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Studies on the metabolism of drug-induced porphyria

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STUDIES ON THE METABOLISM OF DRUG-INDUCED PORPHYRIA

A thesis submitted in fulfilment of the requirements
for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

in accordance with the regulations of
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By

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3. The research on which this thesis is based was commenced in April, 1966, and completed in November, 1968.

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LIST OF ABBREVIATIONS

AIA	Allylisopropylacetamide
ALA	δ-Aminolaevulinic acid
ALA-synthase	δ-Aminolaevulinic acid synthetase
CoA	Coenzyme A
COPRO	Coproporphyrin
COPROgen	Coproporphyrinogen
DDC	Diathoxycarbonyl-1,4-dinhdrocollidine
EDTA	Ethylenediaminetetracetic acid
FAD, FADH	Oxidized and reduced forms of flavine adenine dinucleotide
NAD, NADH	Oxidized and reduced forms of nicotinamide adenine dinucleotide
NADP, NADPH	Oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate
PBG	Porphobilinogen
POPOP	p-bis-(2-)5-Phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
PROTO	Protoporphyrin
PROTOgen	Protoporphyrinogen
S.D.	Standard Deviation
TCA	T..carboxylic acid cycle (Kreb's cycle)
URO	Uroporphyrin
UROgen	Uroporphyrinogen

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

TRIVIAL NAME	SYSTEMATIC NAME	NUMBER
ALA dehydratase	5-Aminolaevulin[illegible]ydrolyase (add 5-aminolaevulinate cyclising)	4.2.1.24
ALA synthetase	Succinyl CoA : glycine N-succinyltransferase	2.3.1.13
Coproporphyrinogen oxidase	Precise mechanism unknown	Unclassified
Glutamate dehydrogenase	L-glutamate : NAD oxidoreductase	1.4.1.2
Haem synthetase (Ferrochelatase)	Protohaem ferro-lyase	4.99.1.1
Malate dehydrogenase	L-Malate : NAD oxido reductase	1.1.1.37
$NADH_2$ - oxidase	Reduced NAD : (acceptor) oxido reductase	1.6.99.3
PBG deaminase (Urogen I synthetase)	Mechanism unknown	Unclassified
Succinyl CoA synthetase	Succinate : CoA ligase (GDP)	6.2.1.4
Uroporphyrinogen decarboxylase	Uroporphyrinogen - III carboxy-lase	4.1.1.37
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CHAPTER I

HISTORICAL AND CLASSIFICATION

a) General Historical Background

The concept of inherited metabolic error leading to disease is about fifty years old, but only within the last decade has interest been thoroughly aroused in such inborn errors. With the steady development of biochemical techniques and their increasing application to clinical medicine, a surprisingly large number of diseases have been shown to arise from familial transmission of some biochemical abnormality. Usually it has been possible to demonstrate an absolute or relative deficiency in a particular enzyme system, and to relate this to the pathogenesis of the disease in a more or less direct manner.

The porphyrias are metabolic disorders, some of which are hereditary in origin. They are disorders of the porphyrin chemistry of the body, the striking clinical expression often being the passage of dark urine. The hereditary forms belong to that larger family of diseases called by Garrod (1923) "inborn errors of metabolism". It is known that the metabolism of the body is regulated by an intricate mosaic of enzymes, the synthesis of which is under genetic control. A defect in this control may result in the defective formation of one or more specific enzymes and thus lead to an inborn error of metabolism. The error may be revealed as an abberration of molecular structure, as in the haemoglobinopathies. It may lead to the inadequate production of

an essential protein as for example in haemophilia, or of an important enzyme such as glucose-6-phosphate dehydrogenase in the red cell. Several of the 'primary' amentias are now disclosed as being in reality inborn errors of metabolism, for example phenylketonaria.

The history of the porphyrias began when Scherer (1841) showed that the red colour of blood was not due to iron. Mulder (1844) carried out a similar study and described a 'purple-red fluid' devoid of iron, which he named 'iron-free haematin'. This red substance was called 'cruentine' by Thudichum (1867), and he defined its spectrum and noted it's remarkable fluorescence. Hoppe-Seyler (1871) found that the iron-free haematin described by Mulder, was a mixture of two substances the main consituent of which he called 'haematoporphyrin' (Greek porphuros-purple). Three years later, Shultz (1874) published clinical details of a case of so-called 'Pemphigus Leprosus' for his doctorate thesis. The urine of this patient was extensively studied by Baumstark (1874) who named two pigments derived from it, urorubrohaematin and urofuscohaematin. It is now clear, that the patient of Shultz and Baumstark was a description of what we now call congenital porphyria, and was in fact the first association of this pigment in urine with a disease in man. The diagnosis was made almost certain by the autopsy record of intense red-brown discolouration of the skeleton.

In 1880, MacMunn described a dark-pigment, which he named urohaematoporphyrin. This was excreted in the urine of a patient with sub-acute rheumatism, who had been taking sodium salicylate. Le Nobel (1887) confirmed this finding, also listing a number of diseases, including lead poisoning, in which he had found the pigment.

Shortly after sulphonal was introduced as a hypnotic, Stokvis in 1889 reported that an elderly woman who had taken sulphonal excreted a dark red urine and had later died. Harley (1890) reported a fatal case of an unusual form of nervous disturbance, associated with dark red urine. For the next twenty to thirty years, many other cases were recorded of 'haematoporphyrinuria' associated with a definite pattern of symptoms. Günther (1911, 1922) carefully listed and classified these individual cases. Garrod (1923) credits Günther with the first recognition that this disease was an 'inborn error of metabolism'.

One of Günther's patients was a man named Petry. Hans Fischer commenced a study of the excreta of this patient and between the years 1915 and 1945 made monumental contributions to porphyrin chemistry. By these researches the naturally occurring porphyrins of excreta, uroporphyrin and coproporphyrin were found to differ structurally from haematoporphyrin, which was considered to be a chemical artefact. Fischer (1915 a,b), Laidlaw (1904) and Schumm (1924) differentiated

the naturally occurring porphyrin of haem itself from haematoporphyrin and the name protoporphyrin was suggested for this by Fischer. Thus it was that the diseases of porphyrin metabolism came to be termed the porphyrias by Waldenström (1937) rather than haematoporphyrias (Günther 1911).

b) Classification

Many authorities in the field of porphyria have attempted at some time or other to arrive at a satisfactory classification of the porphyrias (Table I). However, not until specific enzyme or other defects in the various disorders of porphyrin metabolism have been unravelled will a wholly satisfactory classification be achieved.

Hans Günther (1911, 1922) published the first classification comprising four types of 'haematoporphyria' (Table IA). In his classification it is noteworthy that he recognised acute and chronic forms and that h first defined and named congenital (erythropoietic) porphyria. From a study of 103 cases of acute porphyria in 1937, Waldenström distinguished five clinical sub-types (Table IB). It is now realized that these represent different manifestations of the same disease.

The classification of Schmid, Schwartz and Watson (1954) (Table IC), was the first classification in which in addition to purely clinical manifestations, biochemical findings were taken into account. These authors demonstrated that the porphyrias are clearly divisable into erythropoietic and hepatic types from analyses of the porphyrin and porphobilinogen content of bone marrow and liver. Waldenström (1957) (Table ID) revised his original 1937 concepts, but still held the view that the lesion in acute porphyria was present in all haem synthesizing cells rather than a disturbance of hepatic function. This concept is however, not accepted in more recent classifications. Of some interest in this classification is the recognition of an acquired form of porphyria, termed porphyria cutanea tarda symptomatica.

Watson (1960) (Table IE) suggested an extension of the 1954 classification also clearly recognizing hereditary and acquired forms of hepatic porphyria. The classification recommended by Goldberg and Rimington (1962) (Table IF) has a composition similar to that put forward by Watson, and reflects the difficulties encountered in the classification of these diseases. The more recently published classification of Eales (1963) (Table IG) and Tschudy (1965) (Table IH) are essentially similar. These recognize erythropoietic and hepatic forms, the latter being divided into three major types, viz. acute

TABLE 1

VARIOUS PROPOSED CLASSIFICATIONS OF THE PORPHYRIAS

(Modified after Goldberg and Rimington 1962)

(...)

iii) With systemic disease

e) Acquired

i) Secondary to hepatoma

ii) Secondary to fungicide - turkish epidemic

F. GOLDBERG AND RIMINGTON (1962)

a) Acute intermittent porphyria, Swedish genetic porphyria, pyrollo-porphyria (dominant)

i) Mainfest

ii) Latent

b) Porphyria variegata, mixed porphyria, S.A. genetic porphyria, PCT hereditaria and protocoprophyria (dominant)

i) Cutaneous - little or no acute manifestation

ii) Acute intermittent - no cutaneous symptoms

iii) Various combinations

iv) Latent

c) Symptomatic porphyria, PCT symptomatica, urocoproporphyria, constitutional porphyria

i) Idiosyncratic associated with alcohol, liver disease, systemic disease, drugs, etc.

ii) Acquired - Hexachlorobenzene induced and hepatoma

TABLE .1.

Please see last fiche.

intermittent porphyria, South African genetic porphyria, and symptomatic porphyria. The major subgroup of symptomatic porphyria is recognized as that associated with liver disease and alcoholism, this form occurring in high incidence in the Bantu of South Africa. Experimentally induced porphyrias are recognized as a separate subgroup in the Goldberg and Rimington classification (Table IF) and would presumably fall into the symptomatic porphyria groups of Eales and Tchudy. As indicated previously it is shown that a wholly satisfactory classification will only be arrived at with the detailed elucidation of the metabolic aberrations in these diseases.

c) Porphyria in South Africa

By far the most common types of porphyria found in South Africa today, are the so-called South African genetic type of porphyria variegata found mainly in the White population, and the Bantu type of porphyria which is thought to be an acquired affliction and is called porphyria cutanea tarda symptomatica.

The rarer erythropoietic porphyrias have however, also been reported in South Africa. The first case of congenital erythropoietic porphyria in South Africa was reported by Finlay and Barnes (1950). The same case and a further case in an elderly male were reported and

fully characterized by Kramer and his co-workers (Kramer et al. 1965). Sweeney and his co-workers (1963) first reported erythropoietic protoporphyria in an African and several families have since been discovered (Finlay et al. 1966; Leeming and Kramer, 1968). Hereditary coproporphyria has been reported in a family by Dean, Kramer and Lamb (1969).

In a painstaking and brilliant study, Dean (1963) was able to trace the original forebears of the vast majority of White porphyric subjects in this country. According to Dean, Adriaantjie Jacobs (Adriaantjie Adriaanse) one of a number of orphans sent out by the Dutch East India Company in 1688, as prospective wives for the free burghers, married Gerrit Janz who had come to the Cape in 1685, and were the original forebears of the disease in the White population.

The first record of the disease only appeared in the literature in 1939 (Kooy 1939, Eales and Chair 1931). At present it is estimated that there are about 10,000 White South Africans who have inherited the gene for porphyria variegata, accounting for the highest incidence of porphyria in the world. Porphyria variegata exists in two forms. There is a latent form associated with relatively few clinical symptoms other than photosensitivity, but with the excretion of increased amounts of protoporphyrin and coproporphyrin in the faeces. Protoporphyrin

usually exceeds coproporphyrin in the faeces, and the urine shows no abnormality. The manifest or acute form is associated with acute abdominal pain and neurological disturbances which may result in death. Tachycardia, hypertension, and in some cases skin photosentitivity are marked features of the disease. In addition these patients excrete increased amounts of δ-aminolaevulinic acid and porphobilinogen in the urine in the acute phase.

The incidence of symptomatic porphyria in the Bantu peoples of South Africa is also very high indeed, although accurate figures are not available. The clinical and biochemical features of the condition have been extensively studied and characterized, particularly by Barnes (Finlay and Barnes, 1950; Barnes 1955, 1959), Lamont (1961) and Eales (1963). The features of Bantu porphyria are skin sensitivity, hypertrichosis, excessive pigmentation of the skin, and excretion of greatly increased quantities of uroporphyrin in the urine and increased amounts of coproporphyrin excreted in the faeces. Almost all these patients have deranged hepatic function with siderosis and a history of excessive consumption of alcohol.

CHAPTER II

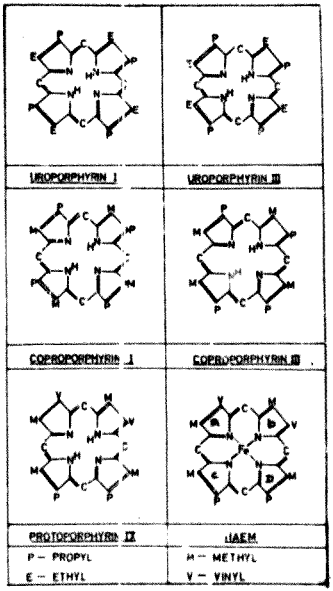
HAEM BIOSYNTHESIS

II. Haem Biosynthesis and the Porphyrins

a) General

Porphyrins are widely distributed throughout the animal and plant kingdoms. In the free form, they occur in small amounts, but the iron complexes of porphyrins, or haems are quantitatively most important and play a vital role in the living cell. Ferroprotoporphyrin IX is the prosthetic group of several important enzymes such as for example, catalase, peroxidase, and cytochrome b, whilst haems derived from prophyrins of slightly different structure, occur in chlorocruorin, cytochrome c, and cytochrome oxidase. The macrocytic rings of chlorophylls a and b are derived from the same parent structure, porphyrin, and recent evidence shows that they are built up in a way comparable to that of animal porphyrins (Bogorad 1955). Chlorophyll-like pigments also occur in brown and red algae and in some bacteria and although experimental evidence is still lacking, it seems likely that their biosynthesis follows the same general pattern. Finally, haemoglobin and myoglobin are two chromoproteins whose prosthetic group is ferroprotoporphyrin IX. Thus functions of oxygen transport, photosynthesis, and intracellular oxidation are dependent on this group of compounds. Porphyrin synthesis would appear to be one of the most fundamental attributes of cells. Relatively simple materials are employed and the steps involved seem to follow a general plan. Although

FIG I.

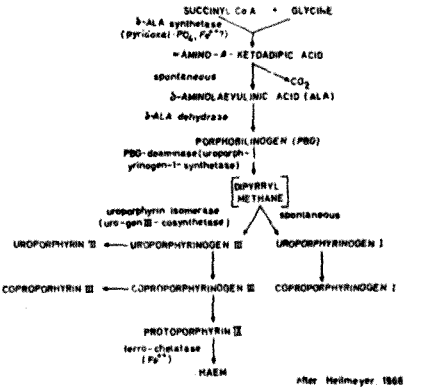

UROPORPHYRIN I
UROPORPHYRIN III
COPROPORPHYRIN I
COPROPORPHYRIN III
PROTOPORPHYRIN IX
HAEM
P — PROPYL
E — ETHYL
M — METHYL
V — VINYL

the bulk of knowledge has been gained chiefly from study of the biosynthesis of the haem of haemoglobin the results are applicable in a much wider context.

As a result of the work of Fischer et al. (1939), of Lemberg et al. (1949) and of MacDonald et al. (1953, 1954, 1956; Jackson and MacDonald 1957; Morsingh and MacDonald 1960 and Tarlton, MacDonald and Baltazzi, 1960), the chemistry of porphyrins is well known, and most of the naturally occurring porphyrins have been prepared. There are many isomeric forms of the porphyrins, namely 4 uroporphyrins, 4 coproporphyrins and 15 pre[illegible]rphyrins. However, only the single protoporphyrins IX, either in the free state, or as the iron complex haem, and only coproporphyrin I and III and uroporphyrins I and III have so far been encountered in nature (Fig. I).

Present knowledge concerning the pathway of porphyrin and haem synthesis has been accumulated during the past 20 years and represents a major accomplishment of modern biochemistry. The final scheme has been pieced together from discoveries made at different times, and there still remain significant details to be filled in. Nevertheless, the way in which this complicated yet vital synthesis is achieved may be regarded as firmly established (Rimington 1958; Heilmeyer 1966) (Fig. II).

FIG II.


SUCCINYL CoA + GLYCINE
δ-ALA synthetase
(Pyridoxal PO_4, Fe^{++})
α-AMINO-β-KETOADIPIC ACID
spontaneous
CO_2
δ-AMINOLAEVULINIC ACID (ALA)
δ-ALA dehydrase
PORPHOBILINOGEN (PBG)
PBG-deaminase(uroporphyrinogen-I-synthetase)
DIPYRRYL METHANE
uroporphyrin isomerase
(uro-gen III-cosynthetase)
spontaneous
UROPORPHYRIN III
UROPORPHYRINOGEN III
UROPORPHYRINOGEN I
COPROPORPHYRIN III
COPROPORPHYRINOGEN III
COPROPORPHYRINOGEN I
PROTOPORPHYRIN IX
ferro-chelatase
(Fe^{++})
HAEM
After Heilmeyer 1966

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

The next step was to establish the distribution of the C and N atoms of glycine in the porphyrin molecule. Protoporphyrin IX is an asymmetric tetrapyrrole consisting of two types of pyrrole units as shown in Fig I through various degradative steps, ring a and b can be converted to methylmaleimide and rings C and D to haematinic acid. On degrading ^{15}N-labelled haem protoporphyrin prepared in the rabbit, (Muir and Neuberger 1949) or duck, or human (Wittenberg and Shemin 1949) by the administration of glycine-^{15}N, it was found that the ^{15}N concentrations of the porphyrin molecule and the two breakdown products were equal. Thus it was established that glycine provided the N-atoms present in all four pyrrole units. The fate of the C atoms of the glycine molecule in porphyrin biosynthesis was elucidated in similar fashion. Using ^{14}C and ^{15}N-labelled glycine and other precursors

Radin et al. (1950) and Muir and Neuberger (1950) found that eight of the C-atoms of glycine were incorporated into the porphyrin molecule, and that four of these labelled C atoms were present in the methene C fraction. Further step wise degradation (Wittenberg and Shemin 1950) revealed that the other 4 C atoms are situated in comparable positions in the pyrrole rings. On the other hand, experiments with carboxy-labelled glycine did not lead to labelling of protoporphyrin or haem (Grinstein et al. 1948, Radin et al. 1950).

