,20-dione sodium hemisuccinate.

TABLE IV.

Drugs which do not produce fluorescence when added to tissue culture.

Dosage range is to the optimum resulting in inviability.

Trade Name.	Generic Name.	Chemical Name.
SEDATIVES:		
Chloral hydrate. 10µg - 10mg		
Mogadon. 1µg - 100µg		1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepine-2-one
Sparine. 5µg - 500µg	Promazine	10,3'-dimethyl,amino, propyl,phenothiazine HCl
Largactil. 5µg - 500µg	Chlorpromazine	2-chloro-10,3'-dimethyl,amino,propyl, phenothiazine HCl
Librium. 5µg - 500µg	Chlordiazepoxide	7-chloro-2-methyl, amino-5-phenyl-3H-1,4-benzodiazepine-4-oxide
Soneryl. 5µg - 500µg	Butobarbitone	
Stelazine. 2µg - 20µg	Trifluoperazine	2-trifluo,methyl,-10,3'-methyl, piperazinyl-4-phenothiazine, dihydrochloride
Melleril. 2µg - 200µg	Thioridazine	Methyl,mercapto-3N-methyl,piperidyl,-2-ethyl-10-phenothiazine HCl
Milontin. 10µg - 10mg	Phensuximide	N-methyl-α-phenyl, succinimide
Celontin. 10µg - 10mg	Methsuximide	N-methyl-α-phenyl, -α-methyl,succimide.

Trade Name.	Generic Name.	Chemical Name.
HORMONES:		
Mixogen. 0.72µg - 72µg + 0.9ng-0.09µg		Oestradiol-monobenzoate and -phenylpropionate; and testosterone-propionate, -phenylpropionate and -isocaproate
Syntocinon. 0.001 - 0.1 i.u.	Oxytocin	Synthetic oxytocin
ANTIBIOTICS:		
Chloromycetin. 10µg - 1 mg	Chloramphenicol	p-nitrophenyl-2-dichlor,acetamidopropane-1,3-diol
ANTIMALARIAL:		
Chloroquine. 5µg - 5mg		Chloroquine phosphate
VITAMINS:		
Redoxon. 10µg - 1 mg	Ascorbic acid	Oxo-3-l-gulofurono-lactone
Vitamin E.	∝ tocopheryl	l-∝- tocopheryl acetate
Folic acid. 2.5µg - 250µg		Pteroyl-glutamic acid
CARDIAC AGENTS:		
Digitalis. 1.2µg - 1.2mg		Digitalis folia
Quinidine. 2µg - 2mg		Quinidine sulphate
Lethidrone. 1µg - 1mg	Nalorphine	N-allyl-nor-morphine
Nepresol. 5µg - 500ug	Dihydralazine	Dihydralazino-1,4-phthalazine-methane-sulphonate

Trade Name.	Generic Name.	Chemical Name.
MISCELLANEOUS:		
Artane. 1µg - 100ug	Trihexiphenidyl	3,1-piperidyl-1-phenyl-1-cyclohexyl-1-propanol HCl
Avafortan. 1ug - 100ug		Avapyrazone
Avertin. 10µg - 1mg		Tribromoethanol
Aldomet. 5µg - 5mg	Methyldopa	Levo-3,3,4-dihydroxyphenyl-2-methylalanine
Dindevan. 0.1µg - 10µg	Phenindione	Phenindione-2-phenyl, indione-1,3-dione
Morphine Sulphate. 6µg - 600µg	Morphine sulphate	
Pethidine. 10µg - 1 mg	Pethidine	Methyl-1-phenyl-4-ethyl ester of carboxylic acid-4-piperidyl-HCl
Phenergan. 2.5µg - 2.5mg	Promethazine	N-2-dimethylamino-2-methyl,ethyl,phenothiazine HCl
Rastinon. 10µg - 10mg	Tolbutamide	N-4-methyl,benzene, sulphonyl,N-butyl urea
Tensilon. 1µg - 1 mg	Edrophonium chloride	3-hydroxy,phenyl,dimethyl,ethyl ammonium chloride.

(ii) The criteria of selection of drugs were governed by:-

(a) the likelihood of their use in acute medical conditions, and

(b) correspondence with the chemical structures already found by Granick to induce porphyrin production in tissue cultures.

In respect of the first consideration, an adequate tranquillizer or analgesic for porphyric patients is still sought. The use of pethidine or morphine, which appear to be safe from the aspect of aggravation of symptoms, carries the risk of addiction. The action of other tranquillizers is difficult to assess in such patients with disorders of porphyrin metabolism.

(iii) The results of screening a large number of drugs are found to have porphyrin-inducing activity are presented in Table III. The arrangement is roughly according to use, and includes the optimal concentration. A second list of those drugs tested in various dilutions to the limit of viability, which had no effect is shown in Table IV. It may be seen that the porphyrin-inducing capacity appears predominantly among tranquillizers, analgesics and antipyretics.

(iv) The results of quantitative porphyrin estimation are shown in Tables V -XVII. Each positive result was repeated once, and results appeared to correlate well with each other. In many cases, three dilutions of each drug were tested.

The tranquillizers meprobromate (Miltown), trimipramine (Surmontil), imipramine (Tofranil) and amitriptylene HCl (Tryptanol)

Table: V Experiments to show the increase in porphyrin production by tissue culture after the addition of Meprobromate

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	2	0·07	1·58	2	0·74	1·32
	4	0·92				
	8	0·59		4	0·58	
Meprobromate 8mg	2	0·02	1·97	2	1·55	1.86
	4	1·03				
	8	0·92		4	0·31	
Meprobromate 800 µg	4	1·53	2·47	2	0·89	1·62
				4	0·26	
	8	0·94		8	0·47	
Meprobromate 80µg	4	1·54	2·33	2	1·60	2·13
				4	0·32	
	8	0·79		8	0·21	

Table: VI Experiments to show the increase in porphyrin production by tissue culture after the addition of Sûrmontil.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	2	0·86	1·22	2	0·37	0·54
	4	0·14		4	0·17	
	8	0·22				
Surmontil 450µg	2	0·67	2·86	2	1·17	2·45
	4	1·02		4	1·28	
	8	1·17				
Surmontil 45µg	2	2·22	3·02	2	1·41	2·16
	4	0·39		4	0·75	
	8	0·41				
Surmontil 4·5µg	2	2·11	2·70	2	1·16	1·56
	4	0·27		4	0·36	
	8	0·32				

Table: VII Experiments to show the increase in porphyrin production by tissue culture after the addition of Tofranil.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	4	0.86	1 87	2	0.64	1.82
				4	0.92	
	8	1.01		8	0.26	
Tofranil 450µg	4	0.26	0 36	2	1.24	2.27
				4	0.91	
	8	0.10		8	0.12	
Tofranil 45 µg	2	1.82	2.70	2	1.08	1.89
				4	0.67	
	4	0.88		8	0.12	
Tofranil 4.5 µg	4	1.24	1.78	2	0.31	0.89
				4	0.37	
	8	0.54		8	0 21	

Table: VIII Experiments to show the increase in porphyrin production by tissue culture after the addition of Tryptanol.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	4	0·93	0·93	2 4	0·50 1·05	1·55
Tryptanol 450 µg	4	0·38	0·38	2 4	0·33 0·39	0·72
Tryptanol 45 µg	2 4	0·70 0·27	0·97	2 4	0·43 1·00	1·43
Tryptanol 4·5µg	2 4 8	0·24 0·72 0·22	1·18	2 4	1·26 0·98	2·24

Table: IX Experiments to show the increase in porphyrin production by tissue culture after the addition of Irgaprim.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	2 4 8	0·82 0·69 1·51	3·02	2 4 8	0·23 0·40 0·31	0·94
Irgaprim 2mg	2 4 8	0·50 1·12 0·36	1·98	8	0·77	0·77
Irgaprim 200 µg	2 4 8	0·93 1·12 1·05	3·10	2 4 8	1·00 1·33 0·28	2.61
Irgaprim 20µg	2 4 8	1·24 2·56 0·68	4.48	2 4 8	0·53 0·29 0·59	1·41

Table: X Experiments to show the increase in porphyrin production by tissue culture after the addition of Allopurinol.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	2	0·07	2·00	4	0·96	1·34
	4	1·40				
	8	0·53		8	0·38	
Allopurinol 1·81mg	2	3·09	4·43	2	0·22	3·75
	4	0·92		4	3·35	
	8	0·42		8	0·18	
Allopurinol 181 µg	2	1·56	2·77	4	1·32	3·55
	4	0·84				
	8	0·37		8	2·23	
Allopurinol 18·1µg	2	0·61	1·51	4	2·26	2·45
	4	0·71				
	8	0·19		8	0·19	

Table:XI Experiments to show the increase in porphyrin production by tissue culture after the addition of Indomethacin

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	4	1·36	1·36	4 8	1·35 0·36	1·71
Indomethacine 650µg	2 4 8	2·94 0·36 0·29	3·59	2 4 8	0·74 1·60 1·45	3·79
Indomethacine 65µg	2 4 8	1·06 0·16 0·50	1·72	2 4 8	0·72 1·16 0·12	2·00
Indomethacine 6·5µg	2 4 8	1·05 0·59 0·29	1·93	2 4 8	1·03 0·41 0·62	2·06

Table: XII Experiments to show the increase in porphyrin production by tissue culture after the addition of Zactirin.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	4	0·15	0·15	4	0·85	0·85
Zactirin 2·5mg+6·5mg	8	0·17	0·17	4	0·75	0·75
Zactirin 250µg + 650µg	4	1·20	1·20	4	1·59	1·59
Zactirin	4	3·77	5·99	4	0·81	1·74
25µg+65µg	8	2·22		8	0·93	

Table XIII Experiments to show the increase in porphyrin production by tissue culture after the addition of Norinyl.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	2	0·16	0·35	4	0·45	0·45
	4	0·19				
Norinyl 200 µg	2	0·79	1.13	2	1·58	3·79
	4	0·34		4	1·58	
				8	0·63	
Norinyl 20µg	4	0·22	0·22	2	2·21	5·55
				4	3·34	
Norinyl 2µg	2	0·06	0·17	2	1·76	3·09
	4	0·11		4	1·33	

Table: XIV Experiments to show the increase in porphyrin production by tissue culture after the addition of Sulphadiazine.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	4	0·72	0·72	4	0·52	0·52
Sulphadiazine 5mg	2 4	0·81 0·93	1·74	2 4	0·61 0·98	1·59
Sulphadiazine 500 µg	4	1·52	1·52	4	1·25	1·25
Sulphadiazine 50µg	2 4 8	0·99 1·22 0·22	2·43	2 4	0·80 1·11	1·91

Table: XV Experiments to show the lack of effect on porphyrin production by tissue culture after the addition of Lactose.

SPECIMEN	COOH gr.	µg porphyrin/ g w.w.	Total
Control	2	0·41	0·74
	4	0·33	
Lactose 40mg	2	0·44	0·77
	4	0·26	
	8	0·07	
Lactose 4mg	2	0·34	0·63
	4	0·29	
Lactose 400µg	2	0·74	0·80
	4	0·06	

Table: XVI

Experiments to show the lack of effect on porphyrin production by tissue culture after the addition of Ambilhar.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	4 8	0·60 0·25	0·85	4	1·56	1·56
Ambilhar 500µg	4 8	0·21 0·09	0·30	4	2·77	2·77
Ambilhar 50µg	4	0·48	0·48	4	1·13	1·13
Ambilhar 5µg	4	0·48	0·48	4	1·00	1·00

were found to increase porphyrin production, as did the analgesics phenylbutazone (Irgaprim), allopurinol (Zyloprim) and indomethacine (Indocid). In view of reports already reviewed of aggravation of the symptoms of porphyria by hormones, the combination substance Norinyl (norethisterone + mestranol) was tested and found to increase porphyrin production. Because some of the earliest reports incriminated sulphonal and its derivatives, sulfadiazine was tested and found to increase porphyrin production.

In view of the content of lactose in many of the tablets used, the effect of lactose in tissue culture was investigated. From fig.XV it may be seen that lactose in varying dilutions has no effect on cultures. Starch, another component of tablets, had been tested by screening and did not cause fluorescence.

The drug, chloroquine, has been found to have a detrimental effect in symptomatic porphyria, possibly excluding it from use. Its addition to tissue culture results in fluorescence. However, amoebiasis occurs frequently in patients who may also take alcohol to excess. For this reason, the recently-introduced drug nitro-thiazolylimidazolidinone (Ambilhar), was investigated. No increased porphyrin formation resulted from addition of this drug to cultures. However, as will be discussed later, it cannot be immediately extra-polated that the drug may be used with impunity in patients with porphyria.

