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TITLE OF THESIS.....Drug-induced Porphyria and the Hepatic Drug-Oxidising System.....

UNIVERSITY.....University of Alberta.....

DEGREE FOR WHICH THESIS WAS PRESENTED.....Ph. D.....

YEAR THIS DEGREE GRANTED.....1971.....

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DATED.....August 10.....1971

NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

DRUG-INDUCED PORPHYRIA AND THE HEPATIC DRUG-OXIDIZING SYSTEM

by

JOSEPH M. CREIGHTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILISOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Drug-induced Porphyria and the Hepatic Drug-Oxidizing System", submitted by Joseph M. Creighton in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date 19th July 1971

ABSTRACT

In previous studies a series of compounds was shown to cause porphyrin accumulation in isolated chick embryo liver cells and in the 17 day old chick embryo liver, but not in mice. To ascertain whether the above observations were due to species or developmental differences the activity of the above series of drugs was investigated in 5 to 7 week old chickens. The fact that this series of compounds was found to be active in the adult chicken demonstrates a species difference in response to porphyria-inducing drugs. Male and female chickens were found to respond in a similar fashion to porphyria-induction by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC).

In previous experiments it has been shown that the levels of hepatic δ-aminolevulinic acid synthetase (ALA-synthetase) are high in rabbit, rat and guinea pig embryos but that they rapidly assumed lower adult levels at the time of birth or shortly thereafter. In contrast we have shown that the level of hepatic ALA-synthetase in 17 day old chick embryos was only one third that of adult chickens. Levels in newborn chickens were found to be intermediate between adult and embryonic levels. It has been reported that newborn rats and rabbits are refractory to induction by porphyria-inducing drugs. In contrast we have shown that ALA-synthetase could be readily induced in newborn chickens.

It has been postulated that drug-induced experimental porphyria results from derepression of ALA-synthetase, which in turn leads to porphyrin and heme formation. The heme is thought to be utilized for the prosthetic group of cytochrome P-450 leading to an increased level of drug-oxidizing activity. The object of our next series of experiments was to test this hypothesis. The presence of drug-oxidizing activity in chick

embryo liver was demonstrated as early as the 11th day of development. This was in accord with the above hypothesis since chick embryo liver cells are responsive to porphyria-inducing drugs at this stage of development. It was shown that the drug-oxidizing activity of liver could be increased in 17 day old embryos by pretreatment with phenobarbital and the porphyria-inducing drug, allylisopropylacetamide (AIA). These facts were also in agreement with the above hypothesis. In further experiments designed to test the above hypothesis a positive correlation was sought between the porphyria-inducing activity of several DDC analogues and their ability to increase drug-oxidizing activity in the liver. Contrary to what was anticipated the administration of DDC and several of its analogues resulted in decreased drug-oxidizing activity. It is thus clear that the sequence of events postulated to occur in response to porphyria-inducing drugs does not apply to these compounds.

In our next series of experiments we investigated the idea that a decrease in drug-oxidizing activity might be linked to the induction of experimental porphyria in the following manner: Porphyria-inducing drugs cause the breakdown of the heme moiety of cytochrome P-450 leading to decreased levels of this cytochrome, decreased drug-oxidizing activity and to derepression of ALA-synthetase. We demonstrated that a decrease in drug-oxidizing activity prior to ALA-synthetase induction was not paralleled by a fall in cytochrome P-450. Consequently the above hypothesis was not supported by our experiments. Neither AIA nor DDC inhibited drug-oxidizing activity by inhibiting the generation or oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and hence the flow of electrons to cytochrome P-450.

Following DDC administration a moderate rise in ALA-synthetase

activity was accompanied by high hepatic porphyrin levels. On the other hand AIA and 5β-pregnan-3α-17α-diol 11,20-dione produced higher ALA-synthetase levels accompanied by considerably lower hepatic porphyrin levels. Thus hepatic porphyrin levels provide a poor index of ALA-synthetase activity and cannot be used as an index of the activity of this enzyme in the chick embryo liver as has hitherto been assumed.

ACKNOWLEDGEMENTS

The research reported in this thesis was conducted under the supervision of Dr. G. S. Marks with the financial support of the medical Research Council of Canada.