The origin of the remaining 26 C atoms of protoporphyrin was likewise traced with the aid of isotopically labelled compounds. Using ^{14}C-acetate labelled either in the methyl of carboxyl position, Shemin and Wittenberg (1951) found that acetate could supply all 26 C atoms in question. Furthermore, in degradative studies of the type mentioned above, they detected equal amounts of radioactivity similarly distributed among three C atoms in each of the two residues representing the two sides of the monopyrrole molecule. It seemed then highly likely that the basic monopyrrole unit was formed by condensation of two molecules of the same compound with glycine (Shemin and Wittenberg 1951). Shemin and Wittenberg (1951) reasoned that this precursor must of necessity be a three or four carbon compound, most probably an unsymmetrical four carbon molecule. In a series of further experiments using other

FIG. III

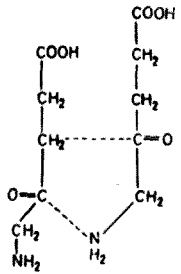


Diagram representing Knorr condensation of 2 molecules of ALA to yield PBG.

labelled intermediates of the TCA cycle including carboxyl-labelled succinate-^{14}C methylene-labelled succinate-^{14}C α-Ketoglutarate-5-^{14}C α-Ketoglutarate-1,2-^{14}C and citrate 1,5-^{14}C, Shemin and co-workers (Shemin and Kumin 1952, Wriston et al. 1955) identified the four carbon compound in question as a derivative of succinate, possibly a succinyl-coenzyme complex which could arise from succinate or α-Ketoglutarate.

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

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

Shemin and Russel (1953) also demonstrated that unlabelled ALA diluted in the radioactivity of haem is synthesized by chick red cell haemolysates from glycine-^{14}C or succinate ^{14}C. This would be expected if the non-radioactive ALA were diluting ALA-^{14}C formed in the incubate from the labelled glycine or succinate. Finally, experiments with ALA-5-^{14}C (Shemin, Russel and Abramsky 1955) and ALA-1,4-^{14}C (Schvffman and Shemin 1955) gave precisely the predicted labelling pattern for haemin, i.e. ^{14}C in the 5 position behaved in the *same fashion* as the C-atom of glycine, and that in the 1 and 4 positions in the same way as the carboxyl groups of succinate.

b) δ-Aminolaevulinate Synthetase (δ-ALA Synthetase)

The formation of ALA from succinyl-CoA and glycine is the first step leading specifically to the biosynthesis of the porphyrins. Neuberger (1961) proposed a mechanism whereby a stable carbanion is formed from glycine and pyridoxal phosphate which reacts with the electrophilic carboxyl carbon atom of succinyl CoA to produce α-amino-β-ketoadipic acid. This compound then loses CO_2 to give ALA. The formation of ALA is carried out by the enzyme δ-ALA synthetase which utilizes pyridoxal phosphate as a coenzyme. α-amino-β-ketoadipic acid decarboxylates virtually spontaneously in neutral solution (Laver, Neuberger and Scott 1959). Thus it is not known whether it occurs as an enzyme-bound intermediate or free in solution, or indeed whether it is an intermediate at all, since it is possible that condensation and decarboxylation occur together in a concerted reaction. This initial step in the porphyrin biosynthetic pathway has been extensively studied in a number of different systems, including micro-organisms (Kikuchi et al. 1958, Gibson 1958, Lancelles 1959), avian erythrocytes, (Brown 1958a, Laver et al. 1958, Granick 1958) and mammalian liver (Granick and Urata 1963, Tochudy et al. 1964, Marver et al. 1966). In each

system the enzymic formation of ALA by condensation of glycine and succinyl CoA has been demonstrated. Kikuchi et al. (1958) Gibson et al. (1958) and Granick and Urata (1963), in examining the induction of guinea pig liver *ALA*-synthetase by DDC clearly demonstrated the intramitochondrial localization of this enzyme.

Brown (1958b,c) working on particulate preparations of chicken erythrocyte showed that ferrous iron was involved at some stage in the synthesis of ALA, but Burnham and Lascelles (1963) working on Rhodopseudomonas spheroides found that iron does not participate in the action of ALA-synthetase from this source. Thus it would appear that iron is not involved in all ALA-synthesising systems.

A requirement for the coenzyme, pyridoxal phosphate is now unequivocal. Shulman and Richert (1957), studying vitamin B_6 deficient ducklings, found a decreased rate of red cell haem synthesis, furthermore, pyridoxal-5-phosphate added in vitro to red cells from these ducklings stimulated haem formation from labelled glycine and succinate. Elder and Mengell (1965) induced a pyridoxine deficiency in a patient suffering from acute intermittent porphyria, and found a decrease in urinary excretion of ALA and PBG. Subsequent pyridoxine loading of the patient markedly increased excretion of these precursors.

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

c) ALA dehydrogenase and the synthesis of PBG

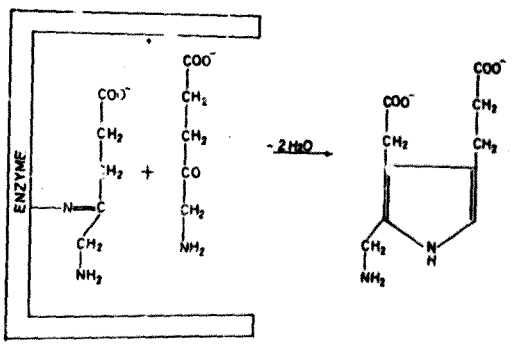
Soon after PBG was first isolated from the urine of a patient with acute intermittent porphyria by Westall (1952) the position of this monopyrrole as a porphyrin precursor was established (Falk et al. 1953, Bogorad and Granick, 1953). Granick (1954) demonstrated that ALA could be enzymatically converted to PBG by chicken erythrocyte extract. Gibson et al. (1955) confirmed this on a highly soluble enzyme preparation from ox liver, Schmid and Shemin (1955) on duck erythrocyte and Nandi et al. (1968) on *Rhodopseudomonas spheroides*.

The cytoplasmic enzyme (ALA dehydratase) which requires reduced glutathione (GSH) for activation before it can effect synthesis of PBG (Gibson et al. 1955, Schmid and Shemin 1955), has now been purified from a number of sources; from ox liver (Gibson et al. 1958), cow liver (Batlle et al. 1967), human erythrocytes (Calisano et al. 1966), yeast (Clara De Barieco, 1967), soybean callus tissue (Tiger et al. 1968), wheat leaves (Nandi and Waygood, 1967), and R. spheroides (Burnham and Lascelles, 1963).

Shemin et al. (1955), on the basis of the positions of the labelled C-atoms in protoporphyrin synthesized from Glycine-2-C^{14} and succinyl CoA, proposed the formation of a monopyrrole by condensation of two molecules of ALA via a Knorr type of mechanism Figs. III & IV. The proposed structure of this monopyrrole was also identical with that put forward by Coulson and Rimington (1953) for PBG. Furthermore, additional support for the condensation idea came from the finding that the molar activity of PBG synthesised from ALA-5-^{14}C was exactly twice that of the latter molecule (Schmid and Shemin, 1955).

The Knorr reaction (Fig. III) involves an aldol condensation followed by the elimination of H_2O between the β-Carbon of one molecule of ALA and the carbonyl group of the second molecule, plus

FIG. IV



The mechanism of PBG synthesis from 2 molecules of ALA according to Nandi and Shemin (1968).

the formation of a Schiff base with elimination of water between the carbonyl group of the first molecule and the amino group of the second.

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

The ALA-dehydratase enzymes in all systems so far examined, are SH enzymes, and, with almost all activity can only be demonstrated in the presence of added thiol compounds. With a number of extracts preincubation with thiol compounds has to be performed for maximum activity to be observed (Gibson et al. 1955; Burnham and Lascelles, 1963). This requirement of highly reducing conditions for assaying

the enzyme, suggests that the oxidation reduction potential in cells may play a part in determining the expressed activity. When the oxidation reduction potential is high the enzyme may only be able to function in vivo at a fraction of its maximum activity (Tait 1968).

d) Porphobilinogen → Uroporphyrinogen III

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

However, Schwartz (1955) working on rat liver homogenate and bone marrow, and Dresel and Falk (1956) working on chicken red cell haemolysates, demonstrated that URO could not replace PBG as substrate for COPRO and PROTO formation. The true intermediates were subsequently shown to be the colourless reduced or hexahydroporphyrins (porphyrinogens), by a number of workers on a variety of different tissues. Bogorad (1955) working on spinach leaves, and Neve et al. (1956) on a haemolysed duck red cell preparation, demonstrated that UROgen and not URO was converted

to haem. Manzerall and Granick (1958) confirmed these findings with rabbit reticulocytes, and Bogorad (1958) on Chlorella preparations. The porphyrin therefore instead of being true intermediates in biosynthesis of haem, represent by-products formed by irreversible oxidation of porphyrinogens (Rimington 1956).

The conversion of four PBG molecules into the tetrapyrrole Urogen III, requires two enzymes, PBG deaminase (UROgen I synthetase) and UROgen isomerase (UROgen III cosynthetase). In the absence of the second enzyme the symmetrical UROgen I is formed. These two enzymes have been separated and partly purified from plant cells (Bogorad 1958). In extracts from animal cells the system which makes the III isomer, after heating to 60°C now only forms the I isomer (Rimington and Booij, 1957) showing that the second enzyme is heat labile. These two enzymes have also recently been separated from extracts of mouse spleen (Levin and Coleman 1967). The first enzyme can be visualized as catalysing the formation of a linear tetrapyrrole that is condensation between the α-aminomethyl group of one PBG molecule and the free α-position of the next with the release of NH_3. Finally, at the tetrapyrolle stage the α-amino-methyl group of the fourth unit condenses with the free α-position of the first unit, to form the porphyrinogen ring. The spatial arrangement of the tetrapyrrole is such that this condensation is favoured rather

than formation of longer linear pyrroles, since models show that the α-aminomethyl group is in close proximity to the free α-position (Tait 1968). This porphyrinogen has a symmetrical arrangement of side groups, i.e. type I. The problem is to account for the formation of the III isomer in which one of the PBG units is inserted 'back to front' as it were. This is obviously the function of the second enzyme, but how it does is unknown. Many hypotheses have been put forward (Bullock et al. 1958; Wittenberg 1959; Bogorad 1960; Mathewson and Corwin 1961; Bogoard 1963; Cornford, 1964), but a number have been ruled out by experimental work and there is no direct evidence to support any of the others. UROgen III co-synthetase by itself has no action on PBG or on UROgen I or on any of the products of PBG deaminase - that is, if it is added to the reaction mixture after PBG and PBG deaminase have been incubated, UROgen III is not formed (Bogorad 1960). No intermediates have been identified, although the presence of some in reaction mixtures has been indicated (Bogorad 1960).

e) Uroporphyrinogen III (UROgen III) → Coproporphyrinogen III (COPROgen III)

Under normal conditions uroporphyrinogens are metabolised in two different ways. They are either oxidized to uroporphyrins, or enzymatically decarboxylated to coproporphyrinogen (Mauzerall and Granick 1958; Bogorad 1958; Hoare and Heath 1958). Normally a

small fraction of the UROgen formed in the cell undergoes irreversible stepwise oxidation to URO and is thereby lost to the haem biosynthetic pathway. The oxidative process has been observed to occur either enzymatically (Bogorad 1958), or by means of photocatalytic autoxidation, sensitised by the product URO (Mauzerall and Granick 1958).

Sequential enzymatic decarboxylation of most of the uroporphyrinogens formed from PBG leads to the formation of a series of porphyrinogens with less than 8 carboxyl groups (Mauzerall and Granick 1958). The enzyme uroporphyrinogen decarboxylase has been studied in preparations from rabbit reticulocytes (Mauzerall and Granick 1958), duck erythrocytes (Neve et al. 1956), Chlorella (Bogorad 1958) and R. spheroides (Hoare and Heath 1956).

Both series I and III are acted upon by this enzyme but at different rates - UROgen I is decarboxylated at only one third of the rate for UROgen III. Partial purification has been achieved, and as far as in known, a single enzyme is responsible. This enzyme is found in the cytoplasm. Isomers I, II, III and IV of uroporphyrinogen are substrates, whereas the corresponding uroporphyrins, are not (Mauzerall and Granick 1958). The exact mechanism of decarboxylation of UROgen (8 carboxyl groups) to coprogen (4 carboxyl groups) is not known. While acetic acid side chains are attacked, the proprionic acid side chains are not. Mauzerall and Granick (1958) and San Martin

de Viale and Grinstein (1968) have been able to isolate porphyrins with 7, 6 and 5 carboxyl groups as well as coproporphyrins when uROgen is incubated with the enzyme. When these porphyrins are reduced to the porphyrinogens and reincubated with the enzyme, further decarboxylation occurs, suggesting that the carboxyl groups are removed one at a time. However, it is not known in what order the acetic acid side chains are decarboxylated or indeed whether there is any preferred order. Considering all these intermediates and their possible isomers there would be altogether 34 different substrates for this enzyme. Since it has not been established with any certainty just how many enzymes are involved, it remains convenient to describe the "collective enzyme" as UROgen decarboxylase (Mauzerall and Granick 1958).

f) Coproporphyrinogen III (COPROgen III) → Protoporphyrin IX (PROTO IX)

The enzymatic conversion of COPROgen III to protoporphyrin IX has been demonstrated and extensively studied in a number of systems, including chicken erythrocytes (Granick and Mauzerall 1958a), Euglena (Granick and Mauzerall 1958b), beef mitochondria (Sano and Granick 1961), and a purified enzyme from mammalian liver mitochondria (Batlle, Benson and Rimington 1965).

The enzyme coproporphyrinogen oxidase (coproporphyrinogenase) is located intramitochondrially, has an absolute requirement for oxygen, and unlike UROgen decarboxylase is substrate specific for COPROgen III and does not act on the I isomer. Only two specific proprionic acid groups are oxidatively decarboxylated to vinyl groups yielding protoporphyrinogen (PROTOgen). Porra and Falk (1964) have found coproporphyrinogen IV to be a substrate, albeit a very poor one.

The exact mechanism(s) involved are still uncertain, but the available evidence suggests that the two proprionic acid side chains appear to be acted on independently since Sano and Granick (1961) found that a porphyrinogen with three carboxyl groups was formed and then disappeared as the reaction proceeded. On the other hand Mauzerall (1964) has published evidence, which suggests that decarboxylation and oxidation by molecular oxygen of the proprionic acid groups are simultaneous.

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

g) Insertion of Metal into Protoporphyrin

Haem is finally synthesised from ferrous iron and protoporphyrin by a particulate enzyme. ferrochelatase (haem synthetase), which is located intramitochondrially. Current available evidence favours the insertion of the ferrous ion at the stage following the oxidation of the hexahydroporphyrin i.e. PROTO IX, although this could occur at the PROTOgen stage. (Mauzerall and Granick 1958, Heikel et al. 1958).

Early studies using avian erythrocyte preparations suggested that the biosynthesis of *haem from PROTO and ferrous iron was enzyme* dependent since the activity was abolished by CN- and significantly inhibited by preheating of the haemolysate for 30 minutes at 56°C (Goldberg et *al.* 1956).

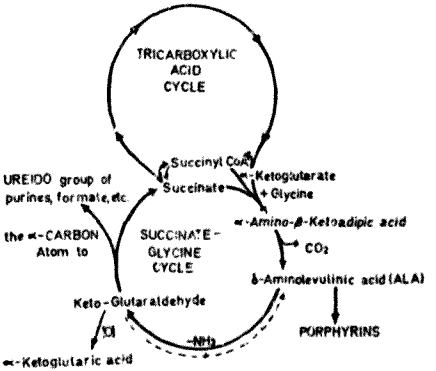
More recently the enzyme has been purified from mitochondria of avian erythrocytes (Oyama et al. 1961), mammalian liver (Porra and Jones 1963), bacteria (Labelles 1964). The enzyme from most cells has a relatively wide specificity for the metal and for the porphyrin, although it is not certain whether all the reactions are catalysed by a single enzyme (Johnson and Jones 1964). Apart from Fe^{2+}, Zn^{2+} and Co^{2+} are used by most systems. However, Porra and Ross (1965) using a cell free extract of Clostridium tetanomorphum, which does not make

haem, but does make vitamin B_{12}, found that cobalt was inserted whereas iron was not. Until recently metal protoporphyrin in complexes, other than haem, were not thought to occur in cells. However, zinc protoporphyrin has been found in yeast, and certain mutants accumulate large amounts, (Pretlow and Sherman 1967). The possible relevance of this is not clear. Addition of reducing substances - ascorbic acid, GSH and cysteine - has been shown to markedly potentiate iron-incorporating enzyme activity in rabbit bone marrow but not in liver preparations (Lockhead et al. 1963).

h) The Succinate glycine cycle (Shemin cycle)

Shemin (1955) discovered that the δ-carbon atom of δ-aminolaevulinic acid could be utilized as a 'one carbon fragment' for the ureido group of purines, β-carbon of serine, and the methyl group of methionine. Shemin thus demonstrated that porphyrin synthesis was not the only metabolic fate of ALA. It could also be converted to formic acid (Nemeth et al. 1957). Since the δ-carbon of ALA is derived from the α-carbon glycine, Shemin et al. (1955) proposed a 'succinate-glycine cycle' to explain these phenomena (Fig. V). Shemin and his co-workers proposed that some of the ALA formed by the condensation of succinyl-CoA and glycine, is transaminated to yield α-ketoglutaraldehyde; the latter is then decarboxylated, yielding the one carbon fragment mentioned

FIG. V



after St.emin (1955)

above, and succinate which re-enters the pool. ALA transaminase activity has subsequently been demonstrated in several different systems (Bagdascarcair 1958; Neuberger and Turner 1963). In addition, Shemin et al. (1955) showed that duck red cell haemolysates on incubation with ALA-δ-^{14}C yields ^{14}C-labelled α-ketoglutaric acid, and Nemeth et al. (1957) subsequently demonstrated the appearance of succinic acid-1, 4-^{14}C in the urine of rats after injection of ALA-1, 4-^{14}C. It seems that this pathway does operate, although Tschudy and his co-workers (Tschudy et al. 1962, Tschudy 1965) have obtained evidence which suggests that in mammalian liver this pathway is probably not very active.

i) The Source of Glycine and Succinyl CoA

i) Glycine: The source of most of this amino acid in animals is probably dietary although glycine can also be formed from serine by the action of hydroxymethyltransferase (Lascelles 1964).

ii) Succinyl CoA: There are four immediate sources of succinyl CoA which are summarized in Table 2. The relative contributions of each of these sources is not known with any certainty, but the major one in animals is probably oxidative decarboxylation of α-ketoglutarate (Lascelles 1964) (Fig. V) (Table 2 reaction 1). α-Ketoglutarate is an intermediate of the tricarboxylic (TCA) cycle and hence it follows that

TABLE II

THE SOURCES OF SUCCINYL-S-CoA

NO.	REACTION	ENZYME
1	α Ketoglutarate + CoA-SH + NAD^+ $\xrightarrow[Mg^{++}]{TPP,\ Lips}$ Succinyl-S-CoA + CO_2 + $NADH_2$	α-Ketoglutarate dehydrogenase (complex)
2	Succinate + GTP (ATP) + CoA-SH $\xrightarrow{Mg^{++}}$ Succinyl-S-CoA + H_2O + Pi + GDP (ADP)	Succinyl-CoA synthetase
3	Acetoacetyl-S-CoA + succinate $\longrightarrow$ Succinyl-S-CoA + acetoacetate	Acetoacetyl-CoA: Succinyl-CoA transferase
4	Methylmalonyl-S-CoA $\xrightarrow[B_{12}]{}$ Succinyl-S-CoA	Methymalonyl-CoA Isomerase

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

Succinate was shown to be an immediate precursor of succinyl CoA (Table 2 reaction 2) by Shemin and Kumin (1952), using carboxyl-labelled succinate-^{14}C which cannot give rise to ^{14}C-labelled succinyl-CoA via an oxidative turn of the TCA cycle. Labbe and his co-workers (Labbe et al. 1965, Kurumada and Labbe 1966, and Labbe 1967) have produced evidence that conversion of pyruvate to succinyl-CoA via a reductive dicarboxylic acid pathway (Fig. IX) involving malate, fumarate and succinate occurs both in normal and porphyric mammalian liver. These aspects will be considered in more detail in Chapter IV. In the third reaction (Table 2), a transfer of CoA from acetoacetyl CoA to succinate does not occur in liver since the relevant enzyme is not found in this organ (Harper 1967).