Table: XVII Experiments to show the increase in porphyrin production by tissue culture after the addition of Amphetamine.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
Control	4	0·22	0·22	2 4	0·02 0·22	0·24
Amphetamine 2·5mg	2 4 8	0·32 0·73 0·49	1·54	2 4 8	0·62 0·71 0·43	1·76
Amphetamine 250µg	2 4 8	0·29 0·89 0·38	1·46	2 4 8	0·71 0·72 0·29	1·72
Amphetamine 25 µg	2 4 8	0·48 0·92 0·50	1·90	2 4 8	0·84 0·92 0·62	2·38

Table:XVIII Experiments to show the appearance of uroporphyrin (8) only after addition of the highest concentration of phenobarbitone to tissue culture.

SPECIMEN	Experiment I			Experiment II			Experiment III		
	COOH gr.	µg porph./ g w.w.	Total	COOH gr.	µg porph./ g w.w.	Total	COOH gr.	µg porph./ g w.w.	Total
Control	2	0·88	1·07	2	0·06	0·12	2	0·33	0·56
	4	0·19		4	0·06		4	0·23	
Phenobar-	2	1·40		2	0·17		2	3·19	
bitone	4	1·37	3·25	4	0·07	0·52	4	0·52	4·06
4mg	8	0·48		8	0·28		8	0·35	
Phenobarb.	2	1·73	2·14	2	0·17	0·46	2	0·62	0·97
400µg	4	0·41		4	0·29		4	0·35	
Phenobarb.	2	1·13	1·45	2	0·19	0·39	2	0·41	0·77
40µg	4	0·32		4	0·20		4	0·36	

Amphetamine was also tested.

It was thought that some significance might be attached to the types of porphyrin induced, especially as coproporphyrinuria is a non-specific result of many diseases. In general, a review of the quantitative results of the drugs tested, revealed no such pattern. However, in the testing of the effect of phenobarbitone, it may be seen from Table XVIII, the appearance of uroporphyrin occurs consistently only with the highest dosage. However, the concentrations of drugs used are in most cases well above those which would be used in therapy, and further testing is required to determine whether the production of porphyrins other than coproporphyrin is merely a dose response, as suggested by the results obtained when using phenobarbitone.

DISCUSSION OF RESULTS SO FAR PRESENTED

(i) The most important feature in the interpretation of the results of drug screening is to stress again that no direct extrapolation to human porphyria can be made. However, it is possible to state that so far, no drug known to aggravate the condition of porphyria in man, has been shown to have a negative effect in tissue culture. Moreover, with regard to the anaesthetic agents, all so far tested, including the non-barbiturate preparation Viadril, have induced porphyrin synthesis. But there are many drugs which have a positive effect on the chick liver explants, yet have been used with safety so far in porphyric patients - e.g. morphine, pethidine,

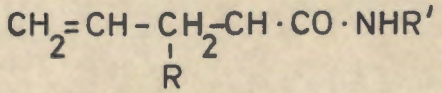
norinyl. Individual variations in patients are known to occur, with, for example, apparent resistance to the development of an acute attack on six occasions of thiopentone anaesthesia, with precipitation of a severe reaction after the seventh induction(41). Such variations are even more likely to occur in tissue culture.

Although it could be suggested that the effect is purely non-specific, the number of agents tested with negative result both by Granick and in this series, tends to dispute this conclusion.

(ii) Recent studies on porphyrin induction by Granick have suggested that the action of drugs is that of effectors in the scheme of haemoglobin biosynthesis as discussed in chapter IV. He has excluded the possibilities that the increased ALA-synthetase which he found in chick embryo liver systems in vivo made porphyric with drugs, was due to a decreased destruction of the enzyme; or that the inducing chemical altered the configuration of the enzyme or blocked a hypothetical inhibitor. He found no evidence either, of enzyme action when drugs were added to isolated mitochondria, or a feed-back inhibition when haem was added to isolated mitochondria.

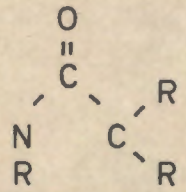
(iii) The hypothesis of competition with haem, stresses the importance of the structures of the porphyrin-inducing drugs. The chemical structure necessary to produce porphyria in one or other experimental form has long been a source of interest. In 1939, the matter was considered by Rimington(300), who recognised a corres-

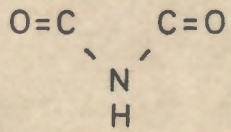
Fig. XVI

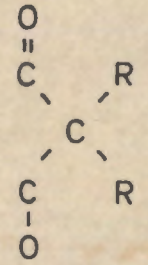


The chemical structure necessary to produce experimental porphyria. (35)

Fig. XVII

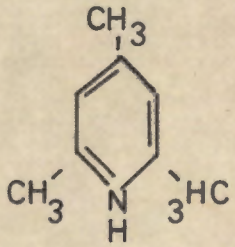


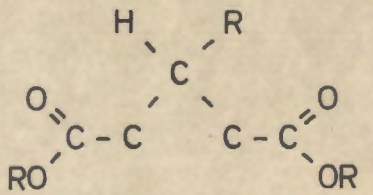




Structures which induce a chemical porphyria (91)

barbiturate class





collidine class

pondence with the structure necessary to produce methaemoglobinaemia. He postulated the presence of "an amino group unsubstituted or potentially free, and preferably in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative, or a reversibly oxidising system such as p- or o-iminoquinone". Subsequent work from the same laboratory(83), and by Labbe, has suggested the importance of the sub-groups of drugs and chemicals(84)(301). The basic structure was described by Goldberg and Rimington(83) (see fig. XVI)- a dialkyl substituted acetamide or acetamide derivative. Labbe(84) proposed in addition, an allyl group as substituent, or a 3-carbon group, preferably branched. This structure was thought to interfere with the function of vitamin B12. Granick has described five structures for this allosteric inhibition(91). Three are related to barbiturate structure and two to collidine design (fig. XVII). The theory that only one site is involved, is confirmed by findings that simultaneous addition of two drugs does not result in any increased porphyrin production compared with the addition of a single agent. Further work on one of the groups proposed by Granick, has been performed by Marks(302)(303). Many analogues of DDC and the corresponding pyridine have been tested for their properties in tissue culture, and their chemical structures have been analysed.

A study of the Fisher-Hirschfelder-Taylor models and of the UV absorption spectra indicated a non-planar relationship between

substituents and the ring, which may be important in determining porphyrinogenic activity. However, this is only an extension of the concept of competition. The work has also involved the investigation of the necessity for free allyl groups which has been described in vivo. This was not confirmed in vitro, but the hypothesis was proposed that the free allyl group endows the molecule with suitable physico-chemical properties which enable it to achieve a significant concentration in the liver cell, when administered to whole animals.

The third possibility remains that the chemicals interfere with the normal repression of the structural gene for haem, resulting in derepression. Estimates of ALA-synthetase activity revealed a 40-fold increase in the mitochondria of guinea-pigs fed DDC and an 8-fold increase when the drugs were injected into the air-sacs of chick embryos confirming one site of drug action. The proposal that the chemicals compete with haem for the site as co-repressor was confirmed when with varying concentrations of haem and AIA, the porphyrin-inducing effect of the latter was blocked by haem(91). It was also suggested that the corepressor site was not absolutely specific for haem, since deuteroporphyrin and manganese protoporphyrin also inhibited porphyrin induction by AIA.

Two other postulates which have been tested and discarded, are (i) that the drugs themselves might in some way act as porphyrin precursors, and (ii) that drugs might interfere with the excretion of bile pigments, resulting in a back-pressure and a build-up of porphyrins(299).

(iv) Granick has also proposed that the action of various drugs in vivo relates to the response by the liver to such drugs(91). This involves the synthesis of detoxifying enzymes. One mechanism of detoxication is by hydroxylation of aliphatic groups or aromatic rings by various oxidases, which depend for their activity on haem, flavin, iron or NADPH2. One of the terminal oxidases has been found to be a cytochrome(304), and increased cytochrome formation has been shown in animals following barbiturate administration(305)(306). It is suggested that a response in man to similar administration of drugs is the formation of more haem to assist excretion of the agents.

This last proposal would explain the fact that many of the drugs tested in this series and found positive, do not conform to the structures derived by Granick. It appears therefore, that while the structural competition with haem by these drugs is a distinct possibility, it must occur in conjunction with another effect. However, it may be that as yet insufficient definition of the structures has been achieved and that further analysis will reveal either additional structures, or a specific planar arrangement.

OTHER PORPHYRIN-INDUCING SUBSTANCES

(i) Alcohol

With respect to the involvement of alcohol in the syndrome of symptomatic porphyria, this substance was added to tissue culture, although it was thought naïve to imagine that its mere addition would

result in fluorescence. As expected, no fluorescence ensued. Because a number of alcoholic beverages have as their base various fusel oils (307), a selection of these compounds was tested in dilution. Again no fluorescence resulted, and a limiting aspect of the culture system became evident - namely that for the establishment of a condition of chronic intoxication analogous to that in man by alcohol, animal experiments could be more profitably exploited.

(ii) Fungus

A surprise discovery was made in 1965 by Keen(308) in this laboratory when a control culture was found to be fluorescing. Of various species tested, only the pigeon liver was found to fluoresce spontaneously. This culture of chick liver explants was contaminated with a fungus, identified as Aspergillus fumigatus. At present, this is being cultivated in larger quantities for possible identification of the active principle. The discovery is of interest in two directions - the first being the hepatotoxic effect in experimental animals of another member of the genus, Aspergillus flavus, and the second concerns the induction of symptomatic porphyria by alcohol, fungal contamination of which may occur under certain circumstances.

It is evident from all these studies that further investigation is required to determine precisely, the nature of the chemical structure which has the ability to stimulate porphyrin synthesis.

This will be considered in more detail after the presentation of additional relevant results in this and the subsequent chapter.

CHAPTER VI THE HYPOTHESIS

"The fact is only half the truth - the remainder depends upon its correct interpretation".
C.F.M. Saint - Another Look Around. 1967.

In chapter V, the production of porphyrins by embryo chick liver in tissue culture with the addition of drugs and chemicals has been confirmed, and the number of drugs which appear to have this effect, has been increased.

The fact that inhibitors of protein synthesis prevent this effect has led Granick(91) to propose that this porphyrin production is the result of induction of the rate-limiting enzyme ALA-synthetase. Further support for this comes from his experiments in the intact chick embryo where increased ALA-synthetase synthesis occurred after exposure to AIA.

The small amount of ALA-synthetase in tissue culture has so far defied direct measurement both in Granick's laboratory and in our own. However it seems reasonable to assume that the increased production of porphyrin in chick embryo liver cells after exposure to certain drugs is the result of increased ALA-synthetase synthesis.

The hypothesis was developed that RNA, especially messenger RNA, was involved in the induction of ALA-synthetase, and it was proposed that one messenger RNA coding for ALA-synthetase in porphyric animals

is formed in such a way that addition of such nucleic acid to a system might result in induction of the enzyme in excess of control systems.

This hypothesis was tested experimentally and the results are recorded in chapter VII.

CHAPTER VII THE TRANSDUCTION OF EXPERIMENTAL PORPHYRIA, USING RNA

"It cannot be seen, cannot be felt, cannot be heard, cannot be smelt. It lies behind stars and under hills, and empty holes it fills" -
"Dark" - J.R.R.Tolkien in The Hobbit, 1937.

EXPERIMENTS USING TRANSFER AND RIBOSOMAL RIBONUCLEIC ACID (RNA)

(i) Materials

Wistar rats of both sexes were used throughout the investigation of the effect of RIA. Female rats were, however, only used where a sex-linked difference in response was suspected. The rats were bred in the Animal House of the University of Cape Town Medical School, and transferred to a room near the laboratories when required. Their cages were of zinc and iron with steel bases, and the temperature and humidity of the room were kept constant.

Allyl-isopropyl-acetamide (AIA) which was received as a gift from Messrs. Roche Products, was given by nasogastric tube as a 12½% w.v. solution in propylene glycol in the dose of 250mg/Kg body weight. 4 doses were given at 12 hour intervals for 48 hours. This dose schedule followed that suggested by Ginsburg(90).

To promote maximal protein synthesis, the rats were starved for the duration of the experiment, but allowed free access to water. Control and experimental animals were matched for weight, and were killed either by guillotining, or by incision of the aorta under light

ether anaesthesia after cardiac puncture and removal of the liver had been performed.

The initial technique used for extraction of RNA yielded fractions of transfer and ribosomal RNA. The method was performed according to DiGirolamo et al(206) and Henshaw, Revel and Hiatt(209).

(ii) Method

After preparation, the rats were killed by decapitation and the livers removed onto cracked ice. Homogenisation was performed in 3 volumes of ice-cold medium (0.25M sucrose; 0.003M CaCl2; 0.05M tris-HCL pH 7.6), and the mixture centrifuged at 15,000g for 15 minutes. The upper four-fifths of the supernatant solution was centrifuged for 90 minutes at 104,000g to separate a microsomal pellet from the soluble supernate. The supernate was aspirated and made 1% with respect to sodium dodecyl sulphate (SDS) before isolation of the RNA with phenol:0.05M tris, pH 7.5 (77:23). This fraction according to various authors(206)(209) contains transfer RNA. (Details of sedimentation constants appear on page 66). A microsomal fraction was similarly isolated from the pellet after suspending it in 0.5 original liver volumes of homogenising medium containing 1% SDS. (See page 66 for sedimentation constants).