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CHAPTER I GENERAL INTRODUCTION

A. Structure and Nomenclature of Porphyrins

Porphyrins are cyclic compounds composed of four pyrrole rings linked together by methylene bridges. From a biological standpoint, one of the most important chemical properties of the porphyrins is their ability to complex with metal ions. The complexes of iron with porphyrins are known as hemes, while the chlorophylls are magnesium-tetrapyrrole complexes. The numbering of the porphyrin ring system is illustrated in Fig. 1 with the simplest porphyrin, viz., porphin. Uroporphyrin is a porphyrin with acetic and propionic acid side chains on each of the four pyrrole rings, (Fig. 2). Since there are four different ways in which these two side chains can be arranged so that each ring contains an acetic and a propionic acid group, there are four possible isomers of uroporphyrin (Fig. 2). Only two of these four possible isomers have been found in nature viz., type I and type III (Fig. 2). The type I isomer has a symmetrical arrangement of substituents about the tetrapyrrole nucleus, while the order of the substituents is reversed on ring D for the type III porphyrin (Fig. 2). When the acetic acid substituents are replaced by methyl substituents coproporphyrin is obtained. Of the four possible isomers only types I and III occur naturally. Protoporphyrin has vinyl, propionic and methyl side chains, and of a possible fifteen isomeric forms only one isomer, protoporphyrin IX (Fig. 3), has been isolated from biological materials (Lascelles, 1964). Under appropriate reducing conditions porphyrins may be converted to porphyrinogens by the addition of six hydrogen atoms. Porphyrinogens occur naturally as intermediates in the biosynthesis of heme.

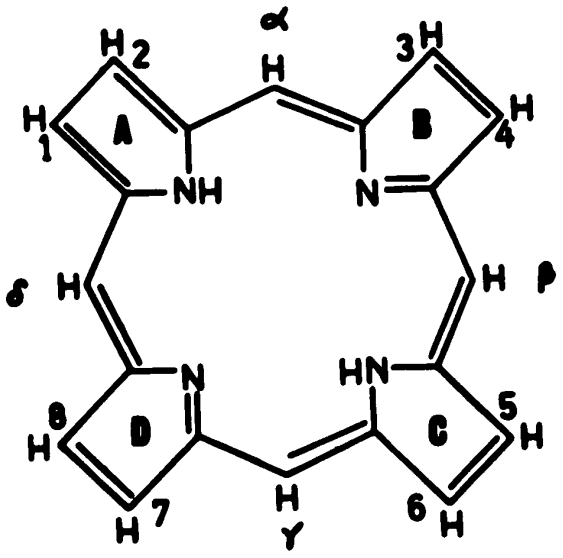
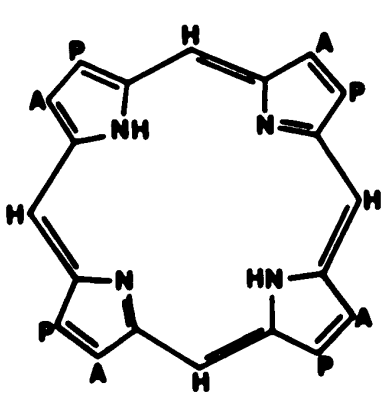
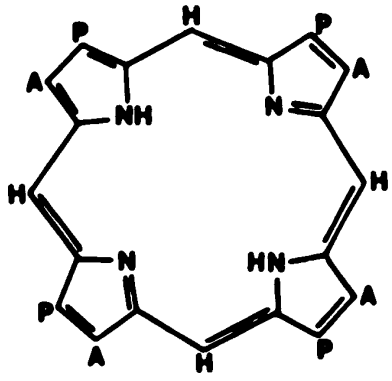


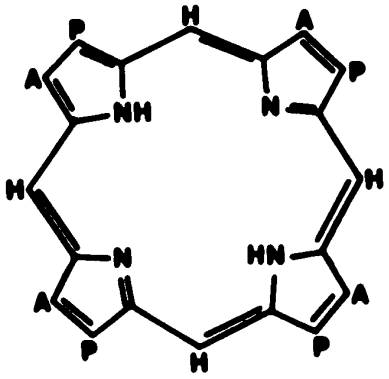
Fig. 1. Structure of Porphin, Showing Numbering of the Porphyrin Ring Structure.