The fourth possible immediate source of succinyl CoA is methyl malonyl CoA which undergoes isomerization under the influence of methylmalonyl CoA isomerase (Table 2 reaction 4). This reaction provides a mechanism whereby proprionate can be converted to TCA cycle intermediates and thereby act as a carbon source for gluconeogenesis. In ruminants, where proprionate is a major source of glucose, this is obviously an important pathway. However, there is at present no information on the relative importance of methylmalonyl CoA as a source of succinyl CoA for haem synthesis in animals and man.

C H A P T E R III

EXPERIMENTAL PORPHYRIA

a) General

Goldberg (1968) has recently written a comprehensive review on experimental porphyria. Experimental attempts, to emulate the picture of porphyria and urinary porphyrin excretion in man, go back to the end of the last century. Stokvis (1895), who made the first observation of 'sulphonal haematoporphyrinuria' in man and also produced the first coproporphyrinuria by the administration of sulphonal (diethylsulphonedimethylmethane) to rabbits and dogs. Fischer and Duesberg (1932) confirmed these findings and also reported trace amounts of ether-soluble porphyrins, now presumed to be uroporphyrin, in the urine of rabbits treated with sulphonal.

Waldenström and Wendt (1939) were unsuccessful in an attempt to produce an experimental porphyria by the non-hypnotic derivative of sulphonal, dimethyl-sulphone-dimethylmethane. Rimington and Hemmings (1938; 1939) found that several sulphonamides, including sulphanilamide, caused a high excretion of coproporphyrin in rats, while Dent and Rimington ( ) observed a marked coproporphyrinuria in rats fed on a diet including oxidized casein. Coproporphyrin III was the predominant porphyrin excreted in the urine of rabbits given lead by Weatherall and Comfort (1952), but in addition tri-penta-and hexacarboxylic porphyrins were detected by paper chromatographic methods. There was also an increase in total faecal porphyrins (Weatherall 1954),

and Schmid, Schwartz and Watson (1950) demonstrated elevations of copro- and proto- porphyrins in the bone marrow and circulating erythrocytes, of lead poisoned rabbits. In addition uroporphyrin I was detected in the circulating blood cells and isolated from bone marrow of lead-poisoned rabbits in the acute stage of intoxication (Schmid, Hanson and Schwartz, 1952).

Schwartz et al. (1952) found that the treatment of rabbits with phenylhydrazine, lead and ultra violet light produced a temporary condition which was chemically similar to congenital porphyria in humans, since the animals excreted much URO I in the urine while the bone marrow URO and COPRO porphyrins were markedly elevated, but the liver porphyrin concentrations were generally normal.

Stimulated by a single report by Duesburg (1932), who reported the presence of porphyria in a patient treated with large amounts of sedormid (allylisopropylacetylcarbamide), Schmid and Schwartz (1952) found that sedormid induced an 'hepatic' form of porphyria in normal rabbits. Large amounts of PBG and uroporphyrin III were excreted in the urine, and the liver contained much PBG, URO- COPRO- and PROTO porphyrins. These observations were confirmed and repeated on many other types of animals (Brockman and Gray 1953; Benard et al. 1953;

Goldberg 1953; Stich 1954; Case et al. 1953; Talman et al. 1955). Goldberg and Rimington (Goldberg 1953; Goldberg and Rimington 1955) found that the clinical assessment of rabbits treated with Sedormid was obscured by the profound hypnotic effect of the drug. They investigated related drugs, one of which might produce the same chemical features in experimental animals, but yet not be hypnotic. They found such a drug to be allylisopropylacetamide (AIA) and showed furthermore that the AIA effect produced in rabbits could also be demonstrated in rats and fowls (Goldberg and Rimington 1955) (Fig. VI).

At the beginning of the century, barbiturates were introduced to medicine, and soon after were associated with the onset of acute attacks of porphyria. Waldenström (1937) put forward the theory that the barbiturates could precipitate latent porphyria into the active form. Early experiments on the effect of barbiturates on porphyrin metabolism were however unsuccessful, but Goldberg (1954) found that of 9 barbiturates given to rabbits, 6 had a significant effect on the urinary excretion of porphyrins, those containing one or more allyl groups being particularly effective. Furthermore, it is also noteworthy that the 3 which had no significant effect have been associated with provocation of the human disease. Moore and his co-workers (1968) have recently demonstrated a significant increase in hepatic δ-ALA

synthetase in rats given pentothal. Solomon and Figge (1959) reported an experimental type of porphyria in mice, rats and rabbits with 3, 5-diathoxycarbonyl- 1,4-diahydrocollidine (DDC). The effect in rats and rabbits is precisely similar to that of AIA and Sedormid, but in mice they also found accumulation of protoporphyrin in the red cell.

The outbreak of cutaneous hepatic porphyria in Turkey brought to light the toxic properties of the fungicide hexachlorobenzene, and stimulated an experimental approach in animals. Ockner and Schmid (1961) showed that rats fed 0.2% hexachlorobenzene in their diet for some time excreted large amounts of uroporphyrin and coproporphyrin and also PBG.

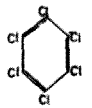
Griseofulvin has been extensively used in the past few years for the treatment of fungal infections in man and animals. Weston-Hurst and Paget (1963) and De Matteis and Rimington (1963) found that it produced a quite remarkable experimental porphyria in mice. Much protoporphyrin is found in the liver and faeces and also in the red cells, and there are increased amounts of δ-aminolaevulinic acid and prophobilinogen in the liver and urine.

Prato et al. (1964) found that the drug 2-allyl-oxy-3-methyl-benzamide produced a disturbance of porphyrin metabolism in man, and

FIG. VI

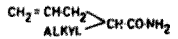
PORPHYRINOGENIC DRUGS

1. HEXACHLOROBENZENE

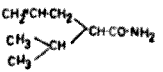


2. SEDORMID AND RELATED SUBSTANCES

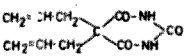
a) Basic formula of dialkyl-substituted acetamides



b) ALLYLISOPROPYLACETAMIDE (AIA)



c) Diallyl-barbituric acid (Dial)



subsequently Battistini et al. (1966) showed that this drug when given to rats produced a marked increase in excretion of urinary ALA, PBG and coproporphyrin and faecal porphyrins. On the whole, the drug studies in experimental animals and liver cell culture have confirmed the clinical evidence of suspected drugs (Granick 1965; de Matteis 1967).

b) AIA-Induced Porphyria in the Rabbit

i) Chemical findings

The administration of AIA to rabbits in a dose of about 200 mg/kg./day was found to cause an immediate rise in the urinary coproporphyrin excretion mounting stepwise until the 4th to 7th day when uroporphyrin and PBG appeared in the urine (Goldberg and Rimington 1962). From this point, the urinary coproporphyrin excretion remained approximately constant, but the PBG and uroporphyrins mounted to reach maximum levels two to three days later. If the drug was continued, uroporphyrin and PBG levels usually fell after several days of maximum excretion. Withdrawal of the drug led to rapid cessation of urinary prophyrin and PBG excretion within forty-eight hours until normal levels of coproporphyrin were regained within four days. Faecal corpo- and proto- porphyrin levels rose and fell in accordance with urinary copropo phyrin.

There was a marked increase in copro- and protoporphyrin levels in the liver, while some rabbits exhibited increased uroporphyrin and PBG as well. The spleen, kidney and bone marrow showed slightly raised levels of copro- and protoporphyrin, while the kidney and plasma of some rabbits showed slightly increased levels of coproporphyrin and PBG.

Goldberg and Rimington (1955), found that in those rabbits treated with AIA which were high excretors of PBG, a fourfold reduction in the urinary excretion of glycine was noted at the end of the drug course as compared with that before the drug was given. Three rabbits which were poor excretors of PBG, and were later shown to have hepatocellular damage, void many amino acids at the end of the drug course.

ii) Clinical findings

Unlike sedormid treated rabbits which lost consciousness for about 10 hours after drug administration, AIA treated rabbit never became unconscious and at the most showed only a slightly dazed appearance for 1 - 1½ hours after drug administration (Goldberg and Rimington 1962).

Limb paralysis of paresis never occurred, and AIA treated rabbits remained active even though excreting amounts of uroporphyrin

as high as 7 mg per day or more for many days. The AIA treated rabbits however quickly lose appetite and weight, but haematological indices remain normal. There is also no evidence of photosensitivity.

c) Hexachlorobenzene poisoning in Rabbits

i) Chemical findings

De Matteis et al. (1961) incorporated 0.5% of hexachlorobenzene into the feed of rabbits and demonstrated increased urinary uroporphyrin excretion after about six weeks. The uroporphyrinuria became progressively more severe and was accompanied by a rise in urinary coproporphyrin. There was no significant rise in urinary ALA or PBG excretion. Massive quantities of uroporphyrin were found in the liver, but only slight increases in coproporphyrin and protoporphyrin were noted. Uroporphyrin was also detected in the serum in appreciable amounts and also in some other organs studied.

The qualitative reaction for porphobilinogen in the liver was negative. On paper chromatography of the URO fraction from liver, Matteis et al. (1961) showed that it was mainly uroporphyrin III with some 'pseudo-uroporphyrin'.

ii) Clinical findings

All the animals studied by Matteis et al. (1961), although varying in amounts of porphyrins excreted, exhibited the same final neurological picture. The animals lost weight, suffered from oliguria and constipation, with diminished intake of food and water. The haemoglobin level remained unchanged. Unless sacrificed earlier, all the rabbits died after 8 - 12 weeks having shown for the last week or so before death, neurological symptoms in the form of tremor and paresis.

d) Relevance of Experimental Porphyria to Human Porphyria

Experimental induction of porphyria in animals by various drugs, produces in some cases a striking chemical resemblance to several forms of porphyria found in man (De Matteis 1967). Thus AIA-induced porphyria in the rabbit produces a chemically similar pattern to variegate porphyria during attacks. This is perhaps the most fundamental similarity between the two conditions and suggests a similar mechanism of porphyrin precursor overproduction. The major difference between the human and the experimental disease, is in the neurological states associated with both. In AIA induced porphyria in the rabbit, there is no evidence, clinical or histopathological, of

any selective damage to nervous tissue, and the main clinical manifestation of acute porphyria i.e. paralysis, hypertension and tachycardia are absent.

On this basis an important question which might be asked is how relevant to the human disease are the results of studies on experimentally produced porphyria in animals. The differences in the two diseases could of course be explained on the basis of a species difference. However it can also be argued that if an animal is exposed to a chemical porphyria for little more than the 2 to 3 weeks, this may not be a sufficient time to produce the clinical manifestations of the human disease, since in man the metabolic abnormality will have been of long standing before the onset of symptoms. In 1960 De Matteis demonstrated both abnormal porphyrin synthesis and disturbances of the nervous system such as paralysis in pantothenic acid deficient rats treated with AIA (De Matteis 1960). Goldberg and Rimington (1962) have suggested that experimentally induced porphyria by drugs such as AIA and sedormid may indeed resemble the acute forms of porphyria in man from the chemical pathological point of view more nearly than has been previously supposed.

Hexachlorobenzene induced porphyria in rabbits has an uncanny chemical resemblence to human acquired symptomatic porphyria, such as

is common in the Bantu people of South Africa. Goldberg and Rimington (1962) have compared hexachlorobenzene poisoning in animals and found the type of lesion produced to resemble the cutaneous porphyria of chronic alcoholics (Conivet and Rimington 1953). Again as in AIA induced porphyria, the neurology associated with the two diseases differ. In hexachlorobenzene poisoning of rabbits, the development of neurological symptoms is a characteristic feature more reminiscent of the human acute porphyrias. However, in an outbreak of porphyria in Turkey which was put down to hexachlorobenzene ingestion, and involved about 5,000 men, women and children (Cam 1959), no neurological manifestations were evident. It would seem therefore that in this case a distinct species difference may be involved and further work will clearly be required to clarify the situation.

C H A P T E R IV

THE EFFECT OF DRUGS ON HAEM BIOSYNTHESIS

a) General

A large body of recent experimental work on the porphyrias has been concerned with the study of control mechanisms governing the rate of haem biosynthesis. The porphyrinogenic drugs have been a major tool in these studies, as mechanisms of action of these porphyrinogenic substances and the control of haem synthesis are intimately connected.

An unequivocal finding to date has been that one of the sites of control is at the stage of δ -ALA synthesis. δ -ALA-synthetase has been shown to be the rate-limiting enzyme for haem biosynthesis in a number of experimental systems. These include the bacterium Rhodopseudomonas spheroides (Lascelles 1960, Burnham and Lascelles 1963), guinea pig liver (Granick 1963, Granick and Urata 1963), chick embryo liver cells (Granick 1966), and chick erythroid precursor cells (Levere and Granick 1965, 1967). The structural diversity of substances capable of including δ -ALA synthetase is extremely wide, and ranges from drugs such as allylisopropylacetamide (AIA) (Granick 1966), to steroids (Granick and Kappas 1967, Kappas and Granick 1968), and structurally small molecules, of which ethanol is an example, (Shanley, Zail and Joubert 1968). This structural diversity has resulted in a number of mechanisms of δ -ALA synthetase induction being proposed.

In 1963 Lascelles, working on the bacterium Rh. spheroides, found that two mechanisms were involved in the control of ALA-synthetase activity. Firstly haem allosterically inhibited δ -ALA-synthetase (i.e. end-product inhibition), and secondly haem repressed the formation of δ -ALA-synthetase (i.e. end-product repression (Lascelles 1960, Burnham and Lascelles 1963).

In 1967, Levere and Granick working on the early differentiating erythroblast of the chick found that haem formation was limited only by a lack of ALA-synthetase which they proposed was under the control of a repressor mechanism. End-product inhibition was also demonstrated in rabbit reticulocytes by London et al. (1964). Granick and Urata (1963) demonstrated a 40-fold increase in the δ -ALA-synthetase activity of guinea-pig liver on treatment with 3,5-dicarbethoxy-1,4, -dihydro-collidine (DDC). Granick (1963) found this increase to be due to induction of enzyme synthesis rather than to activation of performed enzyme. Granick (1966) confirmed these results on chick embryo liver, and furthermore demonstrated, that in this system haem inhibited the induction process, but that no end-product inhibition of δ -ALA-synthetase took place.

b) Granick's Repressor mechanism for δ-ALA synthetase

Granick's findings on guinea-pig liver cells and chick embryo liver led him to postulate a repressor mechanism for δ-ALA-synthetase (Granick 1966) (Fig. VIII). He postulated the presence of a protein aporepressor and a corepressor which is haem. To explain the action of porphyrinogenic substances, he found it necessary to suggest that such substances compete with end-product (haem) for an active site on the aporepressor, which regulates the activity of a structural gene coding for δ-ALA-synthetase. The combination of aporepressor and inducer fails to repress the operator gene for δ-ALA synthethase, consequently leading to accelerated porphyrin biosynthesis.

As already mentioned, (Granick 1966) found that a wide variety of chemicals and drugs including steroids, were capable of inducing excessive porphyrin synthesis in chick embryo liver. The inducing potential of the natural steroids was shown to be equal to or exceeded that of even the most potent porphyrinogenic drugs (Granick and Kappas 1967). Furthermore Kappas and Granick (1968) demonstrated, that the pattern of induction by these natural steroids was very similar to that of foreign drugs and chemicals. This demonstration of porphyrinogenesis in the liver by a group of physiological compounds allowed Kappas

and Granick (1967) to propose that these steroids play a role in normal control of haem biosynthesis. They proposed, that under physiological conditions, the free steroids compete with haem for a site on the aporepressor thereby causing derepression of ALA-synthetase.

Granick's hypothesis, however, is based on evidence obtained from non-mammalian sources, and until more direct evidence is obtained from mammalian liver it cannot be assumed that the hypothesis applies equally to humans.

c. The Labbe Hypothesis

Since all known functions of haem are directly related to oxidative processes, it has been suggested that the state of cellular oxidation might influence the metabolic regulation of porphyrin and haem biosynthesis (Margoliash 1961). Earlier work produced a number of observations, which indirectly linked porphyrin and haem synthesis with biological oxidation. For example, Falk et al. (1959) have shown that decreasing oxygen tension, within limits, would result in increased porphyrin formation by avian erythrocytes in an in vitro system. Rhosopseudomonas spheroides, an organism which can be induced to synthesize porphyrins at a greatly accelerated rate by growing it anaerobically in the light, has been used to study the regulation of

porphyrin synthesis (Lascelles 1959, Shemin et al. 1960, Kikuchi et al. 1961 and Gibson et al. 1962). Most of these studies with R. sphaeroides have been concerned with the tricarboxylic acid cycle (TCA-cycle), and with the activity of ALA-synthetase. However, in an extensive investigation of factors affecting pigment formation in this organism, Cohen-Bazirre et al. (1957) concluded that the rate of porphyrin formation in the cells is governed by the state of oxidation of a carrier in the electron transport system.

Many workers have since suggested that the redox environment in the liver may play an important role in determining both patterns of porphyrin excretion and the extent of porphyrin excretion and the extent of porphyrin formation (Lockwood et al. 1958, Rimington, 1963b). Based on results obtained from work on pyruvate utilisation and related facets of oxidative metabolism in symptomatic porphyric patients, Joubert et al. 1963) suggested a functional deficiency in the mitochondrial energy transfer system resulting in defective oxidation of $NADH_2$ in these patients. Depression of $NADH_2$ oxidation by porphyrinogenic drugs has been demonstrated either directly or indirectly by a number of different workers. Using mouse fibroblast cells grown in tissue culture, Cowger et al. (1963) found decreased oxygen uptake by cells utilizing endogenous substrates which were oxidized largely

FIG. VIII The diagram opposite, illustrates the proposed mode of induction of liver ALA-synthetase by inducing substrates which compete with haem for a site on the aporepressor molecule.