The final RNA fractions were dissolved in 0.1M Na acetate or tris buffer and were added to tissue culture in amounts between 300 - 400 µg RNA phosphorus.

Table: XIX

Experiments to show the increase in porphyrin production by tissue culture after addition of tRNA from control and porphyric rats (method I)

CONTROL				PORPHYRIC			
µg RNA phosph.	COOH gr.	µg porphyrin per g w.w.	Total*	µg RNA phosph.	COOH gr.	µg porphyrin per g w.w.	Total*
216	2 4 8	0·23 0·13 0·06	1·9	182	2 4 8	0·42 0·13 0·03	3·2
557	2 4	0·71 0·44	2·1	677	2 4 8	1·10 0·64 1·05	4·1
386	4 8	0·21 0·32	1·4	345	2 4 8	0·05 0·02 0·48	1·6
228	4 8	0·36 0·09	2·0	275	2 4 8	0·05 0·72 0·19	3·5
384	2 4	0·25 0·05	0·8	342	4 8	0·09 0·45	1·6
234	2 4 8	0·21 0·04 0·03	1·2	143	2 4 8	0·15 0·11 0·04	2·1
509	2 4 8	0·24 0·12 0·26	1·2	703	2 4 8	0·74 0·11 0·38	1·7

MEAN INCREASE : Porphyric - Control :: 1·0 ± 0·61

($p<0·01$)

* µg porphyrin / g wet wt. / µg RNA phosphorus.

Estimation

Estimation of RNA phosphorus was performed by the orcinol reaction as described by Schneider(309). The reagent for this reaction is made up by dissolving 0.25g orcinol (BDH) and 0.125g ferrous chloride in 25ml. conc. HCl. RNA (0.1 ml) was added to 1.4 ml. Na acetate (0.1M pH 6.0) and 1.5 ml. orcinol reagent. After boiling 20 minutes the optical density of the green solution is read at 660mμ, and the content of RNA phosphorus estimated from the formula

$$\frac{(OD + 0.008) - (DNA \times 0.013)}{0.116} .$$

100 μg RNA phosphorus has been found to contain 26.5μg RNA, by correlation of a standard curve for RNA phosphorus with the OD obtained for known amounts of RNA.

In the experiments presented, no attempt was made to add the same amount of RNA on each occasion, and the amount of porphyrin produced was expressed per gram wet weight of liver, and per μg RNA phosphorus added.

(iii) The results of the addition of transfer and ribosomal fractions from control and porphyric rat livers are shown.

Table XIX shows the result of addition of the tRNA fractions from control and porphyric rat livers. In 7 experiments, the mean increase of porphyrin production over control values was 1.0 $\pm$ 0.61 which is significant ($p < 0.01$). In all experiments a small increase

Table: XX

Experiments to show the increase in porphyrin production by tissue culture after addition of rRNA from control and porphyric rats (method I)

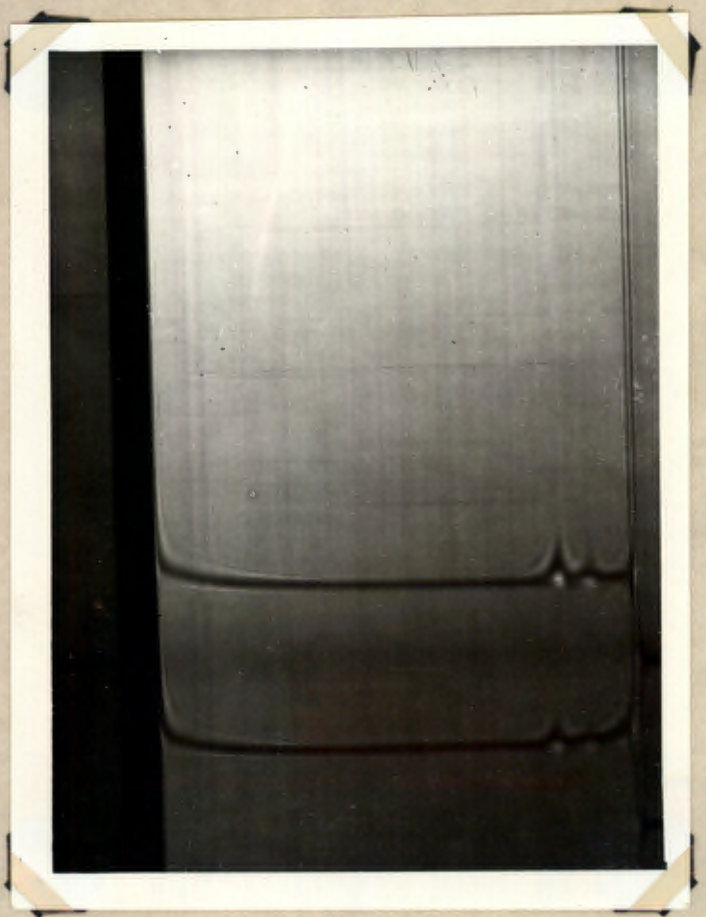
CONTROL				PORPHYRIC			
µg RNA phosph.	COOH gr.	µg porphyrin per g w.w.	Total*	µg RNA phosph.	COOH gr.	µg porphyrin per g w.w.	Total*
330	2 4 8	0·07 0·16 0·07	1·5	242	2 4 8	1·25 0·22 0·11	6·5
654	2 4	0·54 0·32	1·3	725	2 4 8	2·80 0·81 1·16	6·3
621	8	0·43	0·7	609	2 4 8	0·27 0·33 0·75	2·2
289	4 8	0·33 0·46	2·7	369	2 4 8	1·26 0·38 0·63	6·1
312	2 4	0·69 0·05	2·4	441	2 4 8	0·12 0·67 2·17	6·7
261	4 8	0·04 0·16	0·8	535	2 4 8	0·40 0·01 0·81	2·3
597	2 4 8	0·29 0·34 0·22	1·4	304	2 4 8	1·28 0·42 0·71	7·1

MEAN INCREASE: Porphyric - Control :: 3·8 ± 1·7

($p<0·001$)

*µg porphyrin/g wet wt./µg RNA phosphorus

Fig. XVIII



rRNA 28 & 18S

tRNA 28, 18 & 4S

The schlieren patterns of tRNA and rRNA in a Spinco model E ultra-centrifuge at 56,000 r.p.m.

of porphyrin synthesis was noted. In Table XX, the results of the addition of rRNA fractions from control and porphyric rat livers are shown, and it may be seen that the mean increase in 7 experiments is greater, namely 3.8 $\pm$ 1.71 which is significant ($p < 0.001$). The difference between the increased porphyrin synthesis resulting from the addition of the t- and rRNA fractions is also significant ($p < 0.02$).

(iv) The identification of RNA

The identity of the RNA was established by several methods.

(a) sedimentation constants. The sedimentation constants of the 2 fractions were determined in a spinco model E ultracentrifuge with the invaluable assistance of Dr. A. Polsen. The schlieren patterns are shown in fig. XVIII for both fractions of RNA, and from these, the constants were calculated. For the ribosomal fraction, the constants were 18S and 28S, and for the transfer fraction, predominantly 4S with some contamination by ribosomal RNA revealed by the presence of small peaks at 18S and 28S. This contamination has been suspected due to the slight apparent induction of porphyrin synthesis caused by the transfer fraction. (see Table XIX).

(b) absorption spectrum. Further study of the ribosomal fraction showed an absorption spectrum in 0.1M acetate buffer pH 5.2 with a characteristic peak at 258mμ, and an absorbance ratio 258/280 mμ of 1.9.

(c) sucrose gradient. The sedimentation profile in a linear sucrose gradient 5-25% revealed a ratio between 28S and 18S components of 2:1. This is compatible with the known characteristics of ribosomal RNA(310).

Table : XXI

Experiments to show the lack of effect on porphyrin production by tissue culture after the addition of tRNA and rRNA extracted from normal liver to which 200 mg A.I.A had been added just prior to RNA extraction.

SPECIMEN	COOH gr.	µg porphyrin /g w.w.	total	ng p./g w.w/ µg RNA-P
Control	4	0·15	0·15	
rRNA 275µg*	2 4	0·11 0·11	0·22	0·72
rRNA 280µg*	4 8	0·10 0·08	0·18	0·68
tRNA 159 µg*	4	0·14	0·14	0·88
tRNA 163 µg*	4	0·12	0·12	0·76

* expressed as µg RNA phosphorus

(v) Interpretation of results.

This porphyrin induction could be interpreted in several ways other than being the result of stimulus by the RNA:-

(a) The fraction might be contaminated with AIA carried over in the RNA extraction.

(b) The enzyme ALA-synthetase, or other enzymes of the porphyrin biosynthetic pathway might be contaminating the RNA fraction.

(c) Other protein substances of unidentified nature might be present and stimulate porphyrin induction.

(d) Contamination with DNA might have occurred in the course of RNA extraction.

(e) The fraction might contain the porphyrin precursors ALA and PBG carried over from rat liver.

(a) Contamination with AIA was excluded by addition to a normal rat liver (8.0g) the amount of AIA fed to a 200g rat (200mg), and extraction of the two fractions from this liver. Addition of this RNA to tissue culture resulted in no increased porphyrin production (Table XXI).

(b) Estimation of the protein content of a solution of 50µg RNA was performed using the Folin Ciocalteau method(311). There was 55 µg protein present.

The amount of the protein material present was also detected by paper chromatography of the amino acids present after hydrolysis using the method described by Efron et al(312). A sample containing

Table: XXII

Experiments to show the lack of inhibition by trypsin on the increased porphyrin production by tissue culture resulting from the addition of rRNA.

	SPECIMEN	COOH groups	µg porphyrin / g wet wt.	µg porphyrin/g wet wt/µg RNA-P
EXPT. I	Control RNA 300µg*	2 4 8	0·42 0·11 0·22	2·5
	rRNA 375µg*	2 4 8	0·57 0·40 0·75	4·6
	rRNA 375µg* + trypsin 550µg	2 4 8	0·38 0·22 1·18	4·7
	trypsin 550µg	2 4 8	0·24 0·18 0·34	total porphyrin 0·76
II	Control RNA 325µg*	2 4	0·41 0·22	1·9
	rRNA 500µg*	2 4 8	0·59 0·51 0·92	4·0
	rRNA 500µg* + trypsin 550µg	2 4 8	0·61 0·72 0·84	4·3
	trypsin 550µg	4	0·59	total porphyrin 0·59

* expressed as µg RNA phosphorus

approximately 100 µg RNA was hydrolysed with 5 ml. HCl 7.5N at 120°C for 16 hours (1 ml. 7.5N HCl hydrolyses 1 mg protein). The solution remaining was dried under a stream of nitrogen and redissolved in 1.5N NCl. Amounts of the solution from 1 µl to 100 µl were spotted on chromatography paper which was developed in a solvent of butanol: acetic acid:water (12:3:5) for 14-16 hours. The paper was dried at a temperature of 60°C for 15 minutes and then immersed in a solution of 2% ninhydrin in acetone. Further drying at 60°C revealed a trace of glycine which was barely perceptible when compared with a standard solution. This trace was thought to be of little significance in comparison with the amount of RNA present. Granick has shown in tissue culture preparations that glycine alone has no porphyrin inducing effect(91).

(c) Additional evidence that the porphyrin-inducing capacity was not due to protein contamination was obtained when RNA pre-incubated with trypsin (1 mg at 37°C for 60 minutes at pH 7.0) induced porphyrin synthesis as before (Table XXII).

(d) In the course of each calculation for the orcinol reaction the amount of DNA was estimated by the diphenylamine reaction(309). There was always negligible DNA present.

(e) The standard method of ALA and PBG determination used in the laboratory(50) was applied to 1 ml. of a solution containing approximately 100 µg RNA. After adjusting the pH to 6.2 with glacial acetic

acid, the solution was added to an anion column of Amberlite CG400 type I. The column was washed twice with 2 ml. H_2O and these washings added to a cation column of Amberlite CG100 type I. The PBG was eluted from the first column with 2 ml. 1N acetic acid and 0.2 ml. 0.2N acetic acid. The collected eluate was made up to 10 ml. with H_2O, and to 2 ml. was added 2 ml. Erlich's reagent (2% dimethylaminobenzaldehyde in 6N HCl). The optical density was read immediately at 555mμ and the amount of PBG calculated in mg/l from the formula O.D.x 126. The reading, and thus the PBG content, was zero. The cation column was washed with 25 ml. water and with 3 ml. 0.5M Na acetate to wash out retained water. The ALA was eluted with 7 ml. 0.5M Na acetate, and 0.2 ml acetyl acetone was added to the eluate. The solution was made up to 10 ml. with acetate buffer pH 4.6. After shaking, the solution was boiled for 10 minutes to convert ALA to the pyrrole. When cool, 2 ml. Erlich's reagent (2% dimethylaminobenzaldehyde in glacial acetic acid + 16% perchloric acid) was added, and after standing 10 minutes, the optical density was read at 553mμ. The ALA content in mg/l was calculated from the formula O.D.x 45.9. This reading was also zero.