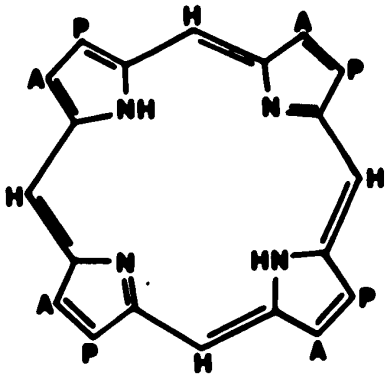
TYPE I



TYPE II



TYPE III



TYPE IV

A = $CH_2 - COOH$

P = $CH_2 - CH_2 - COOH$

Fig. 2. Structures of the Four Isomers of Uroporphyrin.

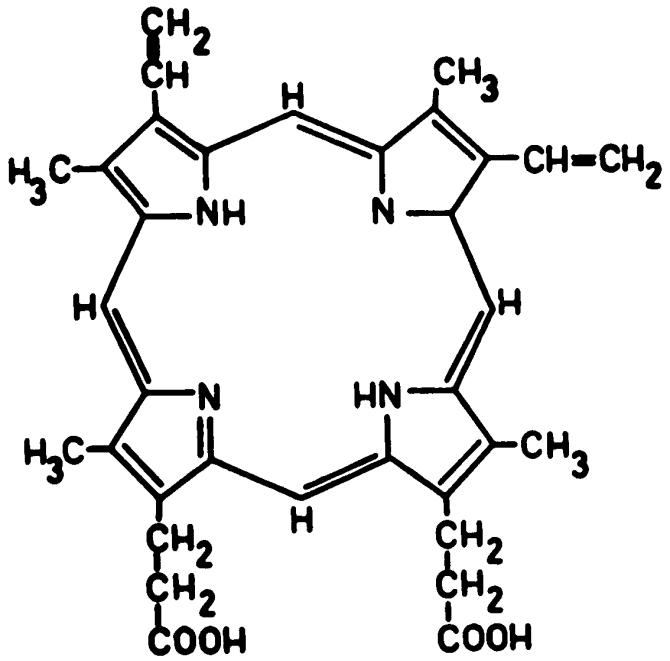


Fig. 3. Structure of Protoporphyrin IX.

B. Biosynthesis of Heme

Studies from the laboratories of Shemin and of Neuberger demonstrated that glycine and succinate contributed all the carbon and nitrogen atoms of the heme moiety of hemoglobin. Moreover, Shemin and his coworkers were able to show the position of each of the carbon atoms of these substrates in the protoporphyrin IX molecule (Shemin, 1956). On the basis of these and other studies (Rimington, 1959; Granick & Mauzerall, 1960) the intermediate steps in the biosynthesis of protoheme were unravelled and are described below.

The initial step in the porphyrin biosynthetic pathway is the condensation of glycine and succinyl CoA to yield δ-aminolevulinic acid (ALA; Fig. 4). The enzyme which promotes this condensation is known as ALA-synthetase, and is found in the mitochrondrial matrix of cells of higher organisms (McKay et al., 1969; Zuyderhoudt et al., 1969). In liver mitochondria succinyl-CoA arises mainly through the oxidation of α-ketoglutarate and to a small extent from succinate via a succinyl-CoA synthetase (Granick & Urata, 1963). The condensation of succinyl-CoA and glycine requires pyridoxal phosphate as a co-factor (Lascelles, 1957; Schulman & Richert, 1957). Kikuchi et al. (1958) and Neuberger (1961) have proposed similar mechanisms whereby pyridoxal phosphate forms a carbanion with glycine, and the carbanion then reacts with succinyl-CoA to produce α-amino-β-oxoadipic acid. α-amino-β-oxoadipic acid decarboxylates virtually instantaneously in neutral solution to give ALA and CO_2 (Fig. 4; Laver et al., 1959). For this reason it cannot be isolated from a biological system nor can it be tested as a possible intermediate.