(OP = Operator gene, G = structural Gene, E_1 - E_7 = Enzymes of the haem biosynthetic pathway, APOREPRES = Aporepressor molecule).

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FIG. VIII

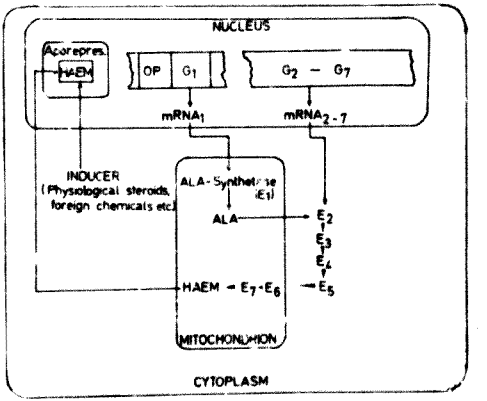

FIG IX

REACTION

a) Malic enzyme
b) Pyruvate carboxylase
c) Malate dehydrogenase

1. Fumarate hydratase
2. Fumarate reductase (Inducible)
3. Succinyl CoA synthetase (Inducible)
4. Succinyl CoA synthetase (Constitutive)
5. Succinyl CoA deacylase
6. Ketoglutarate dehydrogenase

NOTES

a) On administration of porphyrinogenic drugs, reactions 2 and 3 are inducible (Labbe et al. 1965).

b) Reaction 4, 5 and 6 and mitochondrial permeability remain normal (Granick and Urata 1963, Tschudy et al. 1964).

c) Reaction a, b, c, and 1 represent utilization of pyruvate through the reductive pathway, which is increased on administration of porphyrinogenic drugs (Haining and Labbe 1967).

d) The initial site of action of the porphyrinogenic drugs, is the inhibition of $NADH_2$ - oxidase (Cowger and Labbe 1967).

FIG. IX

Diagram representing the Labbe hypothesis

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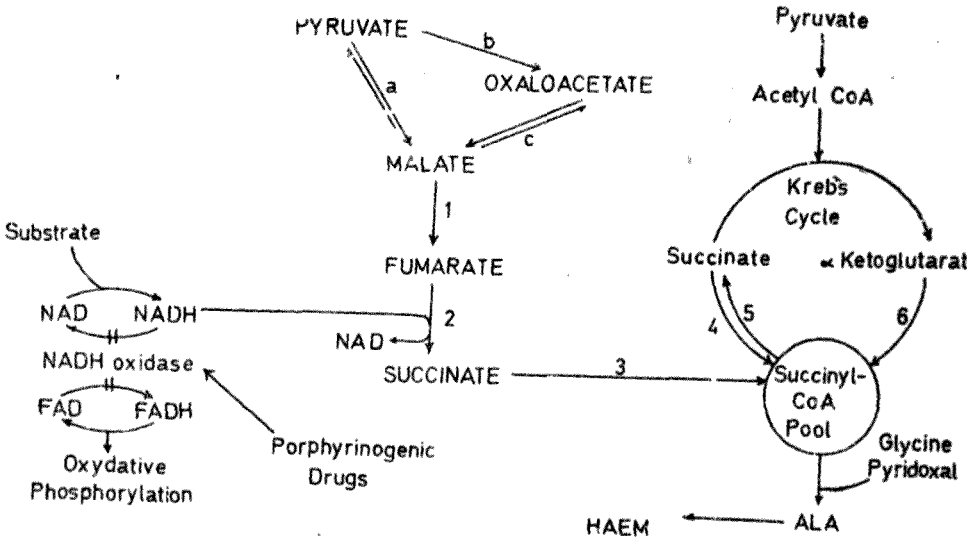
FIG. IX

Diagram representing the Labbe hypothesis



through the mediation of $NADH_2$. Furthermore, these workers showed a decreased cellular ATP concentration in mouse fibroblast cells treated with porphyrinogenic drugs presumably due to a depression o. oxidative phosphorylation, following an inhibition of mitochondrial $NADH_2$ oxidation. Ga dos et al. (1966a) have described an inverse correlation between ATP and porphyrin concentration in the livers of rats treated with Apronal. Gajdos et al. (1966b) also found a consistent decrease in liver ATP after treatment of rats with malonate, a porphyrinogenic substance and known inhibitor of succinate dehydrogenase.

Granick's hypothesis of ALA-synthetase induction is generally accepted today, but the wide structural diversity of substances capable of inducing ALA-synthetase has led to the necessity to postulate the existence of alternative mechanisms of ALA-synthetase induction. One suggestion originally put forward by Rimington (1962, 1963a, 1963b) holds that the common property of porphyrinogenic substances is the ability to change liver redox states. Labbe and his co-workers (Labbe et al. 1965, Kurumada and Labbe 1966, Labbe 1967) have formulated a precise hypothesis on ALA-synthetase induction as a result of increased intramitocholdrial $NADH_2/NAD$ ratios. Labbe has tentatively linked the overproduction of porphyrins or porphyrin precursors to depressed

oxidation of $NADH_2$ (Fig. IX). Labbe and coworkers (1965), observed greater succinate utilization in the form of increased succinyl-CoA synthetase activity in mouse liver mitochondria, after treatment with the porphyrinogenic drugs AIA and DDC. In isotopic experiments using fumurate 1-4-C^{14} Kurumada and Labbe (1965) demonstrated that fumurate reduction and conversion to succinyl-CoA was accelerated in rat liver made porphyric by drug-induction. They furthermore showed that fumurate reduction to succinate was mediated via the enzyme fumurate reductase which is $NADH_2$ dependent. Following on from this work Labbe also carried out experiments in which the incorporation of C^{14}-labelled pyruvate and other labelled dicarboxylic acids into liver haem in normal and porphyric animals was determined. Using these dicarboxylic acids which were labelled in particular positions in the molecule, labelling patterns varied in such a way that permitted selective assessment of the utilization of these acids for haem formation through either the reductive (malate-fumurate-succinate) pathway or through the oxifative (TCA cycle) pathway (Haining and Labbe 1967). These experiments led Labbe to the conclusion that in the porphyric animal, pyruvate conversion to succinyl-CoA and incorporation into haem through the reductive pathway reactions was increased while tricarboxylic acid pathway turnover remained unchanged. Labbe had already furthermore shown, that the enzyme succinyl-CoA

synthetase existed in both a constitutive and an inducable form, and that the effect of drugs such as ALA and DDC was to induce the non-constitutive succinyl-CoA synthetase (Labbe et al. 1965).

In an extensive study Cowger and Labbe (1967) investigated the effect of 30 drugs which included a wide variety of molecular species on $NADH_2$ oxidase purified from beef liver mitochondria and glycolysis in cultured mouse fibroblast cells. Most of the drugs were chosen because of their known association with the precipitation of symptoms in clinical cases of porphyria or their ability to induce porphyria in an experimental system. Their results, indicated a constant relationship between the inhibition of electron transport and the exacerbation of induction of porphyria. These findings supports the thesis of a direct metabolic link between impaired terminal oxidation and porphyrinogenesis.

Based on these findings then, Labbe has proposed that factors which lead to an increase in the intramitochondrial $NADH_2/NAD^+$ ratio stimulate fumurate reductase activity, leading to increased synthesis of succinate, which in turn induces non-constitutive succinyl-CoA synthetase. The succinyl-CoA thus generated, then acts as depressor of ALA-synthetase synthesis by substrate induction (Fig. II).

However, as in the case of Granick's hypothesis (section b), there is virtually no information as to the applicability of the proposal to human prophyria.

d. Drug Detoxification

The mammalian liver has the ability to inactivate potentially toxic compounds and render them suitable for excretion. These substances, which include lipid soluble drugs and naturally occurring compounds (e.g. steroids) are metabolised in the microsomal fraction of the liver by oxidative enzyme systems (Gillette 1963). These enzyme systems require microsomal cytochrome, cytochrome P-450, and are unusual in that they also require atmospheric oxygen and NADPH (Gillette 1963, Conney 1967, Omura et al. 1965 and Cooper et al. 1965).

Marver and Schmid, (1968) have proposed a hypothesis linking the hepatic detoxification mechanisms with the pathogenesis of acute and experimental porphyria. They have based their hypothesis on a number of recent observations. Firstly, haem is involved in the repression of hepatic ALA-synthetase (Granick 1966, Yschudy et al. 1966 and Marver et al. 1966). Secondly compounds that stimulate drug metabolism including the barbiturates, steroids and AIA, induce ALA-synthetase. This subsequently provides the haem needed for

increased formation of microsomal cytochromes. Further evidence for this interdependence is provided by the observation of Marver and Schmid (1968) that haem blocks the drug mediated induction of ALA synthetase and microsomal cytochromes. Thirdly a large number of compounds including AIA, the barbiturates and naturally occurring steroids are capable of producing chemical porphyria in cultured-embryonic chick liver, and they also aggravate genetic porphyria in man. (Granick, 1966, Schmid, 1966, Granick, 1965). However, by contrast, only a few of these compounds, of which AIA is a good example, can induce porphyria in the intact experimental animal. Phenobarbitone does not induce porphyria in the intact animal. Yet both these classes of compounds, represented by phenobarbitone and AIA, induce hepatic δ-ALA synthetase and enhance liver haem synthesis in the intact animal. Marver and Schmid (1968) have explained the difference in the effect of these two classes of compounds by their different effect on the feedback repression mechanism of *ALA synthetase*. Induction of δ-ALA-synthetase results from interference with the normal feedback repression of the enzyme. Phenobarbitone in addition to interference with the feedback control mechanism, also induces the drug detoxification mechanisms in the liver. Thus in the intact animal, the induction of δ-ALA synthetase provides the increased haem needed for synthesis of hepatic cytochromes, and consequently porphyria does not develop.

By contrast, ALA and and some other porphyrinogenic compounds have the capacity to promote haem degradation in addition to stimulating haem synthesis (Marver et al. 1968, Callahan et al. 1968). Marver and Schmid have therefore postulated that these compounds induce δ-ALA synthetase excessively because in addition to interfering with enzyme repression, they enhance the breakdown of haem. In cultured embryonic liver, and in genetic acute porphyria Marver and Schmid have postulated that a constitutional defect may not allow the liver to respond adequately to stimulation by drugs. The defect may be in that one or more components of drug detoxification mechanism are blocked, and therefore detoxification does not take place. Under these circumstances, δ-ALA synthetase is induced excessively, thereby producing porphyria or aggravating pre-existing disease. However, at present, more data is still required to establish whether an operator constitutive mutation or a genetic disorder of detoxification is the underlying defect.

e. The Redox state of the Cell Cytoplasm

The porphyrinogens, being the true intermediates of haem biosynthesis, are highly unstable, and it is therefore understandable that they require reducing conditions to prevent irreversible autoxidation. Heikel et al. (1958) has put forward the suggestion that the excessive

porphyrin appearing in the liver in cutaneous porphyria may be derived from porphyrinogens which have become oxidized and have thus 'escaped' from the biosynthetic pathway. Goldberg & Rimington (1962) have proposed that normally, oxidation-reduction systems in the liver such as NAD^+ - $NADH_2$ and $NADP^+$ - $NADPH_2$ systems preserve an oxidation-reduction climate which keeps the pigment in the reduced state. However, in the diseased liver, the balance of these or other systems is sufficiently upset for oxidation of porphyrinogens to porphyrins to occur. Once formed the porphyrins would accumulate and suffer eventual excretion as useless products.

In the symptomatic porphyria of the Bantu, difficulties have arisen in formulating an approach, in so far as the underlying biochemical basis of the disease is concerned. Since no evidence can be found of genetic inheritance, there is no basis for a hypothesis invoking the enzymes mediating haem synthesis. As a result the postulate that the 'redox state' of the liver underlies the biochemical lesion in symptomatic porphyria has gained some standing.

This situation is however quite different from the invisaged by Labbe (section c) who holds that porphyrin synthesis is promoted by conditions (within the mitochondrion) favouring reduction.

CHAPTER V

PYRIDINE NUCLEOTIDE SYNTHESIS

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PYRIDINE NUCLEOTIDE SYNTHESIS

a) Biosynthesis of the Nicotinamide Nucleotides

The biosynthesis of the nicotinamide nucleotides has been extensively studied in the last twenty years, and it has become obvious, that it is a very complex part of the metabolism of a variety of organisms. More than one biosynthetic pathway seems to be operative and the significance and control of these is still far from understood. However a number of the pathways have been well documented and are summarized in Fig. VII.

Goldberger and Tanner (1922) reported that under normal dietary conditions, the rat did not require nicotinic acid. Working from this observation, Krehl and his co-workers (1945) first demonstrated that there was a metabolic relationship between nicotinic acid and tryptophan, and that in fact tryptophan was the dietary factor which substituted for nicotinic acid in the rat. Subsequently, a number of genetic and isotopic studies were carried out which elucidated the mechanism of this conversion. Rosen et al. (1946) and Singal et al. (1946) demonstrated increased urinary excretion of N-methylnicotinamide after the administration of tryptophan to the rat. Nutritional studies by Hundley (1954) and Henderson et al. (1949) demonstrated that suitable levels of tryptophan could replace the requirement for nicotinic acid

FIG. VII

The enzymes which catalyze the reaction depicted opposite are -

1. Tryptophan pyrrolase
2. Kynurenune firmykase
3. Kynurenine - 3 - hydroxylase
4. Kynureninase
5. 3-hydroxyanthranilic acid oxidase
6. Spontaneous
7.)
8.) Quinolinate transribosylase
9. Desamino - NAD pyrophosphorylase
10. NAD^+ synthetase
11. Nicotinamide deaminase
12. Nicotinic acid ribonucleotide pyrophosphorylase
13. Nicotinamide ribonucleotide pyrophosphorylase
14. NaD^+ pyrophosphorylase

a) The "NAD^+ pathway of Tryptophan metabolism" of Nishizuka and Hayaishi involves reactions 1 - 10

b) The "Preiss-Handler pathway" involves reaction 12, 9 and 10

c) The pathway suggested by Dietrich et al. involves reactions 13 and 14

d) The hypothesis of Greengard et al. involves the entrance of nicotinamide into the "Preiss-Handler Pathway" through reaction

e) The incompletely characterized 'de novo' pathways grouped in reactions 15

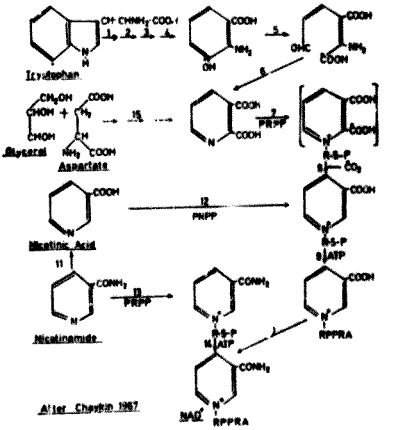
The following symbols are used

P = Phosphate

R = Ribose

A = Adenine

FIG. VII



METABOLIC PATHWAYS LEADING TO THE SYNTHESIS OF NAD+

in the rat, dog, rabbit, pig, monkey, chick duck and several other mammals and birds that they studied. Studies using Neurospora crassa and the rat have led to the compounds kynurenine (Beadle et al. 1947) 3-hydroxykynurenine (Mitchell and Nyc, 1948, Haskins and Mitchell 1949), and 3-hydroxyanthranilic acid (Bonner 1948) being recognized as intermediates in the pathway of tryptophan to nicotonic acid (Fig. VII reaction 1-4). Using isotopic procedures, Heidelberger and other workers found direct evidence for the metabolic relationship between tryptophan and nicotinic acid (Heidelberger et al. 1949a, 1949b, Henderson and Hankes, 1956, Partridge et al. 1952). These workers found, that when labelled tryptophan or 3-hydroxyanthranilic acid was administered to rats, nicotinic acid recovered from the urine and tissues was labelled in the carbonyl carbon position. They furthermore demonstrated, that it was the carbon in position 3 of the indole ring that became the carbonyl carbon of nicotinic acid. Schayer et al. (1949) and Partridge et al. (1952) also demonstrated that the nitrogen atom of the indole ring, was converted to the pyridine ring nitrogen of nicotinic acid.

During the next few years most research was directed at trying to elucidate the mechanism of the conversion of 3-hydroxyanthranilic acid to nicotinic acid. Most attempts were uniformly unsuccessful,

and only the pyridinecarboxylic acids, quinolinic acid and picolinic were found to be derived from 3-hydroxyanthranilic acid and ultimately tryptophan in vitro. However, niether of these compounds gave any indication of being precursors of nicotinic acid in vitro despite the fact that quinolinic acid was found to serve as a precursor of both N-methylnicotinamide in rats (Hankes and Segal 1959), and nicotinic acid in chick embryo tissues (Wilson and Henderson 1960). Recently however, Nishizuka and Hayaishi (1963) found the missing step by which tryptophan is converted to nicotinic acid in rats, These workers demonstrated the conversion of quinolinic acid to nicotinic acid ribonucleotide and not a free nicotinic acid as had been looked for up to that time. They used a soluble rat liver enzyme quinolinate transribosylase which they supplemented with 5-phosphoribosyl-1-pyrophosphate. The 'NAD$^+$ pathway of tryptophan metabolism' which they proposed on the basis of their observations has subsequently been found in a number of organisms (Fig. VII Reactions 1-10) (Kono et al. 1965; Ahmad and Moat 1966; Hadwiger et al. 1963; Yang and Walker 1965; Packman and Jacoby 1965, Andreoli et al. 1963).

The pathway originally proposed by Priess and Handler (1958), for the biosynthesis of NAD$^+$ from nicotinic acid by human erythrocytes and yeast autolysates is still accepted today (Fig. VII Reactions 12, 9 & 10).

Subsequently work has indicated that the 'Priess-Handler' pathway is operative in several mammalian tissues and a number of different organisms including beef liver (Imsande and Handler 1961), E. coli (Imsande 1961) mycobacterium tuberculosis (Dudley and Willet 1964), Ehrlich ascites tumour cells (Grunicke et al. 1966) (Pallinic and Ricci 1965). Priess and Handler discovered the existence of an enzyme nicotinic acid ribonucleotide phyrophosphorylase which converted nicotinic acid to nicotinic acid ribonucleotide. The nicotinic acid ribonucleotide thus produced is converted to deamino-NAD in the presence of ATP, and then amidated in the presence of ATP and glutamine to give NAD^+ (Priess and Handler 1958) (Fig. VII Reactions 12, 9 & 10).