(vi) <u>The effect of RNase upon RNA extracted from rat liver</u>:

RNA was incubated with RNase (in an amount equal to the μg RNA phosphorus in the solution, at 37°C for 60 minutes at pH 5.0), and the mixture added to tissue culture. No porphyrin synthesis occurred.

TABLE XXIII (a)

Experiments to show the inhibition by RNase of the rRNA-induced increase in porphyrin production by tissue culture, and to show the lack of effect of SLS after incubation of RNA with RNase.

µg RNA phos.	rRNA			rRNA + RNase			(rRNA + RNase) + SLS		
	COOH gr.	µg porph./ g w.w.	ng porph/ gw.w./µgRNA	COOH gr.	µg porph/ g w.w.	ng porph/ gw.w./µgRNA	COOH gr.	µg porph/ g w.w.	ng porph/ gw.w./µgRNA
578	2	1·72	6·3	2	0·66	1·5	2	0·90	2·2
	4	0·40					4	0·23	
	8	1·45		4	0·17		8	0·23	
540	2	1·76	5·8	2	0·80	2·1	4	0·85	1·6
	4	0·95							
	8	0·49		4	0·36				
275	2	0·61	5·6	2	0·28	1·8	2	0·28	1·5
	4	0·61							
	8	0·34		4	0·20		4	0·14	
240	2	0·23	3·8	2	0·20	2·2	2	0·15	1·5
	4	0·30							
	8	0·40		4	0·32		4	0·21	

MEAN INCREASE: rRNA – (rRNA + RNase) : 3·5 ± 1·4

(rRNA + RNase) –

(rRNA + RNase) + SLS : 0·2 ± 0·6

rRNA – (rRNA + RNase) + SLS : 3·4 ± 0·9

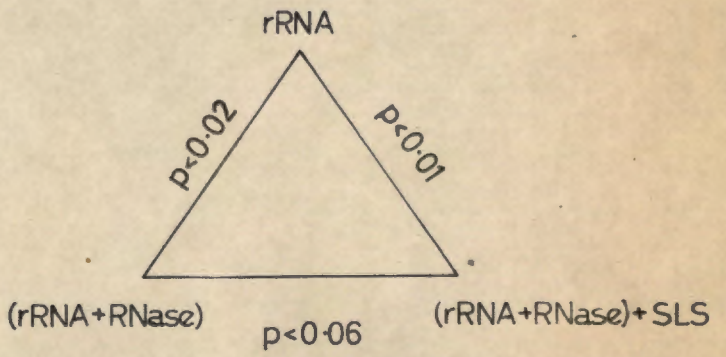


TABLE XXIII(b)

Experiments to show the inhibition by RNase of the rRNA-induced increase in porphyrin production by tissue culture, and to show the effect of incubation of RNase with SLS prior to incubation with RNA.

µg RNA phos.	rRNA			rRNA + RNase			rRNA + (RNase + SLS)		
	COOH gr.	µg porph./ g w.w.	ng porph./ gw.w./µgRNA	COOH gr.	µg porph./ gw.w.	ng porph./ gw.w./µgRNA	COOH gr.	µg porph./ gww.	ng porph./ gw.w./µgRNA
242	2	1·06	6·7	2	0·43	2·3	2	1·10	7.3
	4	0·48					4	0·52	
	8	0·11		4	0·11		8	0·14	
323	2	0·41	5·7	2	0·12	2·0	2	0·88	5.7
	4	0·30		4	0·12				
	8	1·14		8	0·33		4	0·96	
480	2	1·92	5·9	2	0·80	2.3	2	1·46	5.9
	4	0·87					4	0·77	
	8	0·04		4	0·30		8	0·61	
431	2	1·13	4·1	2	0·33	2.2	2	1·41	4.3
	4	0·62					4	0·12	
	8	0·03		4	0·62		8	0·32	

MEAN INCREASE: rRNA – (rRNA + RNase) : 3·4 ± 1·1

(rRNA + RNase) –

rRNA + (RNase + SLS) : 0·2 ± 0·3

rRNA – rRNA + (RNase + SLS): 3·6 ± 1·2

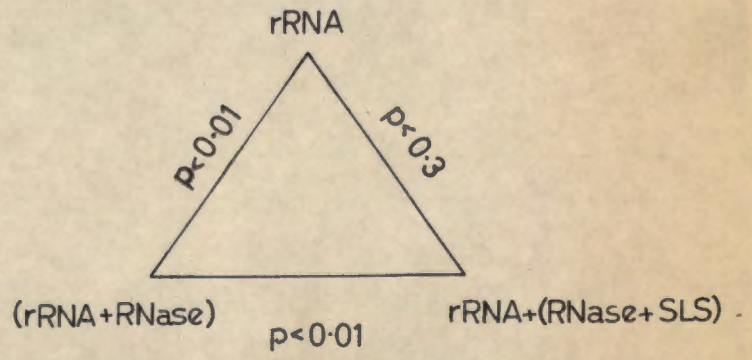


Table : XXIV

Experiments to show the inhibitory effect of RNase upon the increased porphyrin production by tissue culture after the addition of AIA.

Expt. No.	CONTROL			AIA 3mg			AIA 3mg + RNase 500µg		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
I	4 8	0·21 0·36	0·57	2 4 8	0·33 0·70 0·36	1·39	4 8	0·09 0·24	0·33
II	4	0·53	0·53	2 4 8	0·42 0·40 0·94	1·76	2 4	1·03 0·32	1·35
III	8	0·85	0·85	2 4 8	0·54 0·52 0·57	1·63	2 4 8	0·29 0·46 0·18	0·93
IV	4 8	1·22 0·53	1·75	2 4 8	0·19 4·92 0·65	5·76	2 4 8	0·33 1·26 0·45	2·04
V	4	0·23	0·23	2 4 8	0·65 1·30 0·50	2·45	2 4	0·36 0·30	0·66
VI	2 4 8	0·50 0·31 0·41	1·22	2 4 8	0·83 0·70 1·83	3·36	2 4 8	0·82 0·51 0·11	1·44
VII	2 4 8	0·38 0·43 0·99	1·80	2 4 8	0·58 0·48 2·13	3·19	2 4 8	0·37 0·52 0·52	1·41

MEAN INCREASE; AIA - CONTROL :: 1·8 ± 1·1

AIA - AIA + RNase :: 1·6 ± 1·1

CONTROL - AIA+RNase :: - 0·2 ± 0·4

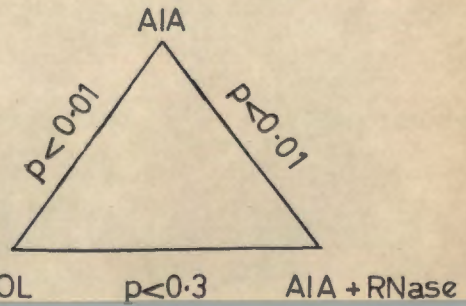


Table: XXV

Experiments to show the inhibitory effect of RNase upon the increased porphyrin production by tissue culture after the addition of AIA ; and the protection afforded by the addition of S.L.S. to the AIA+RNase solution prior to addition to tissue culture.

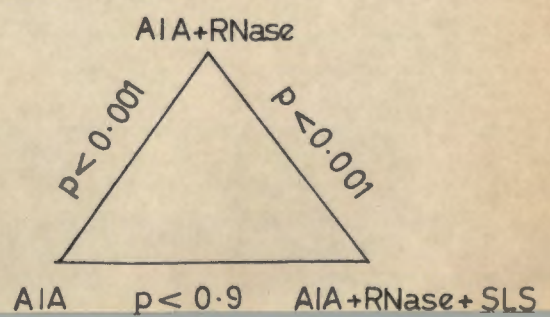
Expt. No.	AIA 3mg			AIA 3mg + RNase 500µg			AIA 3mg+RNase 500µg+SLS 50mg		
	COOH gr.	µg porphyrin/g w.w.	Total	COOH gr.	µg porphyrin/g w.w.	Total	COOH gr.	µg porphyrin/g w.w.	Total
I	2	0·44	2·13	2	0·16	0·93	4	0·30	1·92
	4	0·69		4	0·24		8	1·62	
	8	1·00		8	0·53				
II	2	0·84	2·07	8	0·63	0·63	4	1·85	2·39
	4	0·32					8	0·54	
	8	0·91							
III	2	1·63	2·19	2	0·09	1·77	2	1·55	2·54
	4	0·44		4	1·10		4	0·99	
	8	0·12		8	0·58				
IV	2	1·34	2·70	2	0·67	0·85	2	0·28	2·42
	4	1·21		4	0·18		4	1·63	
	8	0·15					8	0·51	
V	2	0·42	1·76	2	0·26	0·63	2	0·25	1·55
	4	0·40		4	0·07		4	1·10	
	8	0·94		8	0·30		8	0·20	
VI	2	0·54	1·63	2	0·91	0·96	4	0·99	2·75
	4	0·52		4	0·02		8	1·76	
	8	0·57		8	0·03				

MEAN INCREASE: AIA - AIA+RNase :: 1·2 0·4

AIA+RNase+SLS -

AIA+RNase :: 1·4 0·4

AIA - AIA+RNase+SLS :: -1·1 0·8



This inhibition could have resulted from the effect:-

(i) of RNase upon RNA, or

(ii) of RNase upon tissue culture.

The detergent sodium lauryl sulphate was used in 100x concentration to destroy RNase. After the addition of SLS to the pre-incubated RNA + RNase mixture again no porphyrin synthesis occurred. (Table XXIIIa). However, when SLS was added to RNase and this mixture added to RNA, the porphyrin production by tissue culture to which the mixture was added, was increased. (Table XXIIIb).

These experiments showed that the RNase had acted specifically upon the RNA, so that the induction of porphyrin synthesis by the RNA was inhibited.

(vii) <u>The effect of RNase upon the RNA presumed to be induced in tissue culture by AIA</u>.

When RNase was added simultaneously with AIA to tissue cultures, less porphyrin synthesis occurred. (Table XXIV). To exclude the possibility that this was due to an effect of RNase upon AIA, after mixture of RNase with AIA, SLS was added and the mixture added to tissue culture. Porphyrin synthesis occurred in the amount usually seen with AIA. (Table XXV).

From Table XXIV it may be seen that the mean increase in porphyrin synthesis induced by the addition of AIA to tissue culture was 1.80 ± 1.15 which is significant ($p < 0.01$).

With prior incubation of AIA with RNase, there was no significant increase in mean porphyrin production when the control and experimental cultures were compared ($p < 0.2$).

From Table XXV, it may be seen that the addition of SLS to the AIA + RNase mixture protected from the inhibitory effect of RNase and porphyrin synthesis was significantly increased by 1.44 ± 0.38 ($p < 0.001$).

It is possible therefore, that incubation of AIA with RNase alone prevented porphyrin production because of interference with the action of RNA induced in explants by AIA in the tissue culture medium.

EXPERIMENTS USING RIBOSOMAL AND RIBOSOMAL + "MESSENGER"RNA.

It appeared from these experiments that a ribosomal fraction from a porphyric rat liver could induce porphyrin synthesis in tissue culture of chick embryo liver cells. The ribosomal fraction was thought to be that which was already coded for protein synthesis - that is, it had already received the message to code for ALA-synthetase synthesis.

Since the aim was to prove that it is the messenger RNA which is responsible for the effect, a technique was employed for the extraction of a ribosomal plus "rapidly labelled" or messenger fraction.

(i) Method.

This method was described by Parish and Kirby (210), and employs a phenol-cresol mixture in place of phenol as in the first method

The absorption spectrum of rRNA fraction in 5-20% sucrose gradient correlated with the radio-activity in each peak. Each rat was given 1 μcurie C-14 orotic acid 20′ prior to RNA extraction.