ALA-dehydratase catalyzes the condensation of two molecules of ALA

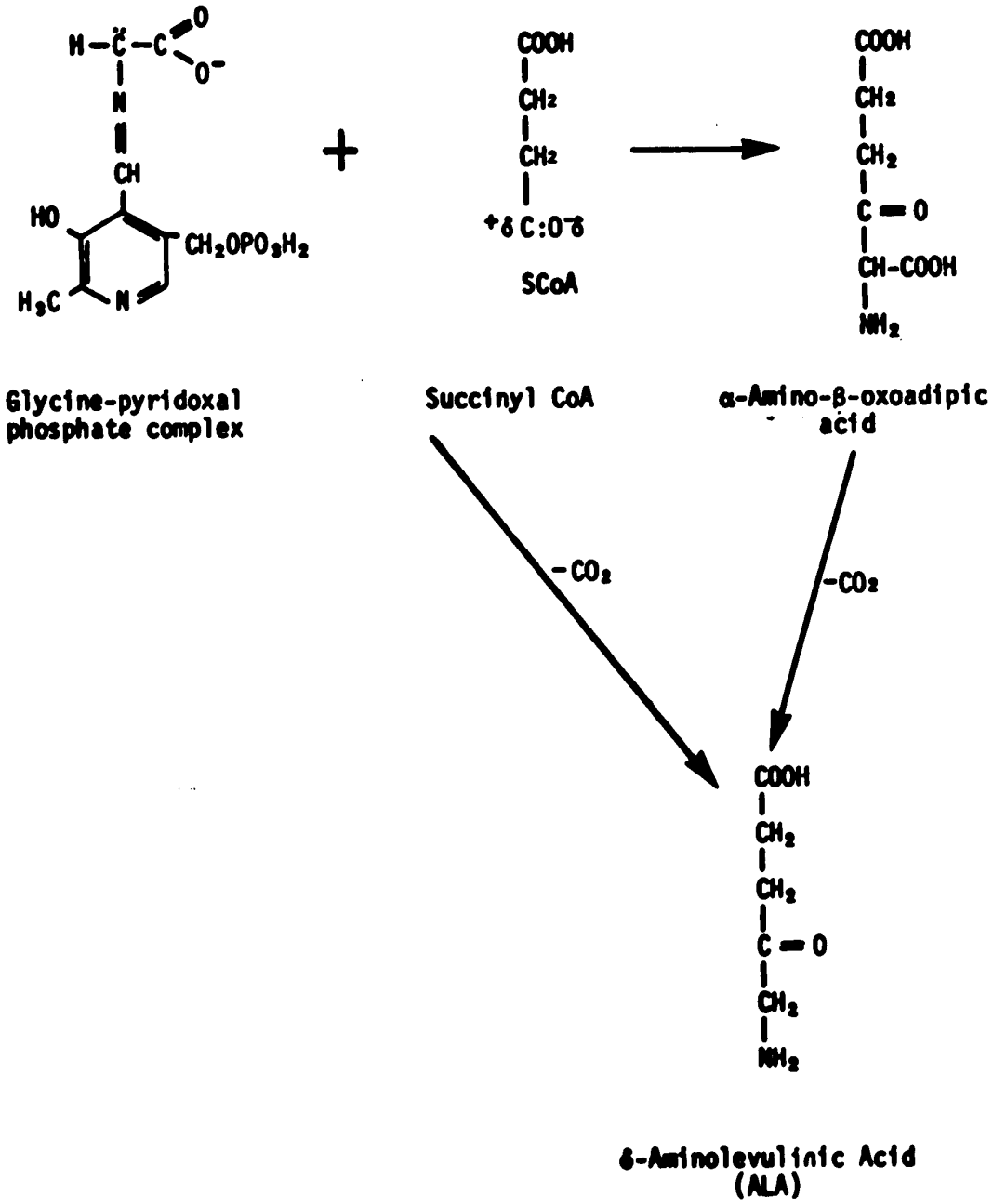


Fig. 4. Heme Biosynthesis: Suggested Mechanism of Formation of Aminolevulinic Acid (ALA).

to yield the monopyrrole, porphobilinogen (PBG; Fig. 5). This enzyme is found in the cytoplasm of the cell, and its activity is markedly inhibited by EDTA (Gibson et al., 1955). In the mechanisms proposed by Shemin (1968; Fig. 5) one molecule of ALA forms a Schiff base with the enzyme resulting in the formation of a stabilized carbanion. This carbanion then combines with the carbonyl carbon atom of a second molecule of ALA by a nucleophilic mechanism. Recently ALA-dehydratase has been isolated from beef liver (Wilson & Dowdle, 1971) and the photosynthetic bacterium Rhodopseudomonas spheroides (van Heyningen, 1971). The beef liver enzyme is zinc dependent, and is inhibited by lead or cadmium.