In addition to the 'Pries-Handler pathway' and the 'NAD pathway of tryptophan metabolism' of Nishizuka and Hayaishi, which incorporates part of the 'Priess-Handler pathway' several alternative pathways of nicotinamide nucleotide synthesis have been put forward. Although in the two pathways already discussed nicotinic acid is the precursor of NAD^+ synthesis, nicotinamide has been known for many years to be an adequate form of the vitamin for meeting the general mammalian dietary requirements for precursors of the nicotinamide coenzymes. Nicotinamide has also been shown to be the predominant form of the vitamin in blood and other mammalian tissue (Chaykin et al. 1965 a,b). Hughes and

Williamson (1953) demonstrated deaminase enzymes, which can convert the amide to the acid, in a variety of bacteria. These enzymes can therefore permit the deamidated nicotinamide to enter into the 'Priess-Handler pathway'. Kirchner et al. (1966) however, in contrast to the findings with the nicotinamide utilizing bacteria, found that very few mammalian cells possessed the ability to convert nicotinamide to nicotinic acid. Greengard and his co-workers (Petrack et al. 1963) did find the deamidase in mammalian liver, but only partially resolved the problem because they found the activity of this enzyme was masked in cell homogenates by an endogenous inhibitor. They suggested that this phenomenon could offer a means for the control of NAD^+ biosynthesis. Recent evidence however obtained by Greengard and his co-workers support their previous proposal that the initial step in the biosynthesis of the coenzyme from nicotinamide in mammalian liver involves the deamination of nicotinamide to nicotinic acid, which is then converted to NAD^+ via the 'Priess-Handler pathway' (Fig VII Reaction 11) (Petrack et al. 1965, Petrack et al. 1963, Greengard et al. 1963).

The discovery by Dietrich and his co-workers (1965, 1966), of a specific nicotinamide ribonucleotide pyrophosphorylase, of widespread occurrence, has raised the possibility of yet another pathway by which

nicotinamide might be converted to NAD^+. This pathway permits the utilisation of nicotinamide without the prior need f.. deamination (Fig. VII Reaction 13).

Finally, much work has been carried out in looking for a possible 'de novo' pathway for the biosynthesis of the pyridine ring. This pathway would utilize relatively small and simple molecules rather than a complex precursor such as tryptophan. At present, derivatives of aspartic acid and glycerol have been implicated as the condensing partners in a reaction yielding quinolinic acid in several organisms (Chandler et al. 1966, Isquith and Moat 1966a, Gross et al. 1965). However, there have been several organisms in which other small molecules have been shown to be preferred precursors, and as yet the 'de novo' pathway(s) is not at all clearly understood (Scott and Hussey 1965, Isquity and Moat 1866b) (Fig. VII Reaction 15).

It seems then, that most cells have potential for synthesizing NAD^+ by more than one metabolic pathway. Since the nicotinamide coenzyme concentrations in these cells are remarkably constant, it seems as though the operation of any given pathway must be regulated with respect to both existing nucleotide levels and the degree of operation of the other pathways (Chaykin 1967). In addition it seems feasible

that the concentrations of nicotinamide coenzymes in certain animal tissues such as the liver might be under the influence of the general metabolic state of the animal as well. For example many of the oxidation-reduction functions of the liver, which are directly related to other metabolic and nutritional factors, could well be under the control of these factors through hormone action, or deranged by drugs or other chemicals which effect these factors.

In summary there seem to be a number of pathways leading to the biosynthesis of the nicotinamide coenzymes in mammalian and other cells. The enzymes of these pathways could be regulated in vivo but the extent to which one or other of the pathways predominate, and the significance of a number of pathways in the same animal or tissue which seemingly all accomplish the same end, is not clear.

b) Nicotinamide-C^{14} (Carboxyl) as the Radioactive Precursor of the Nicotinamide nucleotides

Nicotinamide-C^{14} has been shown to be the precursor of choice when undertaking experiments involving the nicotinamide coenzymes. Stollar and Kaplan (1961) showed that the nicotinamide nucleotides of liver became labelled after administering nicotinamide-C^{14} to the intact

rat. Work has also been published in which nicotinamide has been shown to induce a considerably greater increase in the concentration of liver NAD^+ than does nicotinic acid (Fiegelson et al. 1951; Kaplan et al. 1956; Greengard et al. 1964). Greengard and his co-workers (1966) have shown this apparent contradiction to be due to the fact that nicotinamide has a much longer half-life than nicotinic acid, both in liver and in blood. Coenzyme synthesis is thus permitted to continue for a longer period, following administration of the amide.

(c) $NAD^+ \longrightarrow NADP^+$

The conversion of NAD^+ to $NADP^+$ is carried out by the enzyme NAD kinase (ATP : NAD 2^1- phosphotransferase). The enzyme is Mg^{++} dependent and has been isolated and studied in yeast autolysates (Kornberg 1950), rabbit kidney and liver (Katchman et al. 1951), and pigeon liver, (Wang and Kaplan 1964). The enzyme catalyzes the synthesis of $NADP^+$ by a direct phosphorylation of NAD^+ by ATP in the presence of Mg^{++}. Kornberg and Pricer (1950) have shown that the NAD^+ is phosphorylated at the 5-carbon position of the ribose moiety of the adenine portion of the molecule.

(d) NAD^+ and $NADP^+$ as hydrogen carriers

The coenzyme NAD^+ can be reduced by many different substrates, in the presence of the appropriate enzymes which are known as dehydrogenases.

The same applies to the coenzyme $NADP^+$, but there appear to be fewer enzymes capable of reducing $NADP.^+$ The enzymes are fairly specific for the coenzyme concerned, and those enzymes which reduce NAD^+ do not usually reduce $NADP^+$ and vice versa, though there are exceptions, as for example glutamic dehydrogenase (Williamson et al. 1967). The two coenzymes are also reduced by certain chemical reducers, for example dithionate.

The reduced forms of coenzymes are not oxidized at any significant rates by O_2, or by cytochrome C, or by dyes such as methylene blue. They are however oxidized by many of the dehydrogenases acting in reverse, in the catalysis of anaerobic fermentations, by dyes in the presence of several flavoproteins, by cytochrome C or b_5 in the presence of the respective cytochrome reductases, by cystine, glutathione or lipoamide in the presence of their respective reductases, and by H_2O_2 in the presence of the specific peroxidases. In addition they are also oxidized non-enzymatically by a variety of substances including phenazines, porphyridines and porphyrexides (Schellenberg and Hellerman 1958).

The two coenzymes are widely distributed in living matter. Glock and Mclean (1955), measured the amounts of reduced and oxidized NAD^+ and $NADP^+$ in various tissues of the rat. They found that in most of the different type of cell they studied, the NAD^+ was present in much larger amounts than $NADP^+$, but that the ratio of the amounts

of reduced and oxidised forms was much higher for $NADP^+$ than for NAD^+. For example they found that the total NAD^+ in rat liver was of the order of 570 ug per gm wet weight, with 36% being in the reduced form, in contrast to 210 ug per gm wet weight of $NADP^+$ with 97% being found in the reduced form. Subsequent to this study, many other workers have estimated the distribution of the reduced and oxidised NAD^+ and $NADP^+$ by various methods. Holzer et al. (1958) found higher total values than Glock and Mclean (1955). Using rat liver they found a total NAD^+ concentration of 697 ugms/gm wet weight, with 27.6% being in the reduced form, and 234 ugms/gm wet weight of $NADP^+$, with 97% being in the reduced form. In 1962 Slater and Sawyer using a micro-enzyme method on rat liver obtained similar results to those previously mentioned as far as NAD^+ is concerned. However, they found a total $NADP^+$ concentration of 312 ugms/gm wet weight of liver, with 89% being in the reduced form. Krebs and his co-workers measured the redox state of free nicotinamide adenine dinucleotide in the cytoplasm and mitochondria of rat liver, by measuring the ratio of the concentrations of the oxidised and reduced metabolites of NAD-linked dehydrogenase systems located in the two compartments. They found the ratio $\frac{\text{total NAD}^+}{\text{total NADH}_2}$ to be 7.2 in the cytoplasm and 2.2 in the mitochondria (Williamson et al. 1967).

The only previously published results as to the distribution of NAD^+ in the rabbit liver, are those of Jedeikin and Weinhouse (1955) who found a total of 562 ugms/gm wet weight of NAD^+, with 27.8% being in the reduced form.

e) *Basis for the present study*

The structural diversity of substances capable of inducing ALA-synthetase, and the necessity to postulate the existence of a hypothetical aporepressor in mammalian tissue, have kept speculative as to alternative mechanisms of ALA-synthetase induction other than that postulated by Granick (1966) (Chapter IVb). One suggestion, originally put forward by Rimington (1963), holds that the common property of porphyrinogenic substances is their ability to change liver redox states. Labbe and his co-workers (Labbe, Kurumada and Onisawa, 1965; Kurumada and Labbe, 1966; Labbe 1967) have indeed formulated a precise hypothesis explaining ALA-synthetase induction as a result of increased intramitochondrial $NADH_2/NAD^+$ ratios, as described in Chapter IVc.

However, such evidence as exists of hepatic redox states after administration of porphyrinogenic substances is conflicting. Slater and Ziegler (1966) did not find material changes in $NADH_2/NAD^+$ ratios of the whole liver following administration of a variety of porphyrinogenic

substances to the rat. By contrast Gajdos and his co-workers (1966b, 1969) found increases in the ratio of reduced to oxidized cofactor in the liver of rats after porphyrinogenic substances were administered.

Consequently, the major purpose of this study was to seek more direct evidence in the intact animal, as to the rate of reduced cofactor generation under the influence of a potent porphyrinogenic substance. Such an approach is possible because of the observations of Stollar and Kaplan (1961), Gordon and Reichlin (1962) and Gordon (1963) who showed that nicotinamide-C^{14} is incorporated into the nicotinamide-adenine-dinucleotides. By studying the rate of incorporation in these cofactors and their reduced products, a measure of the rate of reduced cofactor generation is possible.

In the present study two potent porphyrinogenic drugs have been used. Firstly, hexachlorobenzene was administered to the rabbit, because the pattern of porphyrin excretion closely resembles the pattern found in the Bantu symptomatic porphyria. The other drug used was Allylisopropyl-acetamide (AIA) which was administered to rabbits, because in this case the pattern of porphyrin excretion very closely resembles the pattern found in the South African genetic type of porphyria.

METHODS AND MATERIALS

A. METHODS AND MATERIALS

1. Induction of Porphyrin Biosynthesis

Young male and female Albino rabbits (New Zealand strain) were used for all experiments. The rabbits were maintained on a stock diet until commencement of drug administration, and water 'ad libitum'.

a) Hexachlorobenzene (technical grade) was obtained from B.D.H. (Laboratory Chemicals Division). The hexachlorobenzene was administered as part of the diet. Approximately 0.5% hexachlorobenzene was thoroughly mixed in with the stock diet, moistened, pressed into cakes, and dried at 60° - 70°C. The dried cakes were then powdered and approximately 500 g./day fed to the rabbits for 50 - 60 days. (De Matteis, Peter and Rimington 1961). During the hexachlorobenzene feeding, the rabbits were allowed a small amount of greed food (grass, carrots, etc.) and water 'ad libitum'.

b) AIA (2-allyl-2-isopropylacetamide) was kindly supplied as a gift from Hoffmann - La Roche and Company. The AIA was administered by gastric intubation in a dose of 200 mg./Kg. bodyweight per day, for 7 - 10 days (Goldberg and Rimington 1962).

All the rabbits were weighed at the beginning and end of induction period, and urine and stools continually assayed for porphyrins or porphyrin precursors.

2. Introduction of Radioactive Label

Nicotinamide (Carbonyl-C^{14}) with a specific activity of 108 μc/mg was obtained from the Radiochemical Centre, Amersham, England. The nicotinamide-C^{14} was dissolved in 0.9% NaCl solution, and 0.5 ml. of this solution representing an activity of 10 μc was injected into the rabbits through a vein in the ear. The rabbits were sacrificed at appropriate time intervals, by injection of air into a vein in the ear.

3. Extraction of Nicotinamide Nucleotides $NAD^+ + NADP^+$ and $NADH_2 + NADPH_2$ (Gordon 1963).

Immediately after death, the liver was rapidly removed and placed in liquid nitrogen. The whole procedure from death to freezing took no longer than 30 seconds.

Two portions of the frozen liver, weighing 8 gms. each, were taken for the two methods of extraction.

a) Acid extraction of NAD^+ and $NADP^+$

The frozen liver was cut into smaller pieces and homogenized with 6 up and down movements of the pestle in a Potter-Elverjem homogenizer with 5% TCA (5 ml./gm. liver) at 0°C.

The sediment was centrifuged off in a refrigerated centrifuge, washed with a further 10 ml. of 5% TCA (cold) and centrifuged again.

The pooled supernatant's were added to 5 volumes of acetone at -20°C and allowed to stand overnight (12 - 14 hours) at -20°C, in order to precipitate the nicotinamide nucleotides.

The resultant precipitate was collected by centrifugation, washed with 2 changes of fresh cold acetone, and dried under a stream of nitrogen, in an ice bath. The powder was finally washed in one or two changes of cold ether and again dried under a stream of nitrogen in an ice bath until no smell of ether remained.

The resulting slightly yellow-white powder contained the NAD^+ and $NADP^+$, and was stored in the deep-freeze in an atmosphere of nitrogen until used.

b) Alkaline extraction of $NADH_2$ and $NADPH_2$

The reduced forms were extracted with alkali, oxidized, and isolated as the oxidized forms, NAD^+ and $NADP^+$.

Frozen liver was immersed in hot 0.1 M $Na_2 CO_3$ (Temp 80°C) for 30 - 60 seconds, and then homogenized with 6 up and down movements, of the pestle, in a Potter-Elverjem homogenizer with hot 0.1 M $Na_2 CO_3$ (5 ml./gm. liver).

After homogenization, the liver was immediately cooled in an ice bath. The sediment was centrifuged down, and the supernatant removed. The sediment was washed with a further 10 ml. of cold $Na_2 CO_3$ 0.1 M and the sediment again centrifuged down. The combined alkali extract was then incubated in the dark, at room temperature with -

0.4 vols. of phosphate buffer-1M pH 7.4
0.04 vols. of phenozine methosulphate-1 mM
0.36 vols. distilled water

for 20 minutes, in order to oxidize the reduced NAD^+ and $NADP^+$ (Stollar 1960). After incubation, the extract was added to 0.2 of its volume of 100% TCA. The precipitate was centrifuged off in the cold, and the supernatant added to 5 vols. of cold (-20°C) acetone and allowed to stand overnight (12 - 14 hours) at -20°C. The resulting precipitates were collected by centrifugation, washed with 2 changes of cold fresh acetone, 1 or 2 changes of cold ether and dried under a stream of nitrogen after each wash.

The resulting buff-white powder contains the oxidized $NADH_2$ and $NADPH_2$ and was stored in a deep-freeze in an atmosphere of nitrogen until used.

C H R O M A T O G R A P H Y

4. A. Separation of Nucleotides on an ion exchange Resin

1) Preparation of Columns (Hulbert et al. 1954).

The resin used was obtained as Dowex I (Chloride), x 8 or x 10, 200 - 400 mesh. The extremes of fine and coarse particles were removed by sedimentation. In order to prepare the formate form, large columns of the resin (5 cm. x 25 cm.) were washed with 3 M sodium formate until no more chloride ions were eluted off. (Chloride ions tested for in elute, with HNO_3 and Silver Nitrate). When the resin was free of chloride ions, it was washed with copious volumes of water.

A resin column for chromatography was supported in a glass tube by a constriction on which rested a wad of fibre glass. The resin was poured as a thin aqueous slurry and packed with low air pressure in a number of uniform glass columns, with 1 cm. cross section and 10 cm. long.

Before use, the column was washed with 5 to 10 bed volumes of a mixture of 6 N formic acid and 1 M sodium formate (1M Na formate in 6 N formic acid), then with several bed volumes of 88% (23 N) formic acid, and then with deionized water, until the effluent was no longer acidic. (This wash with concentrated formic acid removes considerable coloured material from the resin, and reduces the ultra violet light absorption background of the chromatogram).

A sample (acetone powder dissolved in cold distilled water) was put onto the column at the rate of 0.6 ml. per minute per sq. cm. cross-section and rinsed into the column with about 1 bed volume of distilled H_2O. Before chromatography the column was usually washed with several more volumes of distilled elgastat water, to remove lightly absorbed compounds.

2) Elution

The elution was carried out in two ways - Gradient elution and step-wise elution:

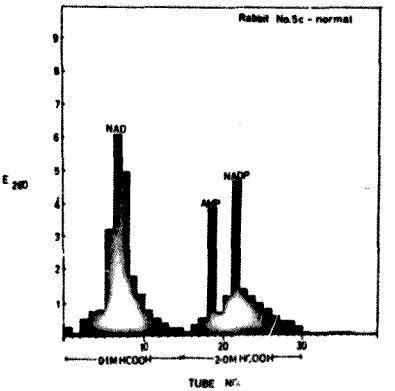
a) Gradient elution. The apparatus for gradient elution consists of a reservoir (½ litre suction flask), (500 ml. Erlenmeyer flask) with magnetic stirrer, and the glass tubing containing the resin column, all joined by, well washed Tygon tubing in an air-tight system.

Air nitrogen pressure applied to the side-arm of the reservoir was used to force the concentrated eluent through a glass outlet tube 'A' into the side-arm of the mixing flask, where the solution from the mixing flask was similtaneously forced through a glass outlet tube into the resin column (Hurlbert et al. 1954). The resin column was protected from being forced dry by addition of 10 ml. water, which also has the effect of a second 'mixer'. The solution was begun with water in the mixing flask, and concentrated eluent in the reservoir. The concentration of the eluent rises smoothly and asymptomatically approaches that of the reservoir solution. The rate of flow eluent, through the column was regulated by air pressure up to several lbs./sq. ins. to give about 0.6 ml. of eluent per minute per sq. cm. of cross-section of the column. The eluate was collected in 5 ml. of fractions.

b) Step-wise elution. This was found to be a much quicker and more simple method. $NADP^+$ and NAD^+ are eluted with quite a large difference in concentration of the eluate, and therefore this method was used in the majority of experiments.

75 ml. of 0.1 M HCOOH eluted off the NAD^+ from the column, and 2.0 M HCOOH the $NADP^+$.

FIG. XII


Rabbit No.5c - normal
NAD
NADP
AMP
E_{260}
9
8
7
6
5
4
3
2
1
10
20
30
0·1M HCOOH
2·0M HCOOH
TUBE NO.

It was found that AMP is eluted between NAD$^+$ and NADP$^+$, when the concentration of formic acid reaches about 1.0 M (Fig. XII)

B. Paper Chromatography of Nicotinamide Nucleotide Fractions

1) Preparation of Samples: The pooled eluates, corresponding to the required nucleotide were subjected to boiling under reduced pressure at 37°C - 40°C to get rid of the formic acid. This process was carried out, until the odour of formic acid fumes were not longer detectable. The samples were re-dissolved in distilled water and if formic acid still detected boiled again. When the formic acid was no longer detectable, the samples were freeze dried and again dissolved in a minimum of distilled water. The samples were then spotted onto Whatman No. 1 chromatography paper. 0.06 ml. (60 µl.) were spotted on with a Sahli adams 20 µl. haemoglobin pipette, and dried under a stream of air.