Fig: XIX

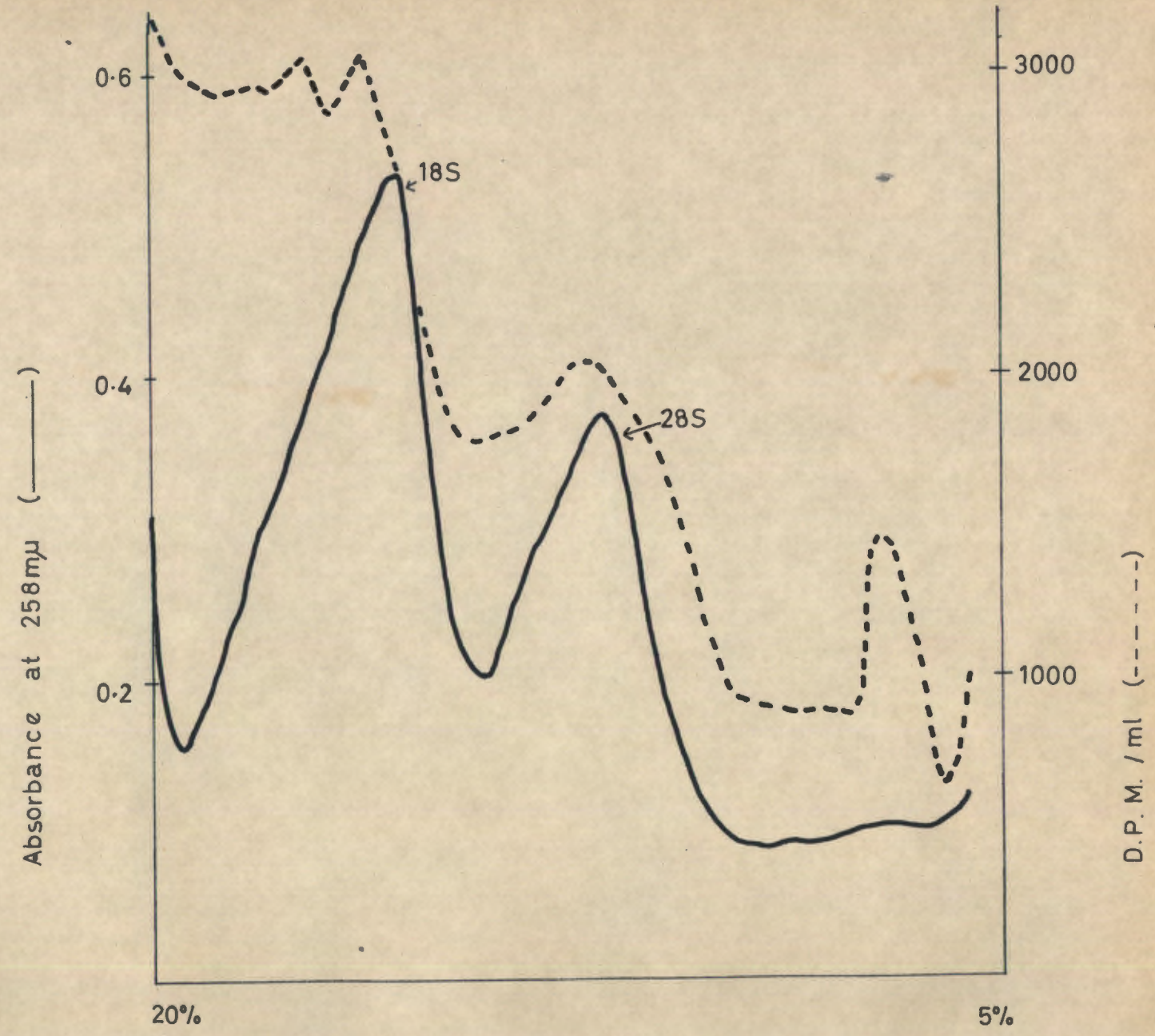


TABLE XXVI

Experiments to show the increase in porphyrin production by tissue culture after the addition of rRNA extracted from livers of rats made porphyric with AIA (method II)

CONTROL				PORPHYRIC			
µg RNA phos.	COOH gr.	µg porphyrin / g w.w.	ng porphyrin / g w.w./µg RNA	µg RNA phos.	COOH gr.	µg porphyrin / g w.w.	ng porphyrin / g ww./µg RNA
191	4	0·20	1·0	204	4	0·91	4·4
364	4	0·64	1·8	245	4	1·29	5·2
324	4	0·20	1·5	170	4	0·31	3·8
	8	0·32			8	0·34	
181	4	0·18	1·9	165	4	0·15	2·6
	8	0·17			8	0·28	
179	2	0·15	1·3	156	2	0·07	3·4
	4	0·05			4	0·33	
	8	0·04			8	0·12	
219	2	0·06	1·2	208	2	0·16	4·8
	4	0·17			4	0·51	
	8	0·03			8	0·17	

MEAN INCREASE: PORPHYRIC - CONTROL 2·6±1·2

($p<0.01$)

TABLE XXVII.

Experiments to show the increase in porphyrin production by tissue culture after the addition of (r+m)RNA extracted from livers of porphyric rats (method II)

CONTROL				PORPHYRIC			
µg RNA phos.	COOH gr.	µg porph./ g w.w.	ng porph./ gww./µg RNA	µg RNA phos.	COOH gr.	µg porph./ g ww.	ng porph./ gww/µg RNA
107	4	0·23	2·2	129	4	1·30	10·0
340	4	1·05	3·1	100	4	1·56	15·6
184	4 8	0·16 0·46	3·4	127	4 8	0·32 1·41	13·6
160	4 8	0·19 0·13	2·0	125	4 8	0·80 0·59	11·1
254	4	0·35	1·4	190	4	2·01	10·5
368	4 8	0·55 0·33	2·4	240	4 8	1·08 0·99	8·6
294	4 8	0·40 0·25	2·2	352	4 8	1·73 0·88	7·4
288	4	0·44	1·5	225	4	2·40	10·6
360	4 8	0·41 0·13	1·5	281	4 8	1·37 1·26	9·4
367	2 4 8	0·29 0·46 0·27	2·8	270	2 4 8	1·08 1·29 0·97	11·9
225	2 4 8	0·07 0·10 0·19	1·6	272	2 4 8	1·56 0·84 0·42	10·4
315	2 4 8	0·55 0·18 0·19	2·9	139	2 4 8	1·18 0·31 0·17	11·9

MEAN INCREASE: PORPHYRIC – CONTROL: 8·5 ± 1·9

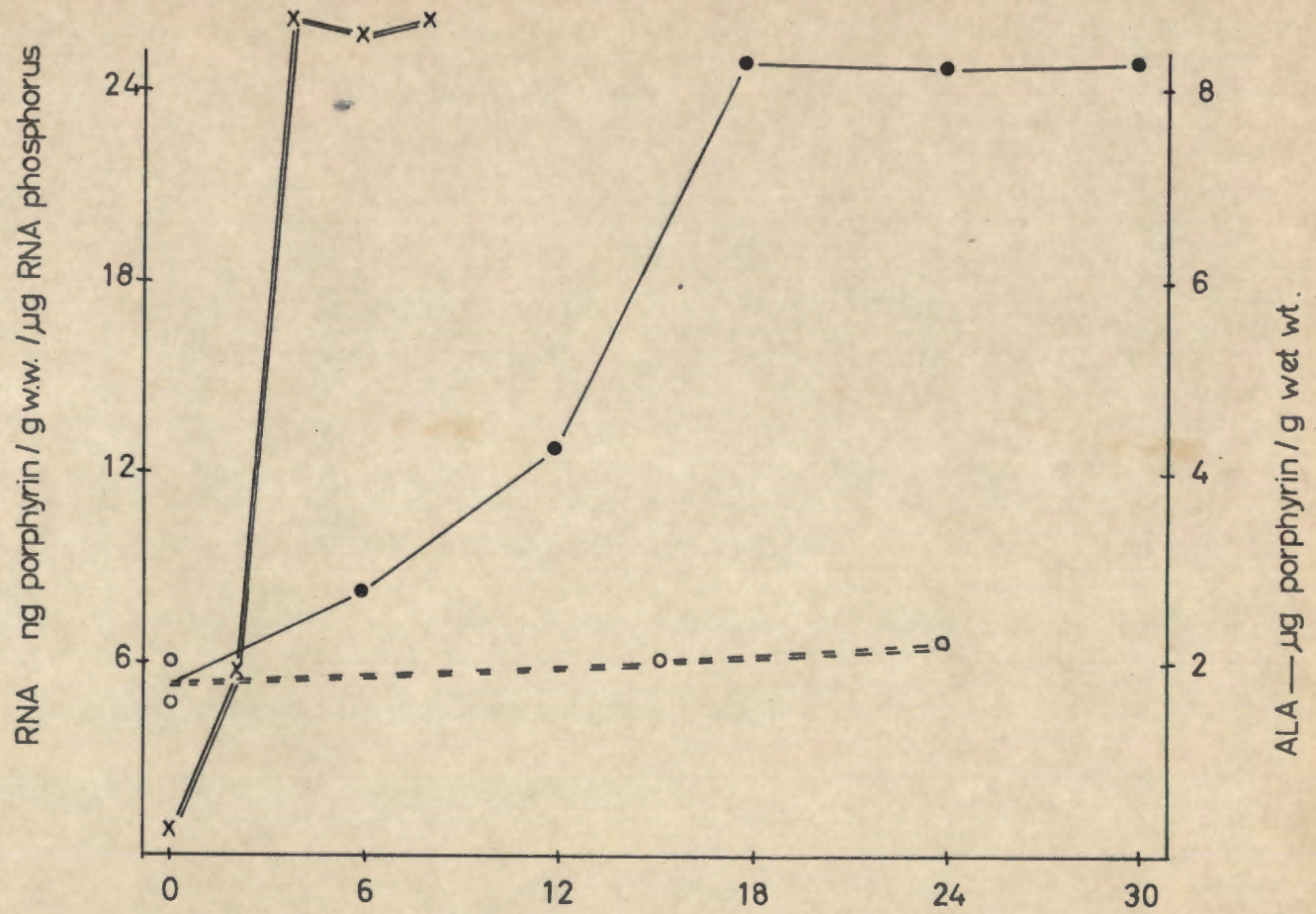
($p < 0.001$)

described, for dissociation of the nucleoproteins and for removal of residual protein and polysaccharide. The dissociation is further aided by increasing concentrations of sodium chloride and the removal of proteins by the addition of 4-amino-salicylic acid. The liver was homogenised in 10 vols. medium (SLS-1g; NaCl-1g; 4-amino-salicylic acid 6g; secondary butanol 6 ml.; H20 100 ml.) and extracted in an equal volume of phenol:cresol mixture by stirring for 20 minutes at room temperature. The mixture was centrifuged at 15,000 rpm for 30 minutes and the aqueous supernate removed. Sodium chloride 2g per 100 ml. supernate was added and the mixture stirred with a half volume phenol:cresol for 10 minutes. After centrifugation at 15,000 rpm for 10 minutes, the supernate was removed and 2 vols ethanol:cresol mixture (9:1) was added. The mixture was left at -4°C overnight, and then centrifuged at 10,000 rpm for 10 minutes. The resultant pellet was dissolved in 0.1M Na. acetate pH 6.0, and the solution made 3.0M with respect to NaCl. After standing for 16 hours at -15°C, the mixture was centrifuged at 6,000 rpm for 10 minutes and washed twice with 3.0M Na acetate pH 6.0. The final pellet could be dissolved in Na acetate 0.1M pH 6.0, or stored under ethanol:water 75:25 + 2g Na Cl. The sucrose gradient analysis of this (r + m) RNA is shown (fig. XIX)

(ii) Results.

The results of the addition of these two fractions are shown. (a) Table XXVI shows the results of 6 experiments with the addition of rRNA from porphyric and control rats to tissue culture using the

Fig: XX Experiments to show the kinetics of the increase in porphyrin productions by tissue cultures after the addition of (r+m)RNA and the addition of ALA



second method of RNA extraction. The mean increase in porphyrin production induced by this fraction is 2.6 $\pm$ 1.16 which is significant ($p < 0.01$). From Table XXVII however, it may be seen that the porphyrin induction stimulated by (r + m) RNA was far greater. The mean increase in 12 experiments is 8.5 $\pm$ 1.89, and this result, and a comparison of the increases produced by rRNA and (r + m) RNA are significant ($p < 0.001$).

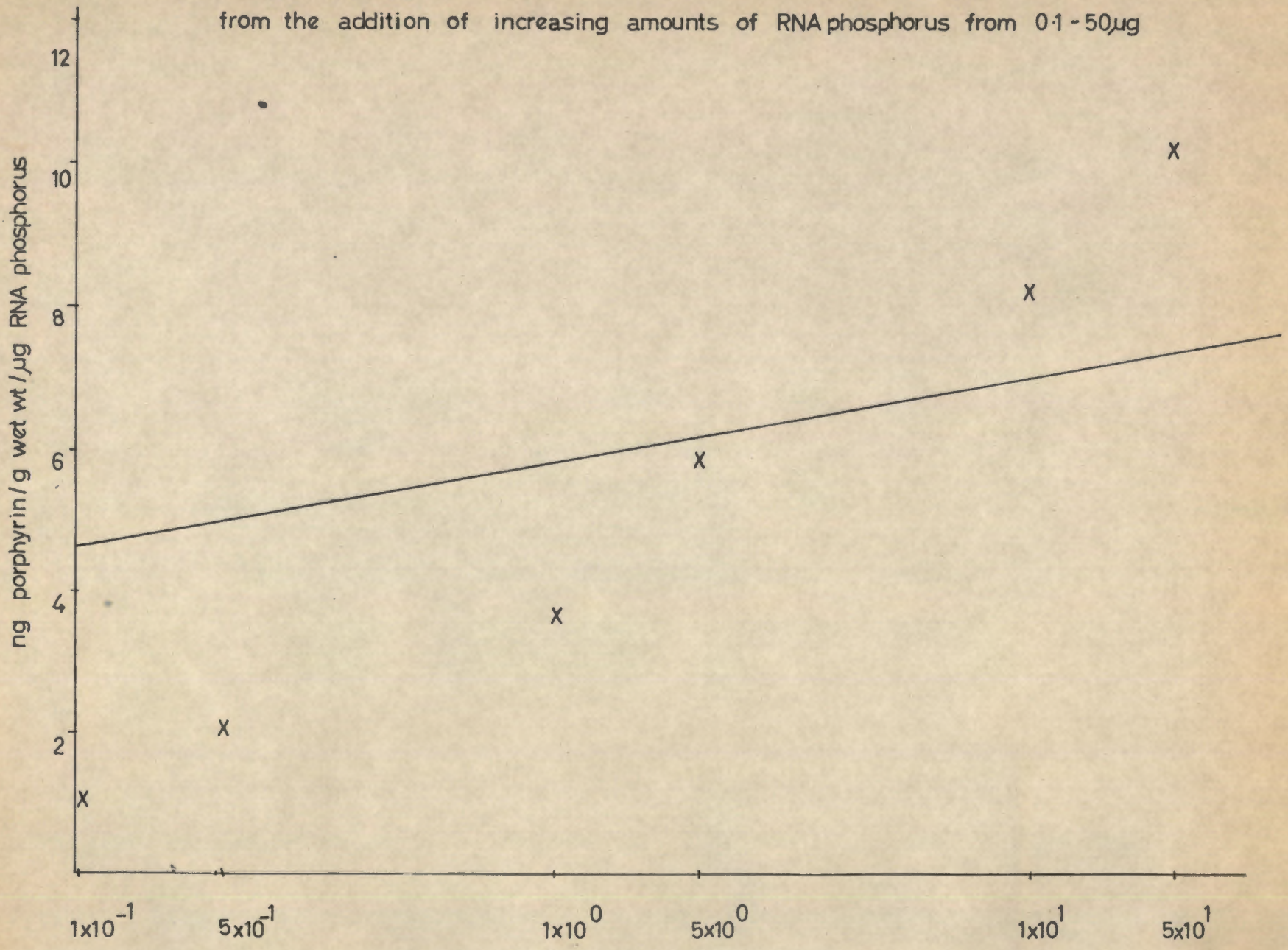
On two occasions the extraction of ribosomal RNA was delayed beyond the customary 42 hours. In contrast to results shown above for the ribosomal fraction alone, the results of addition of this "late ribosomal" fraction also showed increased porphyrin production. This was interpreted as being due to completed incorporation of the message in the ribosomal fraction. In the initial "early" ribosomal fraction, such incorporation had probably not yet occurred.

The fact that increase in porphyrin production induced by a purer fraction of RNA was greater provided some evidence for the actual incorporation of the RNA into the explants in tissue culture. Further evidence was obtained after the performance of a time study.

(b) <u>time study</u> of the rate of increase in porphyrin production following addition of RNA to the cultures. RNA was added and the porphyrins extracted at increasing intervals thereafter. Fig.XX shows the kinetics of the increase in porphyrin production induced by RNA compared with that induced by ALA. The delay of 12 to 18 hours in

Fig: XXI

The increase in porphyrin production by tissue culture resulting from from the addition of increasing amounts of RNA phosphorus from 0·1 - 50µg



attainment of maximal porphyrin production is interpreted as evidence that metabolism of the RNA occurred, whereas in the case of ALA, the immediate precursor of porphyrins was provided and required little metabolism.

(c) Dose response.

Amounts of RNA increasing from 0.1 to 100 µg were added to tissue culture and the porphyrin production response is shown in fig. XXI. The increasing porphyrin production with increasing RNA addition is further evidence suggestive of the incorporation of the RNA.

CONCLUSION

With the exclusion of any contaminating protein material or porphyrin precursors, and with the use of a more refined technique of extraction of a fraction containing messenger RNA, it was felt reasonable to conclude that the increase in porphyrin synthesis could be ascribed to the RNA confirming the hypothesis presented in chapter VI. Since ALA-synthetase is a mitochondrial enzyme, it was assumed that some form of incorporation of the RNA into the explants had taken place. The problem of the proof of entry of RNA into cells, and whether this occurs in a whole or denatured form, has become of great importance in many experiments claiming the effect of "alien" RNA upon cells in culture. Such reports as are presently available, will be discussed in chapter IX.

TABLE XXVIII

Experiments to show the lack of effect of ATP on the increase in porphyrin production by tissue culture after the addition of ALA

Experiment No:	ALA 1µg/ml			ALA + ATP 30µmole/ml		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
I	2	5·94	13·08	2	7·38	15·38
	4) 5)	1·49		4) 5)	1·49	
	7) 8)	5·65		7) 8)	6·51	
II	2	4·77	52·72	2	3·36	52·37
	4) 5)	3 25		4) 5)	1 59	
	7) 8)	44·70		7) 8)	47·22	
III	2	2·00	66·04	2	1·32	58·23
	4) 5)	0·79		4) 5)	10·98	
	7) 8)	63·25		7) 8)	45·93	
IV	2	17·48	33·30	2	15·04	31·18
	4	12·40		4	11·97	
	8	3·42		8	4·17	
V	2	26·04	50·55	2	27·92	52·35
	4	13·61		4	13·01	
	8	10·90		8	11·42	

MEAN INCREASE: ALA − (ALA+ATP) : −1·24 ± 4·07
($p > 0·9$)

TABLE XXIX

Experiments to show the inhibitory effect of ATP on the increase in porphyrin production by tissue culture after the addition of AIA

Expts. No.	CONTROL			AIA 3mg			AIA + ATP 30µm		
	COOH gr.	µg porphyrin per g w.w.	Total	COOH gr.	µg porphyrin per g w.w.	Total	COOH gr.	µg porphyrin per g w.w.	Total
I	2	0·13	0·62	2	0·04	5·25	2	0·23	1·26
	4	0·25		4	0·17		4	0·81	
	8	0·24		8	5·04		8	0·22	
II	2	0·93	1·48	2	0·86	3·32	2	0·65	2·54
	4	0·23		4	0·78		4	0·95	
	8	0·32		8	1·68		8	1·44	
III	2	0·47	0·88	2	3·08	3·15	2	0·97	1·03
				4	0·03				
	8	0·41		8	0·04		4	0·06	
IV	2	0·63	0·82	2	1·20	1·89	2	0·24	0·51
				4	0·23				
	4	0·19		8	0·46		4	0·27	
V	2	0·58	2·80	2	0·58	4·21	2	0·18	0·48
	4	0·43		4	0·48		4	0·21	
	8	1·79		8	3·15		8	0·09	
VI	2	0·44	0·66	2	1·21	1·83	2	0·27	0·81
	4	0·22		4	0·62		4	0·54	

MEAN INCREASE: AIA-CONTROL : 1·96±1·25
AIA-(AIA+ATP) : 2·14±1·27
CONTROL-(AIA+ATP): -0·26±1·07

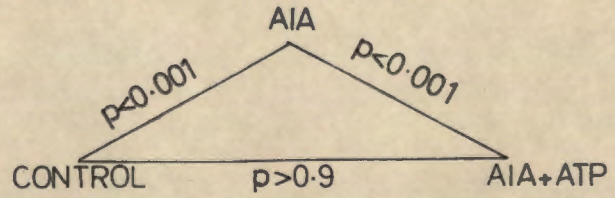


TABLE XXX

Experiments to show the lack of inhibition by ATP on the porphyrin production by control tissue cultures.

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin/ g w.w.	Total	COOH gr.	µg porphyrin/ g w.w.	Total
CONTROL	2	0·15	1·86	2	0·89	2·49
	4	0·22				
	8	1·49		4	1·60	
ATP	2	0·36	1·50	2	0·83	2·81
	4	0·35				
30µm.	8	0·79		4	1·98	
ATP	2	0·62	1·84	2	0·77	2·91
	4	0·74				
30µm.	8	0·43		4	2·14	
ATP	2	0·50	1·78	2	0·84	2·76
	4	0·27		4	0·49	
30µm.	8	1·01		8	1·43	

CHAPTER VIII THE INHIBITORY EFFECT OF ATP:GLUCOSE ON PORPHYRIN SYNTHESIS STIMULATED BY AIA.

"Underlying the extreme complexity we may find a simplicity which escapes us".
F.Gowland Hopkins. 1917.

THE INHIBITORY EFFECT OF ATP IN TISSUE CULTURE

In view of the success claimed by Gajdos in the treatment of patients with AMP (see chapter II), the effect of ATP in tissue culture was tested.

(i) Results

The addition of ATP to cultures in which fluorescence was induced with ALA had no effect on the increase of porphyrins (Table XXVIII). However, in cultures with added AIA, the induction of fluorescence and porphyrin production were reduced almost to control levels by the addition of ATP (Table XXIX). The addition of ATP to control cultures had no effect (Table XXX).

From these results, it may be suggested that the action of ATP occurs at the level of ALA-synthetase or preceding enzymes. As has been mentioned, the role of ATP is complex and especially difficult to interpret in tissue culture systems. However, it might be concerned in two factors of especial interest in porphyrin synthesis. These are:

(ii) Interpretation of results

(a) the activity of ATP in the transport of amino acids to the ribosome in protein synthesis, as described in chapter III. This

effect is of non-specific involvement in the control of all enzyme synthesis.

(b) The roles of oxidative phosphorylation and of NAD in the control of ALA-synthetase have been described in chapter II. The regulation of NAD has been studied especially in connection with the effect of alcohol on the liver (313)(314). An attempt has been made in this regard, to treat fatty liver with ATP (315). NAD has been shown to have roles in the Embden-Meyerhof pathway, the conversion of glucose and fructose to sorbitol, and in the metabolism of glyceraldehyde. This association of NAD and therefore ATP with glucose metabolism may be of significance in the control of porphyrin synthesis.

THE INHIBITORY EFFECT OF GLUCOSE IN VIVO.

(i) The effect of glucose described by Tschudy, has been discussed in chapter II. Administration of glucose apparently reduced the excretion of porphyrin precursors by a patient with acute intermittent porphyria. In a recent publication from the same laboratory, evidence has been presented of the inhibitory effect of glucose administration on the experimental porphyria in rats fed AIA. Using a similar design, an experiment was performed to test the effect in male and female rats, of the administration of glucose with AIA.

(ii) Materials (a) The animals were prepared with AIA as described in chapter VI. Glucose in aqueous solution was given in the dose of 12.5g/Kg with an additional dose 3 hours before commencement of the experiment.

(b) Blood for glucose analysis was collected into tubes containing both heparin and sodium citrate, using a heparinised plastic syringe, as the blood of the rat was found to coagulate particularly rapidly.

This is said to be a protective mechanism due to the social habits of the animals. It was previously ascertained that the presence of heparin did not alter the glucose analysis.

(c) Resins for the determinations of ALA-PBG were prepared as follows.

Preparation of resins for ALA and PBG determinations -

for ALA - Amberlite CG 120 type I - was resuspended 3x in approximately 2 vols. 4N NaOH and stirred on each occasion for 4 hours. It was then washed in a Buchner funnel until the reaction was neutral to litmus, and was resuspended three times in 2N HCl (stirred for 4 hours each). Washing was again performed in a Buchner funnel until the resin was free of chloride as judged by the $AgNO_3$ test. The resin was stored under an equal volume of H_2O.

for PBG - Amberlite CG 400 type I - was resuspended 6x in 4 vols. distilled water. It was then converted to the acetate form in a large column, by running through 1M Na acetate until the eluate was free of chloride as judged by the $AgNO_3$ test, and washed with H_2O until free of Na as judged by the flame test. The resin was stored in 1N HCl.

(iii) Methods

After preparation, the animals were anaesthetised lightly with ether, and cardiac puncture performed for blood glucose analysis. The method of Hagedoorn and Jensen(316) was modified for use on an auto-analyser.

Thereafter, the liver was removed onto cracked ice and divided for ALA-synthetase and liver porphyrin estimations, which were taken as the indices of experimental porphyria.

(a) ALA-synthetase estimations were performed by Mrs. T. Marks using a modification by Dowdle of the method described by Urata and Granick(281).