2) Development of Chromatogram: The chromatograms were developed by ascending chromatography in one direction for 8 - 10 hours with the following solvent Smith 1960).

	Ml	Ml
Iso-butyric acid	100	125
Water	55.8	69.7
Ammonia	4.2	5.25
0.1M (27 g./l) E.D.T.A.	1.6	2.0
	161.6	201.95

After 8 - 10 hours the chromatograms were allowed to dry in air, and were studied for nicotinamide nucleotides under UV light of 254 mu wavelength.

RADIOACTIVE ASSAY

5. Calculation of Concentration of Nucleotide Spot

Six spots were run of the same nucleotide, each with 0.06 ml. of the sample spotted on. Three of the spots were cut out of the paper as discs with a size 14 cork borer and the nucleotide eluted off the paper with 0.01 N HCl. These were read individually at 260 mu in a Unicam S.P. 600 spectrophotometer and the absorbance

measured. The quantity present was estimated using an extinction co-efficient of 18.5×10^{-6} cm^2/mole at 260 mu (P-L Biochemicals Circular OR - 10 (1956)). The average of the three readings was taken as the nucleotide concentration on the remaining three discs which were used for radio active assays.

The radioactivity of the three remaining spots in each case was determined individually and the mean activity calculated. The paper discs were dried in an oven at 60°C and placed on the bottom of standard Packard vials. Fifteen ml. of a standard phosphor (5 gms. PPO and 300 mg. POPOP made up to 1 litre with analytical grade toluene) were added and the discs were then counted in a Packard Tricarb liquid scintillation counter (Series 3000). Quenching was found to be constant throughout the range of quantities of nicotinamide nucleotides counted. The mean activity of these samples was taken as the activity in counts/minute/50 mu moles for that time interval.

5. Equilibration of Extra-mitochondrial and Intra-mitochondrial NAD.

Porphyria was induced in three rabbits, as already described. Three normal rabbits and three porphyric rabbits were injected with 0.5 ml. of nicotinamide-C^{14} (10 μc) in normal saline and then sacrificed after 20 minutes. The livers were rapidly removed and washed in ice-cold 0.25 M sucrose. Weighed aliquots were homogenized in fresh ice-

cold 0.25 M sucrose. All subsequent step were carried out at 0°C. After a preliminary 10 minute centrifugation at 700 x g. the supernatant solution was centrifuged at 6000 x g. for 10 minutes. The supernatant from this centrifugation is referred to as the cytoplasmic fraction, and the pellet as the mitochondrial fraction. The NAD^+ of each fraction was extracted, isolated, and its specific activity determined as described in the text.

7. The ratio of reduced to oxidized nucleotides in the liver of rats

The ratio of the specific activities of reduced to oxidized nucleotides were determined in the livers of normal rats of the Wistar strain after intraperitoneal injection of nicotinamide-C^{14}. The ratios were determined at various time intervals by the methods described, except that only one small portion of liver was used per extraction.

QUANTITATIVE ESTIMATION OF HEPATIC NICOTINAMIDE NUCLEOTIDES

A. Extraction of Nicotinamide Nucleotides

Modified Method of M. Klingenberg (1963)

1) NAD^+ and $NADP^+$ - Materials

Perchloric ($HClO_4$) - Materials 0.6 M 8.3 ml. $HClO_4$ (Sp. 1.67) diluted to 100 ml. with distilled elgastat water.

Dipotassium hydrogen phosphate (K_2HPO_4) - 1 M. 17.4 g. K_2HPO_4 dissolved in distilled elgastat water and made up to 100 ml.

Potassium hydroxide (KOH) - 3 M.

Method:

After sacrifice, the liver was removed and immediately, 'quick-frozen' by placing into liquid nitrogen. In the frozen state the livers were placed in a mortar, containing liquid nitrogen and ground to a fine powder. The oxidized nucleotide in this fine powder was extracted as follows:-

A stoppered centrifuge tube containing 2 ml. of Perchloric acid (0.6 M) was weighed, and to this was added approximately 0.8 gms. of frozen liver powder. The tube was then re-weighed and the ratio of acid to tissue adjusted to 5:1 (approx. 4 ml. acid). The stoppered centrifuge tube was then shaken vigourously in order to extract the nicotinamide nucleotides.

This was then centrifuged for 5 mins. at 3000-5000 xg. and 2 ml. of supernatant removed and placed in a clean centrifuge tube. To this supernatnat, which is cooled on ice, 0.2 ml. of 1 M. K_2HPO_4 was first added, and then ± 1.2 ml. 3 M. KOH (per 4 ml. $HClO_4$) until

the pH reached 7.2 - 7.4. While the alkali was being added, the tube was shaken vigourously to prevent any local concentration of alkali which may denature the oxidized nucleotides.

The $KClO_4$ was centrifuged down, and the clear supernatant used for analysis.

2) $NADH_2$ and $NADPH_2$ - Materials

Alcoholic KOH - (0.5 N) 2.8 gms. KOH dissolved in a mixture of equal parts by volume of ethanol (abs.) and distilled elgastat water; made up to 100 ml. with this mixture. Triethanolamine -Hcl - Phosphate (0.5 M Triethanolamine, 0.4 M KH_2PO_4, 0.1 MK_2HPO_4). 9.3 g. triethanolamine hydrochloride, 5.44 g. KH_2PO_4, and 1.74 g. K_2HPO_4) dissolved in distilled elgastat water, and made up to 100 ml.

Method:

A stoppered centrifuge tube containing 2 ml of alcoholic - KOH was weighed, and to this was added approximately 0.8 g. frozen liver powder. The tube was quickly stoppered, re-weighed and the ratio of alkali - tissue adjusted to approximately 5:1 (approx. 4 ml. - KOH). The tube was shaken vigorously (vortex stirrer) for a few seconds, so that the alcoholic KOH covered all parts of the centrifuge tube. The alcoholic KOH destroys all the DPNase thus preventing

decomposition of $NADH_2$ after neutralization. The tightly stoppered tube was then heated for 60 seconds in a 70°C water bath and vigourously shaken again. The extract was then cooled by placing in an ice-bath and neutralized to approximately 7.8 with triethanolamine -HCl - phosphate (amount calculated before hand - approx. ± 0.5 ml. per ml. of extract). The triethanolamine-HCl phosphate was added very slowly with vigorous shaking to prevent local concentration, and denaturing of reduced nucleotides. The extract was allowed to stand for 20 mins. at room temperature to obtain flocculation of the denatured protein. The extract was then centrifuged at 20,000 - 40,000 g. for 10 mins. and the almost clear supernatant used for analysis. In order to prevent auto-oxidation the solution was flushed as often as possible with nitrogen and tightly sealed.

B. Colorimetric estimation of Nicotinamide nucleotides
Method of Slater and Sawyer (1962).

Materials:

1. Phenozine methosulphate - 1 mgm/ml. Freshly prepared and kept in dark.

2. Dichlorophenol indophenol - Stock solution - 35 mgms/200 ml. Diluted 10 x with water before use.

3. Phosphate Buffer - 0.1 M pH 7.4.

4. Tris Buffer - pH 8.0 0.1 M.

5. Glucose-6-phosphate - sod. salt 0.1 M (Obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A.).

6. Absolute Ethanol A.R.

7. Glucose-6-phosphate dehydrogenase - 1 mgm/ml. in 3.3 M ammonium sulphate solution pH 6.0 obtained from C.F. Boehringer-Soehne, Mannheim (Germany).

8. Alcohol dehydrogenase - 30 mgms/ml. in 2.4 M ammonium sulphate pH 8.0 obtained from C.F. Boehringer-Soehne, Mannheim (Germany).

Method:

i) NAD^+

Dichlorophenol indophenol	2.5 ml.
Phenozine methosulphate	0.05 ml.
Phosphate buffer	0.5 ml.
Absolute ethanol	0.1 ml.
Acid extract (Diluted 1.5)	0.1 ml.

1. The following reagents were mixed in a 1 cm silica cuvette and the change in extinction at 600 mμ followed against distilled water, until decrease/min. was small and constant.

2. 0.05 ml. Alcohol dehydrogenase was then added to one cell, mixed and the change in extinction between this cell and another dichlorophenol indophenol cell measured at 10 or 20 min. intervals in a Unicam spectrophotometer. The difference is proportional to the amount of NAD^+ present in the acid extract.

ii) $NADP^+$

Dichlorophenol indophenol	2.5 ml.
Phenozine methosulphate	0.05 ml.
Tris buffer	0.5 ml.
Glucose-6-phosphate	0.1 ml.
Acid extract (undiluted)	0.2 ml.

1. The following ingredients were mixed in a 1 cm silica cell and the change in extinction followed at 600 mu against distilled water until decrease/min. was small and constant.

2. 0.05 Glucose-6-phosphate dehydrogenase was added to one cell, mixed, and the change in extinction between this cell and another dichlorophenol indophenol cell measured at 10 - 20 min. intervals in a Unicam spectrophotometer.

iii) $NADH_2$ - $NADPH_2$

The estimations of reduced di and triphosphopyridine nucleotides in the alkaline extract were carried out using the same conditions as for NAD^+ and $NADP^+$ respectively. With the alkaline

extract however, a larger period of equilibration was required before adding the specific enzymes to allow the reduced nucleotide to be oxidized by phenazine methosulphate. Thus, in the alkaline extracts containing $NADH_2$ and $NADPH_2$, the reduced forms were converted non-enzymatically to NAD^+ and $NADP^+$. The amounts of NAD^+ and $NADP^+$ were then measured by adding ADH and swichenferment respectively.

RESULTS

B. RESULTS

1. Induction of Porphyrin Biosynthesis

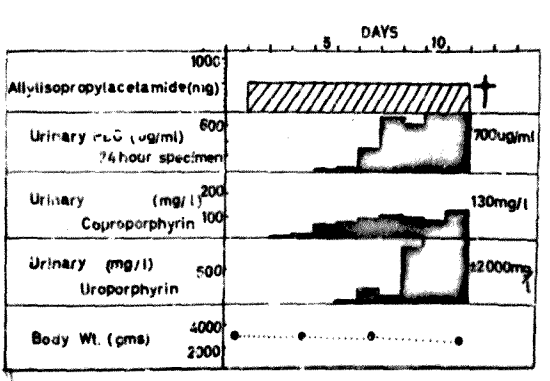
a) Hexachlorobenzene: The induction of porphyria by hexachlorobenzene administration as detailed in the text was not successful. From a series of 48 rabbits, only one excreted large amounts of porphyrins in the urine and stools, after six weeks of drug administration. The majority of the remaining rabbits died before six weeks, showing neurological symptoms, or from Pasteurella infection, possibly due to decreased resistance to the organism caused by the drug. The condition of the porphyric rabbit and porphyrin excreted are shown in Fig. X. After 50 days of hexachlorobenzene administration, the rabbit showed 25% loss of weight, was very weak, and showed hyperexcitability and signs of terminal paralysis. Urinary PBG was raised, and extremely large amounts of uroporphyrin and coproporphyrin were found. Faecal Copro- and Protoporphyrins were only moderately raised.

b) Allylisopropylacetamide (AIA): The induction of porphyria was very successful using AIA, and nearly 100% induction was obtained. The results of a typical induction period are shown in Fig. (XI). The rabbits receiving AIA showed some weight loss,

FIG. 2

RABBIT Hx 23	BEFORE DRUG ADMINISTRATION	AFTER 50 DAYS DRUG ADMIN.
Weight	2·4 Kgms.	1·8 Kgms.
Urine PBG	nil	25·2 ug/ml
Uroporphyrin	nil	18,000 ug/ml
Coproporphyrin	nil	1,700 ug/ml
Faeces		
Coproporphyrin	nil	20ug/gm. dry wt.
Protoporphyrin	nil	21ug/gm dry wt.
General condition	Healthy	Very weak, terminal ataxia, emaciated, hyperexcitability, fatty acid degeneration of the liver

FIG. XI


Rabbit AIA 5c
DAYS
5
10
1000
Allylisopropylacetamide(mg)
600
Urinary PBG (ug/ml)
24 hour specimen
700ug/ml
200
Urinary (mg/l)
100
Coproporphyrin
130mg/l
500
Urinary (mg/l)
Uroporphyrin
2000mg/l
4000
Body Wt. (gms)
2300

but remained relatively alert and healthy throughout the drug course. Coproporphyrin appeared in the urine on the 2nd or 3rd day, reached a peak of around 100 mg/litre on the 8th day, maintaining this level until drug administration ceased or the animal was sacrificed. Urinary PBG and uroporphyrin appeared in the urine on the 5th or 6th day and increased enormously by the 9th or 10th day when the animal was sacrificed.

2. Incorporation of Nicotinamide-C^{14} into Hepatic NAD^+, $NADH_2$, $NADP^+$ and $NADPH_2$ of Normal and AIA-induced Porphyric Rabbits

After injection of 10 µc of nicotinamide-C^{14} intravenously, the specific activity of the hepatic NAD^+ of normal rabbits increased progressively with time until 15 minutes, when the incorporation of the label reached a maximum level and then fell slightly by 25 minutes (Fig. XIII). At each time period, the specific activity of the $NADP^+$ was considerably less than that of NAD^+, indicating the relatively slow rate of equilibrium of $NADP^+$ with NAD^+, in agreement with the data and findings of Gordon (1963), and Stollar and Kaplan (1961). In contrast to Gordon (1963), who found that the increase in incorporation of nicotinamide-C^{14} into $NADP^+$ did not vary significantly between 2.5 and 20 minutes, we found a small but progressive increase with time

FIG. XIII

THE RATE OF INCORPORATION OF NICOTINAMIDE - C14 INTO HEPATIC NAD ▨ AND NADP ▩ OF NORMAL RABBITS THE HEIGHT OF EACH BAR REPRESENTS 3 RABBITS, EACH ESTIMATED IN TRIPLICATE ± STD. ERROR

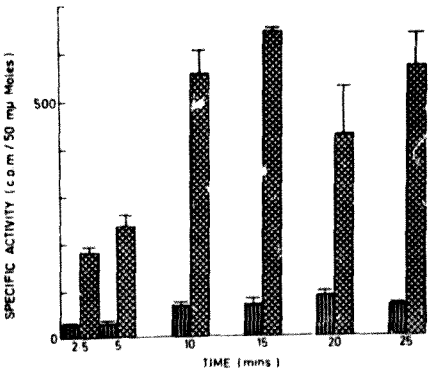


FIG. XV

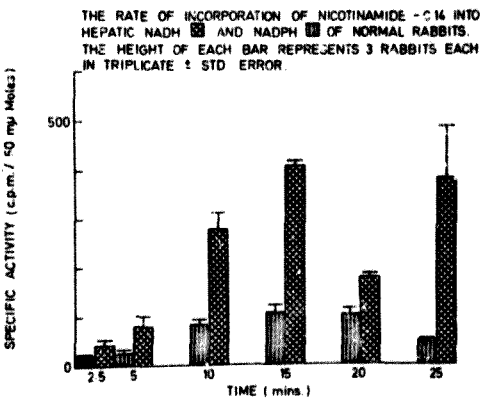
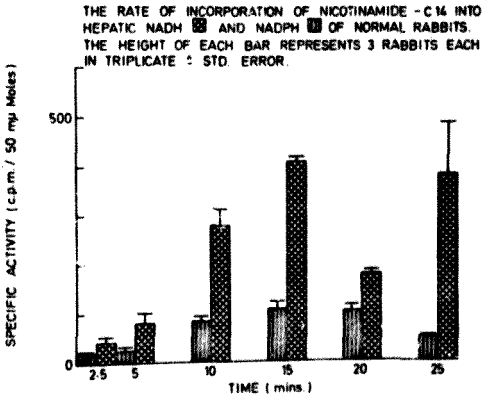

THE RATE OF INCORPORATION OF NICOTINAMIDE - C 14 INTO HEPATIC NADH AND NADPH OF NORMAL RABBITS. THE HEIGHT OF EACH BAR REPRESENTS 3 RABBITS EACH IN TRIPLICATE ± STD ERROR.
SPECIFIC ACTIVITY (c.p.m./ 50 mμ Moles)
500
0
2.5
5
10
15
20
25
TIME (mins.)

FIG. XV


THE RATE OF INCORPORATION OF NICOTINAMIDE - C 14 INTO HEPATIC NADH AND NADPH OF NORMAL RABBITS.
THE HEIGHT OF EACH BAR REPRESENTS 3 RABBITS EACH IN TRIPLICATE ± STD. ERROR.
SPECIFIC ACTIVITY (c.p.m. / 50 mμ Moles)
500
0
2·5
5
10
15
20
25
TIME (mins.)

in specific activity of $NADP^+$. The incorporation in nicotinamide-C^{14} into hepatic NAD^+ and $NADP^+$ of AIA-induced porphyric rabbits followed a pattern similar to that seen in normal animals (Fig. XIV). The incorporation of the label with time, into the reduced nicotinamide adenine dinucleotides of both groups of animals followed a pattern similar to that of the oxidized NAD^+ (Figs. XV & XVI) except that at all time intervals the specific activity was less than that of the oxidized NAD^+. In contrast however the incorporation of the label into reduced $NADP^+$ increased quite considerably in time in both groups of animals as compared to the oxidized forms (Figs. XV & XVI).

3. The ratio of the specific activity of $NADH_2/NAD^+$ and $NADPH_2/NADP^+$ of various times after intravenous administration of nicotinamide-C^{14} to normal and AIA-induced porphyric rabbits

The ratio of the specific activity of $NADH_2/NAD^+$ in normal rabbits increased with time from approximately 0.21 at 2.5 minutes to 0.32 at 5 minutes to 0.68 at 25 minutes after injection of nicotinamide-C^{14} (Fig. XVII). By contrast, in porphyric rabbits the rate of generation of $NADH_2$ was significantly greater, the ratio of the specific activity of $NADH_2/NAD^+$ being approximately 0.88 at 2.5 minutes and 0.85 at 5 minutes after injection of nicotinamide-C^{14} (Fig. XVIII) (at 2.5 minutes $P < 0.01$ and 5 minutes $P < 0.001$).