After rough weighing, the livers were washed in ice-cold buffer (KCl 0.14M, phosphate 0.05M), and then homogenised by mincing with scissors and in 2 volumes of tris EDTA (0.15M tris in 0.02M EDTA pH 7.2), using a Potter-Elvehjem-type homogeniser with a teflon pestle. One ml. of homogenate mixed with 1 ml. medium (0.6 ml. H20; 0.2 ml. glycine 1.0M, pH 7.4; 0.2 ml. Na. succinate, 0.5M) was incubated in an agitating water bath at 37°C for one hour. At the same time approximately 5 ml. homogenate was removed into stoppered tubes for later phosphorus determination, and a blank was prepared from 1 ml. homogenate, 1 ml. medium and 2 ml. 10% trichloracetic-acid. Incubation of the test samples was terminated by the addition of 2 ml. 10% T.C.A. After standing for 10 minutes to allow complete precipitation of proteins, and centrifugation at 3,000 rpm 2 ml. clear supernatant was added to 0.3 ml. 1N NaOH and 0.5 ml. Na. acetate, pH 5-6. The mixture was layered on a column of Dowex-1-acetate (1x3 cm) to adsorb PBG. The column was washed with 6 ml. H20, and the washing which contained amino-acetone and ALA, was combined with 1 ml. acetate buffer pH 4.6 and 0.5 ml. acetyl acetone. Boiling of this mixture for 10 minutes

converted amino-acetone and ALA to pyrrole forms. When cool, the mixture was added to a fresh column of Dowex-1-acetate, and the amino-acetone pyrrole eluted with n-butanol equilibrated with 0.01M NH4OH (approx. 10 ml.) The column was washed with 5 ml. 1M acetic acid, and the ALA-pyrrole eluted with 4 ml. glacial acetic acid:methanol 1:2. This solution was made up to 5 ml. with glacial acetic acid. An equal volume of Erlich's reagent was added and the optical density read after 15 minutes, at 552mμ in a Zeiss spectrophotometer. The blank was passed through a column and read in the same way. A standard solution containing 2.03 mg ALA was also prepared and read. ALA-synthetase was calculated according to the formula

$$\frac{2.03 \times 167.4 \times \text{sample O.D.}}{\text{standard O.D.}}$$

(167.4 = molec.wt. of ALA-HCl.)

and was expressed as mμmole/g x 10^{-6}.

It has been stressed(90)(317) that results of enzyme estimation expressed on a wet weight basis do not reflect the total enzyme activity of the whole organ. A choice of reference base should be directed towards reducing the influence of metabolically inert substances on the apparent enzyme activity with accurate assessment of the amount of tissue homogenised. Variations in liver glycogen, fat and especially porphyrin could be anticipated, as noted by Ginsburg(90), all of which could alter the ratio of active tissue to storage material. In these experiments, total tissue phosphorus was chosen as reference base. The estimation according to the method of Schaffer et al(318) is

relatively simple and gives an accurate steady base. Previous laboratory determination of total phosphorus in a number of rat livers had shown that on average, 3.1 g. phosphorus was present per gram of liver. Consequently, the phosphorus determinations were performed on samples of the homogenates used for ALA-synthetase determinations, and final enzyme levels expressed as

mμmole x 10^{-6}/mg total phosphorus/3.1 = mμmole/gram liver.

(b) Liver porphyrins were extracted using a method devised by Dr.G. Sweeney(319). After rough weighing, the liver was homogenised in 50 ml. acetone using a Virtis "45" homogeniser. One ml. conc. HCl. was added and the mixture shaken vigorously for 2 minutes. After filtration through a small Buchner funnel, the residue was returned to the flask to be shaken again with 25 ml. acetone and 2% conc. HCl, and then filtered. Half of the combined filtrate was added to an equal volume of water, and the pH adjusted to 2.0-2.5 with saturated sodium acetate. The porphyrins were extracted into 1.5 acetone:acid volumes of ethyl acetate, and then into a half volume of ethyl acetate:butanol 1:1. The three extracts were combined and washed once with water containing 0.005% iodine solution (1% in ethanol). A half volume petroleum ether (BP 40-60°C) was added and the porphyrins extracted into 1.5N HCl until UV-light screening was negative. This volume was recorded and the O.D. of the solution read on a Zeiss spectrophotometer between 380 and 430mμ. The porphyrin content was calculated from the formula:-

TABLE XXXI

Experiments to show the effect of glucose administration on various parameters tested in rats made porphyric with AIA.

Estimation	CONTROL (propylene glycol)		AIA (250mg/Kg x 4)		AIA +glucose (12·5g/Kg x 4)	
	m.	f.	m.	f.	m.	f.
Liver porphyrins µg/g	0·63 ±0·4	0·75 ±0·6	9·1 ±2·9	7·1 ±1·5	3·5 ±0·7	2·7 ±1·5
ALA synthetase mµM/g x 10^6	53·1 ±17·2	53·5 ±10·5	158·7 ±82·6	227·8 ±69·6	86·7 ±17·0	92·3 ±24·3
Blood glucose mg%	80·0 ±12·0	75·0 ±12·0	66·0 ±9·0	56·0 ±20·0	84·0 ±5·0	157·0 ±75·0

All results are expressed as a mean of 6 experiments ± standard deviation.

TABLE XXXIa

Mean increases and significances to accompany Table XXXI

Mean		ALA-synthetase	Blood glucose	Liver porphyrins
AIA-CONTROL	m	90·8 ± 8·9 p<0·001	-15·0 ± 1·4 p<0·05	8·3 ± 8·4 p<0·1
	f	176·3 ± 7·6 p<0·001	-20·5 ± 4·7 p<0·001	6·3 ± 1·7 p<0·001
AIA+glucose-CONTROL	m	37·5 ± 2·9 p<0·05	11·5 ± 11·9 p<0·1	2·7 ± 0·8 p<0·001
	f	38·8 ± 2·4 p<0·02	245·0 ± 74·9 p<0·001	2·1 ± 1·9 p<0·05
AIA-AIA+glucose	m	55·4 ± 6·2 p<0·01	18·0 ± 7·5 p<0·01	5·6 ± 2·7 p<0·01
	f	35·6 ± 6·6 p<0·01	101·0 ± 64·0 p<0·01	4·3 ± 0·3 p<0·01

Significances of ratios between results from male & female rats.

m/f ratio	ALA-synthetase	Blood glucose	Liver porphyrins
AIA	p <0·05	p < 0·4	p <0·2
AIA-glucose	p <0·7	p < 0·1	p <0·3

$$\frac{2\text{x}O.D._{max} - (O.D._{430} - O.D._{380}) \times \text{vol.} \times 563 \times 10^{-3}}{1.67 \times E_{mol.} \times \text{wt. liver}} = \mu g/g \text{ wet wt.}$$

563 = molec. wt. protoporphyrin

$E_{mol} = 2.75 \times 10^5$

This formula was originally suggested by Sveinsson and Rimington(320) to account for any background interference with optical density.

(v) Results

It may be seen from the results (Tables XXXI, XXXIa) that animals fed AIA became markedly porphyric with elevated levels of ALA-synthetase and liver porphyrins, and diminished blood glucose levels. The results are expressed as a mean of 6 experiments $\pm$ standard deviation. The mean increases in ALA-synthetase and blood glucose in both males and females are highly significant, as also is the increase in liver porphyrin production by female rats, (for all, $p < 0.05$). For the increase in liver porphyrin production by male rats the significance was $p < 0.05$.

The addition of glucose increased the blood glucose levels in both sexes. Despite the fact that blood for glucose analysis was taken 12 hours after the last administration of glucose, the levels in female rats were above the control levels. Administration of glucose to male rats returned the blood glucose to control levels.

In comparing the results for liver porphyrin and ALA-synthetase estimations in AIA and AIA + glucose-fed rats, a significant decrease

in both parameters is noted ($p < 0.05$). A significant increase occurred in blood glucose levels of glucose-fed rats ($p < 0.01$).

The comparison of all results for male and female rats does not show such significance. Thus although the administration of AIA results in marked experimental porphyria, and glucose appears to protect animals from this effect, no significant difference is noted in the protective effect of glucose in male and female animals. It should be stressed however, that this protective effect was afforded only by feeding massive doses of glucose. Extrapolation to human treatment would require the administration of 5Kg glucose to a 70Kg man.

(v) Discussion.

The inhibition of various enzymes by glucose was first described in 1942(321). The influence of the presence of glucose during growth on the enzymic activities of E. coli was noted. Since then, the effect has been observed upon an increasing number of enzymes(332). Inducibility is neither a sufficient nor a necessary condition for the effect - many inducible enzymes form in the presence of glucose (323). Also the effect is not entirely specific for glucose and has been noted with other closely related compounds (324).

Hypotheses which have been proposed to explain the glucose effect include:-

(i) interference with the transport mechanism for the inducer of the enzyme (325).

(ii) that the formation of glucose-degrading enzymes pre-empt the internal supply of amino-acids and nucleotides (326)(327).

(iii) diminution of the level of inorganic phosphorus in the cell preventing synthesis of inducible enzymes (328).

(iv) catabolite repression - viz. accumulation of catabolites of glucose which repress the formation of other enzymes(323).

(v) the action of glucose or a derivative of glucose as a co-repressor(329).

It has recently been postulated by Tschudy(329A) that the inhibition by glucose may in fact be mediated through the mechanism of RNA. This postulate is based on experiments by Nakada and Magasanik which showed that synthesis of the mRNA specific for β galactosidase in E. coli was inhibited by glucose. In animal experiments, it has been shown that glucose administration inhibits the induction of the hepatic enzymes ornithine transaminase and threonine deaminase(331). The theory of catabolite repression has also been postulated for the effect of glucose upon ALA-synthetase. However, as stressed by Rimington, no biosynthetic pathway from glucose to porphyrin has been described. Nevertheless, the fact remains, that one precursor of porphyrins, viz. succinate, is directly derived from glucose via the citric acid cycle. The similar origin of ATP appears to be relevant to porphyrin metabolism in view of:-

(i) the inhibitory effect of ATP on the induction by AIA of increased porphyrin synthesis in tissue culture.

(ii) a possible role of AMP in the treatment of human porphyria.

(iii) the probable connection which appears in the role of ethionine with diminution of glucose and hepatic ATP levels, and the elevation of porphyrin excretion. The production of increased porphyrin synthesis by the addition of ethionine to tissue culture has also been noted (Table XXXIII).

TABLE XXXII

Experiments to show the inhibitory effect of feeding glucose with AIA to rats; (r+m)RNA* was extracted from the livers of rats fed AIA and rats fed AIA + glucose.

AIA				AIA + glucose			
µg RNA phos.	COOH gr.	µg porphyrin/ g w.w.	ng porphyrin/ µg RNA/g w.w.	µg RNA phos.	COOH gr.	µg porphyrin / g w.w.	ng porphyrin µg RNA/g w.w.
165	4 8	0·41 0·55	5·8	325	4 8	0·14 0·49	1·8
326	4 8	0·60 0·94	4·6	175	4 8	0·14 0·12	1·6
103	2 4 8	0·20 0·13 0·45	7·6	187	4 8	0·05 0·37	2·2
123	2 4 8	0·08 0·09 0·85	8·3	330	4 8	0·11 0·22	1·0
121	4 8	0·59 0·47	8·8	190	4 8	0·41 0·04	2·0
282	2 4	0·97 0·72	6·0	230	4	0·30	1·3
319	2 4	1·12 0·48	5·0	332	4	0·56	1·7
304	2 4 8	0·42 0·99 0·45	6·1	234	4 8	0·17 0·21	1·6
153	2 4 8	0·49 0·31 0·72	9·9	188	2 4 8	0·04 0·11 0·24	2·1

MEAN INCREASE AIA – (AIA + glucose): 5·2 ± 0·7

($p<0·001$)

*after extraction, RNA was added to tissue culture, and the porphyrins induced were estimated as described in the text.

Table: XXXIII Experiments to show the increase in porphyrin production by tissue culture after the addition of Ethionine

SPECIMEN	Experiment I			Experiment II		
	COOH gr.	µg porphyrin / g w.w.	Total	COOH gr.	µg porphyrin / g w.w.	Total
Control	4	0·30	1·48	4	0·70	0·70
	8	1·18				
Ethionine 1 mg	4	0·79	2·03	4	0·20	1·23
	8	1·24		8	1·03	
Ethionine 100 µg	4	0·64	1·69	2	0·27	1·01
				4	0·05	
	8	1·05		8	0·69	
Ethionine 10µg	4	0·67	1·29	4	0·25	0·76
	8	0·62		8	0·51	

THE EFFECT OF GLUCOSE ON RNA.

To investigate the postulate that the inhibitory effect of glucose upon ALA-synthetase production was mediated through the mechanism of RNA, an experiment was conducted using rats prepared with AIA and with AIA and glucose as described on page 76.

Extraction of RNA was performed using the second method described (see page 72) and the ribosomal + messenger fraction was added to tissue culture.

The results are shown in Table XXXII and show significantly less porphyrin synthesis in cultures exposed to RNA extracted from the livers of rats fed both AIA and glucose, as compared with porphyrin synthesis in cultures exposed to RNA extracted from the livers of rats fed AIA alone. The mean decrease by glucose was 5.2 ± 0.74 which is significant. ($p < 0.001$).

Discussion

These experiments suggest that the inhibitory effect of glucose upon the increased ALA-synthetase activity induced in rats by the administration of AIA, may be mediated through the mechanism of RNA.

Mechanisms other than that of catabolite repression for this inhibition have not yet been described, and it is postulated that one such mechanism may be an action upon RNA. However, this action

is not yet explicable. The involvement of ATP both in protein synthesis and in glucose metabolism brings this substance to mind, but there is no evidence to support this yet.

CHAPTER IX A DISCUSSION OF THE EXPERIMENTS PRESENTED AND IDEAS FOR FUTURE RESEARCH

> "Each man is a new power in Nature. He holds the key of the world in his hands. Nature offers every morning her wealth to man - she is careful to leave all her doors ajar - towers, hall, storeroom and cellar. In her hundred gated Thebes, every chamber is a new door."
> R.W.Emerson - The Natural History of Intellect.

The experiments reported in this thesis have been concerned with the induction of increased porphyrin synthesis in tissue culture by drugs, and with an investigation of the possible role of RNA in this phenomenon. Many problems remain to be solved in both these fields, but the solution of one is intimately linked with the further clarification of the other. The following ideas are put forward as a basis for future research.

PORPHYRIN BIOSYNTHESIS

(i) Further chemical structures which induce porphyrin synthesis may emerge after sufficient drugs have been tested. Intensive study of these structures may lead to an even clearer understanding of their mechanism of action but as stressed before, caution must be exercised in the extrapolation of these results to human porphyria.

(ii) Study of the action of ethionine may yield information about the induction of porphyrin synthesis by drugs, and the effect of such substances upon RNA. This analogue of methionine was found to produce fatty liver in experimental animals probably through the

mechanism of diminishing hepatic ATP(332). Such animals became markedly hypoglycaemic(333), the effect being more obvious in female rats than males. Glucose protected rats fed ethionine from developing fatty liver and hypoglycaemia. Noting the diminished levels of hepatic ATP, Gajdos investigated ethionine-fed rats for porphyria(334). His results showed increased excretion of porphyrins in urine and faeces, and increased levels of liver porphyrin. He claimed however, that no increase of ALA-synthetase production occurred, although no results are presented. Indeed, preliminary results from our laboratory suggest that there is increased synthesis of ALA-synthetase.

Ethionine has also been shown to have various, as yet ill-defined effects upon nucleotides(247)(250). Combination of the techniques described in this thesis for testing the ability of RNA to increase porphyrin synthesis, together with further knowledge of the mechanisms of action of ethionine in porphyrin induction and in RNA synthesis, and the effect of glucose upon this, may prove a fruitful field of study

(iii) Recent work has confirmed that the action of many drugs upon the liver is that of increasing the smooth endoplasmic reticulum(335)(336)(337)(338). This indicates the stimulus of the synthesis of enzymes concerned with endogenous substances(335). Phenobarbitone especially, has been shown to have this effect. These changes are associated with increased NADPH-dependent drug detoxication and increased microsomal cytochromes(339). As described in chapter V,

Granick(91) has suggested that increased haem synthesis might occur in drug detoxication, since one of the drug detoxicating oxidases is a cytochrome requiring haem for its activity(305)(305A). It has also very recently been suggested by Labbe that all defects in porphyrin metabolism are at the level of mitochondrial electron transport and energy generation, with the increase in porphyrin synthesis as a secondary occurrence(268).

Various other drugs capable of ALA-synthetase induction in vitro have been shown individually to have other effects which may be associated with porphyrin biosynthesis. Analogues of salicylate have been found to uncouple oxidative phosphorylation(340); anabolic steroids stimulate microsomal enzymes in similar fashion to phenobarbitone and polycyclic hydrocarbons(338), with associated accelerated metabolism of glucose to glucuronic, L-gulonic and ascorbic acids(341). Salicylates and cinchophen derivatives have been shown to inhibit incorporation of amino acids into protein(342). These are probably all non-specific effects.

It is possible that some of the above-mentioned effects, and more as yet to be described, may operate in the induction of ALA-synthetase.

(iv)More recent suggestions that enzymes other than ALA-synthetase may be rate-limiting in porphyrin biosynthesis, viz. succinyl-Co-synthetase and fumarate reductase, require additional experimental

evidence similar to that which has been provided for ALA-synthetase. The synthesis of one enzyme, or in fact of all mentioned above, may be increased in porphyria.

Proof of the action of RNA in the synthesis of porphyrin in tissue culture would be aided by the direct demonstration of elevated levels of ALA-synthetase in these cultures. The amounts of this enzyme produced in cultures are very small and difficult to measure by any form of column separation since the ALA fraction is lost. Both Tschudy (329A) and Granick(91) have described micromethods for measurement, but neither of these has proved entirely satisfactory when applied to tissue culture. Direct measurement of this or any enzyme of the porphyrin biosynthetic pathway in such small quantities will probably require the use of radio-active precursors. Attempts to measure ALA-synthetase activity using C14-glycine are presently in progress in our laboratory.

While the results of some of the experiments reported in this thesis have been interpreted as indicating increased synthesis of ALA-synthetase, the evidence for this is indirect as previously stated. However, if increased porphyrin production is due to another enzyme, the conclusions drawn about the roles of drugs and the action of RNA are equally valid. Further, the inhibitory effect of glucose, acting as it appears to, via RNA, can probably be applied to other enzyme systems.

(v) The suggestion of Kikuchi(58) that a specific inhibitor of ALA-synthetase is present in Rps. sphs. requires further investigation

both for therapeutic reasons and for conclusive proof of the action of RNA.

(vi) The search for a therapeutic agent is of greatest importance in the problem of porphyria and the role of drugs. While the mechanism of porphyrin induction by drugs via RNA has perhaps been clarified by the work described here, the only suggestion of therapy is that of glucose. Unfortunately, direct extrapolation from animals to man suggests that such therapy would be unwieldy, but with one success claimed by Tschudy(102) in this regard, further experimentation with patients seem justified. The same justification seems applicable to the use of AMP which Gajdos(106) has advocated.

In occasional patients, treatment with progesterone derivatives may be of value, and a preliminary survey is in progress in Cape Town.

The use of glutathione has been reported to delay the induction of experimental porphyria in mice by Griseofulvin(178), and further work in this direction may be rewarding in view of the number of enzymes in porphyrin biosynthesis which depend on SH groups.

(vii) It is thought that the metabolism of tryptophan may be disordered in porphyria(343). Increased tryptophan pyrrolase has been found in Sedormid-fed animals(344). Of interest in relation to this finding, and the hypothesis discussed in this thesis, are the facts that indole-acetic acid increases mRNA synthesis(345), and that the omission of tryptophan from the diets of mice results in

disaggregation of the hepatic polyribosomes(346). Particular significance has been attached to this in view of the fact that this was the only amino acid of which the exclusion had this effect.

Tryptophan remains the one amino acid in the genetic code which has only one coding triplet(221) and it is suggested that its presence regulates hepatic protein synthesis at the level of translation of the message via RNA(346). This may be of even greater significance in a situation as envisaged for disordered porphyrin metabolism where the control of the production of mRNA for ALA-synthetase is already unstable.

PROTEIN BIOSYNTHESIS

Further experiments are necessary to support the conclusion that the induction of ALA-synthetase synthesis is indeed stimulated by the addition of RNA. These are:-

(i) the further purification of the RNA fraction added, and

(ii) evidence that the RNA has entered the explants.

(i) Since a metabolite of AIA is a possible contaminant, further work is necessary to determine the catabolic pathways of the drug in man and animals.

As mentioned in chapter VII, the RNA is considered to be essentially pure, but that protein may be present in a small amount. In recently described experiments(347) using a ribonucleoprotein complex from deer reticulocyte ribosomes combined with rabbit reticulocyte

ribosomes, globin synthesis was stimulated in a cell-free system to which the complex was added. It was shown that both ribonucleoprotein and RNA were necessary for this synthesis, and it is felt that a protein fraction may similarly be necessary for the action of RNA as described in the experiments reported in this thesis.

Purification of the RNA fraction may be achieved by passage through a Sephadex column which removes much of the oligonucleotide contamination. Addition of such a fraction should result in greater porphyrin production if greater entry of mRNA occurs. Comparison of the increases of porphyrin production achieved by the addition of RNA or ALA to tissue cultures, reveals that the full capacity for porphyrin production is not exploited by RNA. This is probably due to limitation of incorporation of RNA by permeability barriers at the cell membrane. It is also known that the presence of RNase on the cell membrane may inhibit entry of RNA, and the addition of protamine sulphate(347) or histone(252) has been advocated.

The dose response of porphyrin production to addition of RNA, or the kinetics of the induction of ALA-synthetase by RNA are only indirect evidence for the entry of RNA into the cell.

(ii) Entry of RNA could be shown directly by autoradiography or with the use of RNA labelled with C14-uridine. If such RNA was added to tissue culture, extraction of an intracellular particle, e.g. ribosomes from the tissue culture, and demonstration of the radio-activity

entirely in that fragment, would confirm the postulate that RNA had entered the explants. Such experiments are planned.

Various workers have postulated the entry of alien RNA into cells. Initially most work was done with labelled bacterial RNA, entry of which was noted by the development of infectivity(349) or the induction of bacterial enzyme synthesis(192)(350). However, the demonstration of entry of alien non-bacterial RNA has proved more difficult. Suggested evidence has been the increase of protein synthesis(351), the induction of enzyme synthesis(352-356), and the demonstration of the effect of RNA on ascites tumourcells(357)(358).

Further experiments are required to show whether the hypothesis in this thesis is in fact correct, and whether this may be the first demonstration of the induction of enzyme synthesis in mammalian cells by alien mammalian RNA.

SUMMARY.

This thesis is concerned with an investigation into the drug-induced porphyrias in animals and in tissue culture.

As an introduction to the subject, the first two chapters describe the historical background to classification of the diseases of porphyrin metabolism, and further details of the syndromes which occur. A brief resumé of porphyrin biosynthesis is given, with especial attention to the first enzyme in the biosynthetic pathway, viz. ALA-synthetase.

Since it has been suggested that the increased porphyrin production which occurs in tissue culture after the addition of various drugs is the result of increased ALA-synthetase activity, chapter III is concerned with recent advances in the field of protein synthesis and its control.

In chapter IV, combination of the data on porphyrin and protein biosynthesis results in a description of the control of porphyrin metabolism. This is also concerned principally with the regulation of ALA-synthetase synthesis, but other control mechanisms are considered.

The chief experimental material used was a tissue culture system of chick embryo liver explants. The induction of porphyrin synthesis in these cultures was tested. Screening of 69 agents in frequent use in medical conditions, showed that 38 caused fluorescence of the explants when these were viewed under an ultra-violet microscope.

Quantitative experiments with 12 drugs are described,

and the increased amounts of porphyrin produced are tabulated. These results are discussed with respect to the mechanism of drug action. The proposal that this mechanism is one of derepression of the operon for ALA-synthetase is considered, and from this, the hypothesis is developed that a suitably coded RNA fraction from a porphyric animal, when added to tissue culture, might induce ALA-synthetase and therefore porphyrin synthesis.

Various fractions of RNA from the livers of rats fed Allyl-isopropyl-acetamide (AIA), using 2 different methods to extract the RNA, were added to tissue culture, and the porphyrins produced were quantitated. A ribosomal fraction of RNA induced more porphyrin formation that a transfer fraction, but further purification of the ribosomal RNA showed that a ribosomal plus "messenger" component induced most porphyrin synthesis, while a ribosomal component without "messenger" did so to a lesser degree. These results are presented in chapter VII. Evidence for the purity of RNA is given and it seemed reasonable to conclude that the RNA was of acceptable purity. It is possible, however, that a very small amount of protein might be present, and that the component being dealt with is a ribonucleoprotein complex.

A time-study of the kinetics of porphyrin production, and a dose response curve suggested that the RNA was actually incorporated into the metabolism of the cells.

The inhibitory effects of ATP and glucose were investigated - the former in tissue culture and the latter in experimentally porphyric animals. Results presented in chapter VIII show that the simultaneous addition of ATP to tissue cultures with AIA, results in less porphyrin synthesis than addition of AIA alone. The mechanism of action of ATP possibly via protein synthesis is considered.

Glucose was found partially to inhibit the increased porphyrin synthesis resulting in rats from the administration of AIA. The levels of liver porphyrins, blood glucose and ALA-synthetase in rats fed AIA alone and in rats fed AIA plus glucose are compared in the second part of chapter VIII.

The inhibitory effect of glucose was thought to occur via the mechanism of RNA and this postulate was investigated using RNA from the livers of rats fed AIA alone and from rats fed AIA plus glucose. A diminished porphyrin induction by the latter RNA is shown, and the whole evidence for the theory of catabolite repression of enzymes and ALA-synthetase in particular, is discussed at the end of the chapter.

The last chapter deals with a consideration of the experimental data presented and extension of the design to further research in the fields of porphyrin and protein biosynthesis.

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