FIG. XIV

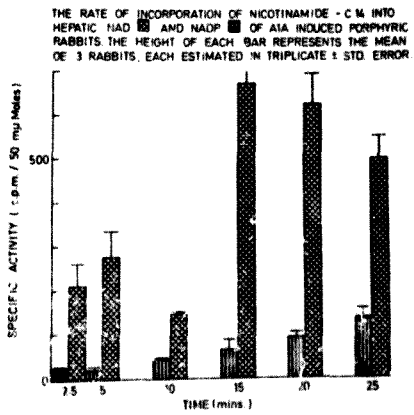

THE RATE OF INCORPORATION OF NICOTINAMIDE - C 14 INTO HEPATIC NAD AND NADP OF AIA INDUCED PORPHYRIC RABBITS. THE HEIGHT OF EACH BAR REPRESENTS THE MEAN OF 3 RABBITS, EACH ESTIMATED IN TRIPLICATE ± STD. ERROR
SPECIFIC ACTIVITY (c.p.m. / 50 mμ Moles)
500
0
2.5
5
10
15
20
25
TIME (mins.)

FIG. XVI

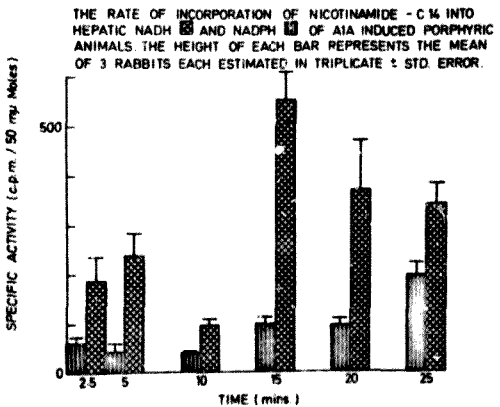

THE RATE OF INCORPORATION OF NICOTINAMIDE - C 14 INTO HEPATIC NADH AND NADPH OF AIA INDUCED PORPHYRIC ANIMALS. THE HEIGHT OF EACH BAR REPRESENTS THE MEAN OF 3 RABBITS EACH ESTIMATED IN TRIPLICATE ± STD. ERROR.
SPECIFIC ACTIVITY (c.p.m. / 50 mμ Moles)
500
0
2.5
5
10
15
20
25
TIME (mins)

FIG. XVII

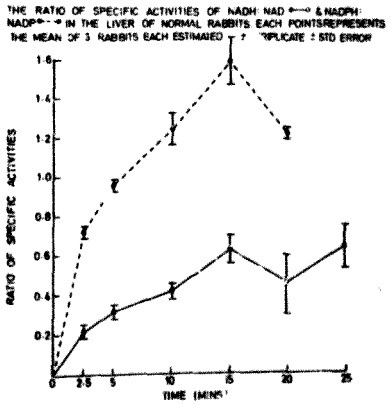

THE RATIO OF SPECIFIC ACTIVITIES OF NADH: NAD ⟷ & NADPH: NADP ⟷ IN THE LIVER OF NORMAL RABBITS EACH POINTS REPRESENTS THE MEAN OF 3 RABBITS EACH ESTIMATED ? TRIPLICATE ± STD ERROR
RATIO OF SPECIFIC ACTIVITIES
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2.5
5
10
15
20
25
TIME (MINS)

FIG. XVIII

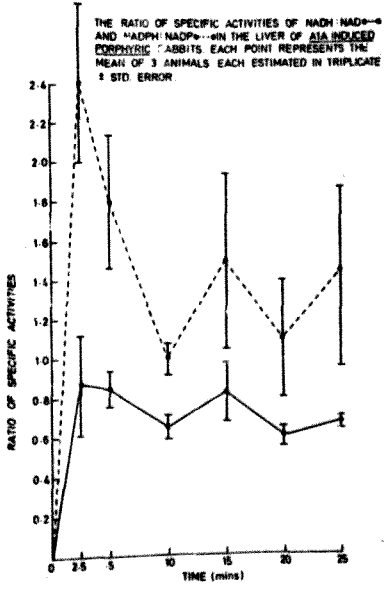

THE RATIO OF SPECIFIC ACTIVITIES OF NADH:NAD+--+ AND NADPH:NADP+---+IN THE LIVER OF A1A INDUCED PORPHYRIC RABBITS. EACH POINT REPRESENTS THE MEAN OF 3 ANIMALS EACH ESTIMATED IN TRIPLICATE ± STD. ERROR.
RATIO OF SPECIFIC ACTIVITIES
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
2·5
5
10
15
20
25
TIME (mins)

FIG. XIX

THE RATIO OF THE SPECIFIC ACTIVITIES OF NADH: NAD o—o & NADPH: NADP •---• IN THE LIVER OF NORMAL RATS. EACH POINT REPRESENTS THE MEAN OF ONE RAT ESTIMATED IN TRIPLICATE.

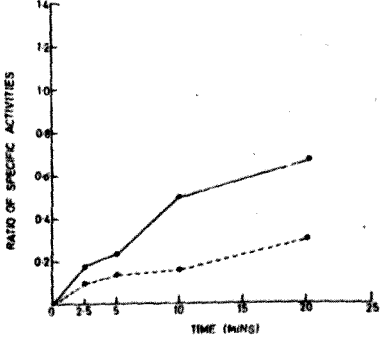


TABLE 3

THE EQUILIBRATION OF NICOTINAMIDE DINUCLEOTIDES IN LIVERS OF NORMAL AND PORPHYRIC RABBITS

The livers were removed and frozen 5 minutes after administration of nicotinamide - C^{14}. The values represent the mean of 3 animals, each estimated in triplicate, ± Std. error

CONDITION OF RABBITS	SPECIFIC ACTIVITY				RATIOS	
	NAD	NADP	NADH	NADPH	NADH: NAD	NADPH: NADP
NORMAL CONTROL	235±39	78±28	26±6	25±7	0·30±0·04	0·96±0·07
AIA - INDUCED PORPHYRIA	277±66	23±6	239±46	[illegible]±16	0·85±0·07	1·80±0·35
HEXACHLORO - BENZENE - INDUCED PORPHYRIA (1 exp.)	411±23	240±6	115±6	155±5	0·58±0·021	1·35±0·04

TABLE 4

Equilibration of intra- and extramitochondrial NAD in livers of normal and AIA-porphyric rabbits

Condition of rabbits	Specific Activity of NAD				a/b
	Mitochondrial Fraction (a)		Cytoplasmic Fraction (b)		
	c.p.m./50mμ Moles				
Normal animals	51·6 47·5	49·5	51·7 52·5	52	0·951
AIA-induced porphyrics	47·7 53·4	50·5	50 54·5	52·5	0·967

The rate of generation of $NADPH_2$ from $NADP^+$ in normal rabbits was more rapid than that of $NADH_2$ from NAD^+, as indicated by the higher ratios of the specific activity of $NADPH_2/NADP^+$ at all times up to 20 minutes (Fig. XVII). In the porphyric rabbits the generation of $NADPH_2$ was again more rapid than that of $NADH_2$, and furthermore the generation of $NADPH_2$ was significnatly higher than in normal rabbits (Fig. XVIII) (at 2.5 minutes $P \ll 0.001$ and at 5 minutes $P \ll 0.001$).

In contrast to the rapid rate of generation of $NADPH_2$ from $NADP^+$ in normal rabbits, the rate of generation of $NADPH_2$ in normal rats was much slower, being even slower than the rate of generation of $NADH_2$ from NAD^+ in these rats (Fig. XIX). The latter finding is in accord with the data of Gordon (1963) in normal rats.

4. The equilibration of nicotinamide dinucleotides in the liver of normal Hexachlorobenzene-induced and AIA-induced and porphyric rabbits at 5 minutes

Five minutes after administration of nicotinamide-C^{14} normal rabbits the ratio of specific activities $NADH_2/NAD^+$ was 0.30 ± 0.04 and that of NADPH/NADP 0.96 ± 0.07. By contrast the ratio of the specific activities of NADH/NAD and NADPH/NADP were significantly increased in the liver of the rabbit made porphyric with hexachlorobenzene (Table 3). This table also shows the ratios obtained in the AIA-induced porphyric animals for comparison.

5. Equilibration of intramitochondrial and extramitochondrial NAD in livers of normal and porphyric rabbits

Twenty minutes after injection of nicotinamide-C^{14} in two normal and two porphyric rabbits, the specific activity of mitochondrial NAD^+ was almost equal to that of extramitochondrial NAD^+ (Table 4) indicating a fairly rapid rate of equilibration between the two compartments. These findings are in contrast to those obtained in rats by Gordon (1963), who found that the specific activity of mitochondrial NAD was a little over one third of that of extramitochondrial NAD 20 minutes after injection of the label.

6. Quantitative estimation of pyridine nucleotides in normal and ALA-induced porphyric rabbit livers

The results of this study are summarized in Table 5. It can be seen, that ALA produces a significant change in NAD^+ and $NADH_2$ under the conditions used, with a marked trend towards increased reducing potential ($P<0.001$ for both NAD^+ and $NADH_2$). The ratio of $NADH_2$ increases from 0.279 ± 0.095 in the normal rabbit, to 0.405 ± 0.042 in the porphyric rabbit representing an increase of 45% ($P<0.001$). The sum of $NAD^+ + NADH_2$ decreases to a small but not highly significant degree in the porphyric rabbit. ALA also produces a small change in $NADP^+$ and a significant increase in $NADPH_2$ ($P<0.02$ for $NADP^+$ and $P<0.001$ for $NADPH_2$). The ratio of $NADPH_2$ to $NADP^+$ increases from 11.05 ± 1.94 in the normal rabbit to 20.31 ± 1.83 in the porphyric rabbit, representing an increase of 83.8% ($P<0.001$). The total

oxidized and reduced nucleotides (i.e. $NAD^+ + NADP^+ + NADH_2 + NADPH_2$) does not change ($P \leq 0.5$). Of perhaps further interest, is that the ratio of $NADH_2 + NADPH_2$ to $NAD^+ + NADP^+$ increases from 0.547 in the normal rabbit, to 0.836 in the porphyric rabbit. The only previously published figures as to the ratio of $NADH_2$ to NAD^+ in rabbit liver, are those of Jedeikin and Weinhouse (1955). These workers obtained lower quantitative amounts of oxidized NAD^+ than those obtained in the present study, and consequently a higher $NADH_2$ to NAD ratio, but still not as high as those obtained with porphyric rabbits in the present study (Table 5). A possible explanation for these differences is the use of different techniques and experimental conditions.

7. The calculation of 'turnover times' of $NADH_2$ in the livers of normal and ALA-induced porphyric rabbits

From the equilibration data (Figs. XVI & XVII), it should be possible to calculate 'turnover times' for $NADH_2$ in the liver cells of normal and porphyric rabbits, providing the following assumptions, according to Cordon (1963) can be made.

i) The rate of labelling of nicotinamide nucleotides must not be limited by the specific activity of the C^{14} source. In the present experiments the specific activity of the nicotinamide-C^{14} was 3.96×10^6 c.p.m./50 mu Moles, which greatly exceeds that of the nicotinamide nucleotides at all time intervals.

TABLE 5

QUANTITATIVE ESTIMATION OF THE NICOTINAMIDE NUCLEOTIDES IN THE LIVERS OF NORMAL AND PORPHYRIC RABBITS

CONDITIONS OF RABBITS	NAD	NADH	NADP	NADPH	NAD + NADH	NADP + NADPH	TOTAL NUCLEOTIDES	NADH / NAD	NADPH / NADP	NADH + NADPH / NAD + NADP
	μ Moles/gm. wet weight							Ratios		
NORMAL* (This investigation)	551.8 ± 39.0	154.4 ± 6.54	16.04 ± 5.46	126.1 ± 8.3	706.2 ± 31.8	172.1 ± 7.98	878.3 ± 29.6	0.279 ± .095	11.05 ± 1.94	0.547
PORPHYRIC** (This investigation)	472.3 ± 44.6	190.3 ± 16.7	10.93 ± 2.33	213.7 ± 15.7	662.6 ± 40.1	224.6 ± 12.1	887.2 ± 24.3	0.405 ± .042	20.31 ± 1.83	0.836
Significance of difference	P<0.001	P<0.001	P<0.02	P<0.001	P<0.02	P<0.001	P<0.5	P<0.001	P<0.001	P<0.001
NORMAL (Dudeikin and Veinhouse 1955)	406	156	-	-	562	-	-	0.384	-	

* These figures are the means ± S.D. obtained from 10 observations i.e. 5 rabbits estimated in duplicate, using two different portions of liver.

** These figures are the means ± S.D. obtained from 10 observations i.e. 5 rabbits estimated in duplicate, using two different portions of liver. These rabbits were treated with ALA as detailed in the text.

ii) The concentration of each of the hepatic nicotinamide nucleotides should not change during the course of the experiment. Gordon (1963) has demonstrated no detectable change in hepatic NAD^+ and $NADP^+$ after injection of 0.5 mg of nicotinamide to the rat. In the present experiments the order of 0.1 mg of nicotinamide is injected into the much larger rabbit, and it is therefore felt highly unlikely that any change in hepatic NAD and NADP takes place.

iii) The immediate precursor molecule NAD-C^{14} should be distributed uniformly in a homogenous compartment where the hydrogen transfer between oxidized and reduced nicotinamide nucleotide occurs. In the present study rapid equilibrium was found to take place between mitochondrial and cytoplasmic NAD^+. Although it was not possible to isolate sufficient $NADH_2$ to measure the intramitochondrial rate of generation it is reasonable to expect that if mitochondrial permeability barriers to NAD^+ are small in the rabbit, the same will be true of the reduced cofactor (Table 4).

iv) The rate of loss of C^{14} from any of the nicotinamide nucleotides should be negligible relative to the exchange between oxidized and corresponding reduced forms. The data indicate the rate of

conversion of NAD^+-C^{14} to NADP-C^{14} is slow relative to the rate of conversion of NAD^+-C^{14} to $NADH_2$-C^{14} (Fig. XIII). If there were appreciable breakdown ot the labelled nicotinamide nucleotides, excretion of the label in the urine might be expected. Since in the present study none of the label was detected in the urine of either normal or porphyric rabbits, after one hour it seems unlikely that there was significant loss of C^{14}-labelled nicotinamide nucleotides in the first 25 minutes.

Zilversmit, Entenmann and Fishler (1943) have derived the equation $\frac{\text{specific activity of product}}{\text{specific activity of Precursor}} = n\left(\frac{1}{n} - 1 - e^{-1/n}\right)$ for the situation when the specific activity of the immediate precursor varies linearly with time. A plot of the ratio of reduced to oxidized nicotinamide nucleotides which represents the ratio of the specific activity of the product to that of the precursor, is seen in Figs. XVII & XVIII. For each experimentally determined ratio n may be calculated from the above equation. If n is known for the ratio at each time interval, t, then the 'turnover time' (t_T) can be calculated from the equation $t = \frac{t_T}{n}$ ($t_T = n.t$).

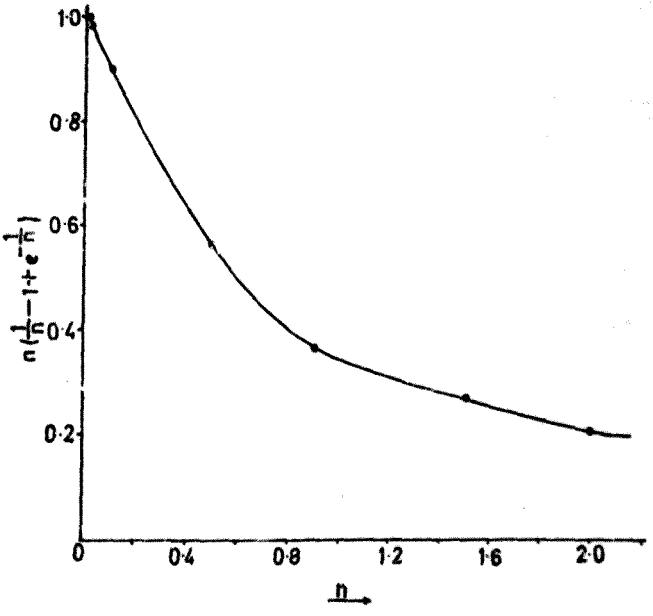
If one works out the ratio arbitrarily for assigned values of n, an exponential curve may then be plotted, (Fig. XX) from which the values of n for the experimentally determined ratios may be read off.

The calculated average 'turnover time' for $NADH_2$ in the liver of normal rabbits in the present study is 5.82 ± 0.566 minutes. In the AIA-induced porphyric rabbit liver however, an average 'turnover time' cannot be worked out for the reason that by 2.5 minutes equilibrium had already been established i.e. we did not and could not make the observation early enough to obtain a range of 'turnover times'. Therefore in this instance 'turnover time' has been calculated at 2.5 minutes. This result is 0.27 minutes, but bearing in mind the consideration that equilibrium has already been reached, it would seem more correct to report the results as $<<$ 0.27 minutes.

8. The ratio of the specific activity of $NADH_2/NAD^+$ and $NADPH_2/NADP^+$ at various times after the administration of nicotinamide-C^{14} to the normal rat

The ratios of the specific activity of $NADH_2/NAD^+$ in the liver of normal and porphyric rabbits compared very favourably with those obtained by Gordon (1963) in the liver of normal rats. However, the ratios of the specific activity of $NADPH_2/NADP^+$ in the liver of normal and porphyric rabbits were in direct contrast to those previously obtained in normal rats (Gordon 1963). In order then to check the present methods and techniques, the experiments were repeated in normal rats. The results (Fig. XII) confirm Gordon's previous finding of a lower $NADPH_2/NADP^+$ ratio in normal rats.

Fig. XX

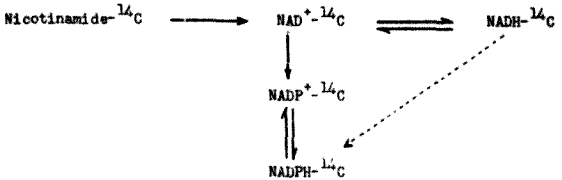


The exponential curve plotted from arbitrarily assigned values of n, against the ratio calculated from Zilversmit et. al (1943).

DISCUSSION

a) General

The experimental approach in the present study is based on the assumption that the following reactions and equilibria are valid.



Results obtained in the control rabbits are entirely consistent with the above schemes as far as the incorporation of radioactivity from nicotinamide into the oxidized cofactors are concerned (Fig. XIII). Furthermore, as is clear from Fig. XVII, NAD^+ equilibrates in time with $NADH_2$ in that the ratio of the specific activities gradually approach unity. By contrast the ratio of specific activities of $NADPH_2/NADP^+$ exceeds unity after 10 minutes. This finding is clearly inconsistent with proposed prior labelling to $NADP^+$ which subsequently equilibrates with $NADPH_2$. Hence the results in the present study on the rate of generation of $NADPH_2$ from $NADP^+$ do not indicate the full picture, and we seem to be measuring two different things in $NADH_2$ and $NADPH_2$ generation.

b) $NADH_2$ Generation

Such evidence as exists on hepatic redox states after administration of a porphyrinogenic substance are conflicting. A number of workers have measured total NAD^+ and $NADH_2$ levels in rat ascites cells and the livers of animals after administation of porphyrinogenic drugs. Kohen (1963) found that amylobarbitone, a typical $NADH_2$-oxidase inhibitor, caused a rise in $NADH_2$ confined to the mitochondrial in aerobic cells, and that anaerobiosis or addition of glucose increased $NADH_2$ in the extramitochondrial region of the cells. Gajdos et al. (1966b) found that an increased $NADH_2/NAD^+$ ratio accompanied porphyrinogenesis in mouse liver after administration of porphyrinogenic substance, and Gajdos and Gajdos Török (1969) found a significant increase in $NADH_2$ concentration in the livers of rats 6 hours after a single dose of AIA. By contrast Slater and Ziegler (1966) found no significant change in $NADH_2/NAD^+$ ratio in the livers of rats made porphyric by a number of different drugs.

In the present study, the results obtained in the control rabbits are consistent with the scheme as represented at the beginning of the discussion as far as the incorporation of radioactivity from nicotinamide into the oxidized nucleotides are concerned (Fig. XIII), and further, that NAD^+ equilibrates in time with $NADH_2$ in that the ratio of the specific

activities gradually approaches unity (Fig. XVII). It is also clear from the results (Figs. XIII and XIV) that AIA does not affect the rate of C^{14} incorporation into liver NAD^+ and consequently any change in the incorporation of radioactivity into $NADH_2$ must be affected by a change in the rate of equilibration between NAD^+ and $NADH_2$. The administration of AIA reduced the time taken for equilibration between oxidized and reduced NAD^+ to less than 2.5 minutes as compared with the control rabbits which took 20 minutes before equilibration was approached. According to Zilversmit et al. (1943) the 'turnover time' of a substance in a tissue is the time required for the appearance or disappearance of an amount of that substance equal to the amount of that substance present in the tissue. In the present study, the 'turnover time' for $NADH_2$ was significantly affected by AIA, being reduced from 5.82 ± 0.57 minutes in the control rabbits to $\ll 0.27$ minutes in the liver of rabbits, treated with AIA. It is, however, important to recognize that the 'turnover time' calculations are approximate because of possible deviations from the assumption that have been made. For this reason the values have their greatest usefullness in a comparative rather than in an absolute sense.

It was a reasonable expectation that if the rate of $NADH_2$ generation is markedly increased, as presently found, then the quantitative ratio

of $NADH_2/NAD^+$ would increase in response to AIA, and therefore the quantitative studies were undertaken. The results (Table 5) have confirmed this expectation, as the administration of AIA, significantly increased the ratio of $NADH_2/NAD^+$. AIA does not affect the total oxidized and reduced NAD^+ to a significant extent, although there is a slight decrease in response to the drug. Total liver cofactor (I.E. NAD^+ + $NADH_2$ + $NADP^+$ + $NADPH_2$) is not altered at all by AIA. These results support the finding of Gajdos and his co-workers (Gajdos et al. 1966b, Gajdos and Gajdos-Török 1969), but do not confirm the results obtained by Slater and Ziegler (1966). In view of the possibility that the high specific activity of the $NADH_2$ in AIA-induced porphyric rabbit could be due to a decrease in NAD^+ synthesis, the quantitative results (Table 5) are of importance. In contrast to Slater and Ziegler (1966) who found decreased total nucleotides (i.e. NAD^+ + $NADH_2$ + $NADP^+$ + $NADPH_2$), in the present study the total nucleotide concentration in both the normal and the AIA-induced prophyric rabbit was the same (Table 5). Therefore a decrease in NAD^+ synthesis in rabbit liver under the influence of AIA does not take place under the experimental conditions used in this study.

In the rabbit, rapid equilibration between cytoplasmic NAD^+ and mitochondrial NAD^+ was found (Table 4). This is quite unlike the findings in the rat (Gordon 1963) where minimal equilibration between

cytoplasmic and mitochondrial NAD^+ occurred even after 25 minutes. It must be assumed that this is a species difference, and although it was not possible to isolate sufficient $NADH_2$ to measure the intramitochondrial generation, it is reasonable to expect that if mitochondrial permeability barriers to NAD^+ are small in the rabbit, the same will be true for reduced cofactor. Krebs and his co-workers, working on rat kidney cortex, rat liver and mouse liver have studied the redox state of the cytoplasm and mitochondria, and the generation of extramitochondrial reducing power (Krebs et al. 1967, Williamson et al. 1967). They have suggested that the $NAD^+ + NADH_2$ pool in the external compartment of the mitochondria communicates freely with the $NAD^+ + NADH_2$ pool in the cytoplasm. Furthermore, they postulate that a transfer of $NADH_2$ is probably not direct, but mediated via special carrier mechanisms such as the malate-oxaloacetate system.

From the observations in these experiments it is clear that in the intact animal a potent porphyrinogenic substance profoundly influences the rate of reduced cofactor generation, and consequently the quantitative $NADH_2$: NAD^+ ratio in the liver. In the particular species used there is every reason to believe that the situation demonstrated for the whole liver, is valid in the intramitochondrial compartment as well. These

are highly significant metabolic deviations and since the effect of AIA on the haem biosynthetic pathway in the liver is not in doubt, it seems reasonable to suppose that there is some relationship between these two metabolic events. For this reason the hypothesis proposed by Labbe (1967) as to the mechanism whereby increased intramitochondrial $NADH_2/NAD^+$ ratios could give rise to induction of ALA-synthetase, is worthy of thorough testing.

c) Hexachlorobenzene-induced porphyria

The very interesting suggestion put forward by Rimington (Rimington 1962, 1963a, 1963b) that the redox environment in the liver may play an important role in determining both the pattern of excretion and extent of accumulation of porphyrins in symptomatic porphyria, prompted the attempts in this study to measure turnover rate of $NADH_2$ and $NADPH_2$ in hexachlorobenzene poisoned rabbits as this type of experimental porphyria closely resembles the symptomatic porphyria found in humans. This study was not successful in that only one rabbit excreted increased amounts of porphyrins and porphyrin precursors out of a total of 48 rabbits studied. Therefore no conclusions as to the result can validly be drawn.

However, it is interesting to note that although a decreased reducing climate in the liver might have been expected on the basis of Rimingtons theory, in fact quite the reverse was found in the single rabbit studied

in that hexachlorobenzene produced an increase in $NADH_2/NAD^+$ and $NADPH_2/NADP^+$ specific activity ratios at 5 minutes (Table 3). This result may indicate that the mechanism of hexachlorobenzene-induced porphyria is also compatable with the Labbe hypothesis.

d) $NADPH_2$ Generation

A possible explanation for the very high ratio of the specific activity of $NADPH_2$ NADP at early times after injection of the label in the normal rabbit (Fig. XVII) is the operation of a kinase with $NADH_2$ as substrate similar to the kinase described in yeast (Kornberg 1961, Bernofsky et al. 1968, Bernofsky 1969) and in *Saccharomyces cerevisiae* (Middleton and Apps, 1969). Such a kinase is absent from pigeon liver (Wang and Kaplan 1954, Apps 1968, 1969, 1970) does not function in the rat (Gordon 1963, Oka and Field 1968), could not be detected in spinach, (Yamomoto 1966) or Azobacter (Chung 1967), and no published information as to its existence in the rabbit could be found. The present work suggests that as far as $NADPH_2$ generation is concerned, we may be measuring $NADH_2 \longrightarrow NADPH_2$. If this reaction is fast compared with $NADPH_2 \longrightarrow NADP^+$, $NADPH_2/NADP^+$ ratios have quite a different meaning in that substrate does not determine the ratios.

In their calculation of 'turnover time' which have been used in the present study with respect to the $NADH_2/NAD^+$ ratios, Zilversmith et al. (1943) have illustrated the 'specific activity-time' relationship of a precursor to its product. Their illustration, is reproduced in Fig. XXI. These authors point out, that during the early interval after the administration of a labelled substance, the specific activity of a precursor of a compound must be higher than that of the compound itself. From Fig. XXI it can be noted that the specific activity of the precursor (S.a.A.) is greater than that of the product (S.a.B.). In time the specific activity of the precursor becomes constant, and then gradually begins to fall, while the specific acitvity of product increases and eventually equilibrates with the precursor. However, in time, the specific activity of the product will exceed that of the precursor. Applied to the present case this could mean a rapid formation of $NADPH_2$ from $NADP^+$ in normal rabbit liver, and a relatively slow breakdown of $NADPH_2$.

In summary then, what are felt to be valid observations on the ratios of the specific activity of $NADPH_2/NADP^+$ in normal and porphyric rabbits have been obtained in the present study. However, these observations may not be consistent with prior labelling of $NADP^+$. Some possible

Fig XXI

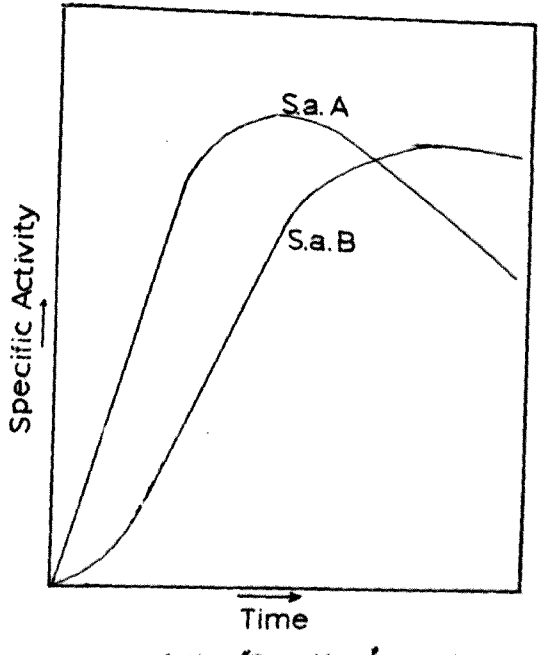


Illustration of the 'S.a.-time' relation of precursor A and product B

after Zilversmit et.al.(1943)

explanations have been put forward, but a further study is necessary to clarify the $NADPH_2$ generation findings.

Whatever the explanation for the high $NADPH_2/NADP^+$ specific activity ratios may be, the ratio in the porphyric rabbit is still significantly higher than in the normal rabbit (Figs. XVII & XXIII $p \ll 0.001$ at 2.5 and 5 minutes). A possible explanation is in the role that this nucleotide plays in the detoxification mechanism of the liver (detailed in Chapter IV). Reduced nicotinamide adenine dinucleotide phosphate has been shown to be an essential component of detoxification mechanism. In recent years many workers have shown oxidative enzyme systems concerned with detoxification to be dependent on atmospheric oxygen and reduced nicotinamide adenine dinucleotide phosphate (Gillette 1963, Gillette et al. 1967; Omura et al. 1965; Cooper et al. 1965; Conney 1967).

Of further interest, are the studies of Conney and Burns (1968), who showed that drugs such as phenobarbital stimulated non-specifically the activity of almost every $NADPH_2$/oxygen dependent microsomal enzyme that they studied. Furthermore, studies by Gillette et al. (1957) showed the presence in liver microsomes of an enzyme system that oxidised $NADPH_2$ to $NADP^+$ ($NADPH_2$-oxidise), with the resultant formation of peroxide as a product of the reaction. These investigators postulated a mechanism

whereby phenobarbital and other drugs stimulated non-specifically the oxidation of a wide range of substances in the liver microsomes by inducing the synthesis of the $NADPH_2$-oxidase system. The increased amounts of active oxygen formed from $NADPH_2$ could then be utilized for the oxidation of drugs by specific enzymes in liver microsomes.

In the present study equilibrium between $NADPH_2$ and $NADP^+$ in the normal rabbit is approached at about 15 minutes (Fig. XVII). The administration of AIA results in equilibrium being already reached at 2.5 minutes (Fig. XXIII). Furthermore, at equilibrium the ratios in the rabbits administered AIA are poised at a higher reducing level than the ratios in the normal rabbit (Figs. XVII & XVIII).

The expectation of a higher quantitative ratio of $NADPH_2/NADP^+$ in response to AIA was borne out by the qualtitative study summarized in Table 5. The $NADPH_2$: $NADP^+$ couple which in the normal rabbit is poised the side of reduction ($NADPH_2/NADP^+$ ratio 11.05 $\pm$ 1.94) has been demonstrated before by many laboratories e.g. rat liver (Slater et al. 1964, Glock and McLean 1955). The administration of AIA in our studies, increases the reducing potential of the $NADPH_2$: $NADP^+$ couple to 20.31 $\pm$ 1.83. The total of $NADP^+$ + $NADPH_2$ was increased under the influence of AIA. These results are in contrast to those obtained in

rat liver by Slater and Ziegler (1966). These workers found little change in the ratio $NADPH_2/NADP^+$ in rat liver under the influence of AIA. Furthermore, a significant decrease in the total $NADP^+ + NADPH_2$ was observed, in rat liver under the influence of AIA. These conflicting results could perhaps be explained on the basis of species differences, or perhaps on the differences in experimental condi ions.

It seems reasonably clear then, that the administration of drugs such as phenobarbital and allylisopropylacetamide to an intact animal, results in stimulation of a number of systems all requiring $NADPH_2$. The results obtained in this study confirm the increased requirements for reduced $NADP^+$, after the administratuon of a potent porphyrogenic sunstance such as AIA to the intact rabbit.

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SUMMARY

1. Porphyrinogenesis was stimulated in rabbits using the drugs Hexachlorobenzene and allylisopropylacetamide.

2. The ratio of the specific activity of $NADH_2$ to NAD^+ and $NADPH_2$ to NADP was measured at various time intervals after the administration of nicotinamide-C^{14} to normal and porphyric rabbits. It was found that AIA produced a significant acceleration in the generation of $NADH_2$ and $NADPH_2$. The results obtained from one rabbit suggest that hexachlorobenezene also increased the rate of $NADH_2$ and $NADPH_2$ generation.

3. Rapid equilibrium between cytoplasmic and mitochondrial NAD^+ was observed in both normal and AIA-induced porphyric rabbit liver.

4. AIA was found to significantly influence the quantitative amount of reduced NAD^+ and $NADP^+$ in rabbit liver. This consequently results in increased $NADH_2$: NAD^+ and $NADPH_2$: $NADP^+$ ratios. However, the total nicotinamide nucleotides in the liver remained unchanged.

5. Using the calculations of Zilversmit et al. (1943) AIA was found to significantly increase the 'turnover time' of $NADH_2$ in the rabbit liver.

6. The ratios of the specific activity of $NADPH_2/NADP^+$ and $NADH_2/NAD^+$ at various time intervals after the administration of nicotinamide-C^{14} to normal rat liver calculated by our techniques are entirely consistent with the results obtained by Gordon (1963).

7. The results have been interpreted as evidence for a link between increased reduced nicotinamide nucleotide generation and porphyrin or porphyrin precursor overproduction under the influence of a potent porphyrogenic drug.

8. The results confirm the increased requirements for $NADPH_2$ under the influence of a porphyrinogenic drug.

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TABLE 1

VARIOUS PROPOSED CLASSIFICATIONS OF THE PORPHYRIAS

(Modified after Goldberg and Rimington 1962)

A. [illegible]

1. Haematoporphyria Acuta
2. Haematoporphyria Acuta Toxica
3. Haematoporphyria Chronica
4. Haematoporphyria Congenita

B. [illegible]

1. Porphyria Congenita
2. Porphyria Cutanea Tarda
3. Porphyria Acuta:
   a) Latent
   b) Abnormal form
   c) Nervous form
   d) Classical acute porphyria
   e) Comatose form commencing as (b)

C. [illegible] (1956)

1. Porphyria Erythropoietica
2. Porphyria Hepatica
   a) Intermittent acute type
   b) 'Cutanea tarda' type
   c) Mixed type

D. [illegible]

1. Congenital Porphyria
2. Porphyria Cutanea Tarda Symptomatica
3. Porphyria Cutanea Tarda Hereditaria (Protocoproporphyria)
4. Acute Intermittent Porphyria

E. [illegible] (1960)

1. Porphyria Erythropoietica (recessive)
2. Porphyria Hepatica
   a) Hereditary acute intermittent (dominant)
      i) Manifest
      ii) Latent
   b) Hereditary, mixed or variegate group (dominant)
      i) Cutaneous with little or no acute manifestations
      ii) Acute intermittent - no cutaneous symptoms
      iii) Various combinations
      iv) Latent
   c) Hereditary cutaneous ?
   d) Constitutional or idiosyncratic (porphyria cutanea tarda)
      i) Chemicals, especially alcohol
      ii) Idiopathic
      iii) With systemic disease
   e) Acquired
      i) Secondary to hepatoma
      ii) Secondary to fungicide - turkish epidemic

F. GOLDBERG AND RIMINGTON (1962)

1. a) Congenital (Erythropoietic) Porphyria
   b) Erythropoietic Protoporphyria
2. Acute Intermittent Porphyria
3. Cutaneous Hepatic Porphyria
   a) Hereditary Forms
      i) Porphyria cutanea tarda hereditaria
      ii) Mixed porphyria
      iii) Porphyria variegata
   b) Acquired Forms
      i) Porphyria cutanea tarda symptomatica
      ii) Bantu porphyria
      iii) Turkish porphyria
      iv) Porphyrin - producing hepatic adenoma
4. Experimentally - induced porphyria

G. ELLES (1963)

1. Erythropoietic porphyrias
   i) Congenital erythropoietic porphyria
   ii) Protoporphyria erythropoietica
2. Hepatic porphyrias
   i) Swedish genetic porphyria; pyrolloporphyria AIP
   ii) S.A. genetic porphyria; protocoproporphyria, PCT hereditaria, mixed porphyria, porphyria variegata
   iii) Symptomatic porphyria: Urocoproporphyria, PCT symptomatica, acquired porphyria hyperporphyrismus
      a) Idiosyncratic-associated with alcoholic liver disorders, systemic disorders, drugs, chemicals
      b) Acquired -
         i) Hexachlorobenzene-induced
         ii) Hepatoma of liver
         iii) Others ?

H. TSCHUDY (1965)

1. Erythropoietic Porphyrias
   a) Congenital erythropoietic porphyria (recessive)
   b) Erythropoietic protoporphyria (dominant)
2. Hepatic Porphyrias
   a) Acute intermittent porphyria, Swedish genetic porphyria, pyrolloporphyria (dominant)
      i) Manifest
      ii) Latent
   b) Porphyria variegata, mixed porphyria, S.A. genetic porphyria, PCT hereditaria and protocoproporphyria (dominant)
      i) Cutaneous - little or no acute manifestation
      ii) Acute intermittent - no cutaneous symptoms
      iii) Various combinations
      iv) Latent
   c) Symptomatic porphyria, PCT symptomatica urocoproporphyria, constitutional porphyria
      i) Idiosyncratic associated with alcohol, liver disease, systemic disease, drugs, etc.
      ii) Acquired - Hexachlorobenzene induced and hepatoma

Author Becker David Morris
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