The conversion of four molecules of PBG to the tetrapyrrole uroporphyrinogen III is also accomplished in the cytoplasm of the cell (Fig. 6). Bogorad (1958 a, b) separated two enzymes from plant sources which were necessary for this conversion. One of these enzymes, uroporphyrinogen I synthetase, converted PBG into uroporphyrinogen I. The second enzyme, uroporphyrinogen III cosynthetase, had no capacity for catalyzing PBG consumption when incubated alone with this substrate. However when uroporphyrinogen III cosynthetase was incubated together with uroporphyrinogen I synthetase and PBG, uroporphyrinogen III rather than I was formed.

Coproporphyrinogen III is formed in the cytoplasm (Fig. 6) by the decarboxylation of the four acetic acid side chains of uroporphyrinogen III (Mauzerall & Granick, 1958). An enzyme which brings about this decarboxylation has recently been isolated from chicken erythrocytes (Tomio et al., 1970). The purified enzyme was heat labile, and its activity was enhanced by EDTA.

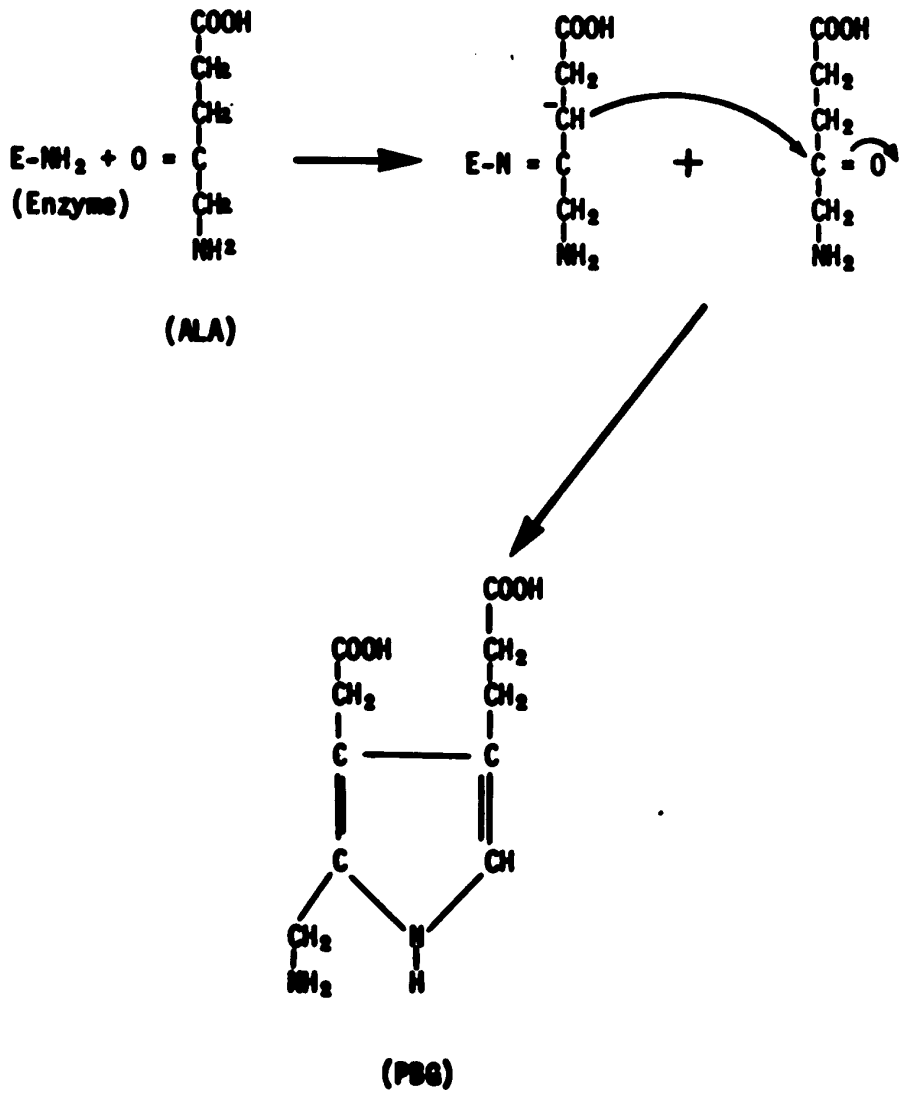


Fig. 5. Heme Biosynthesis: Summary of Proposed Mechanism of Porphobilinogen (PBG) Formation.

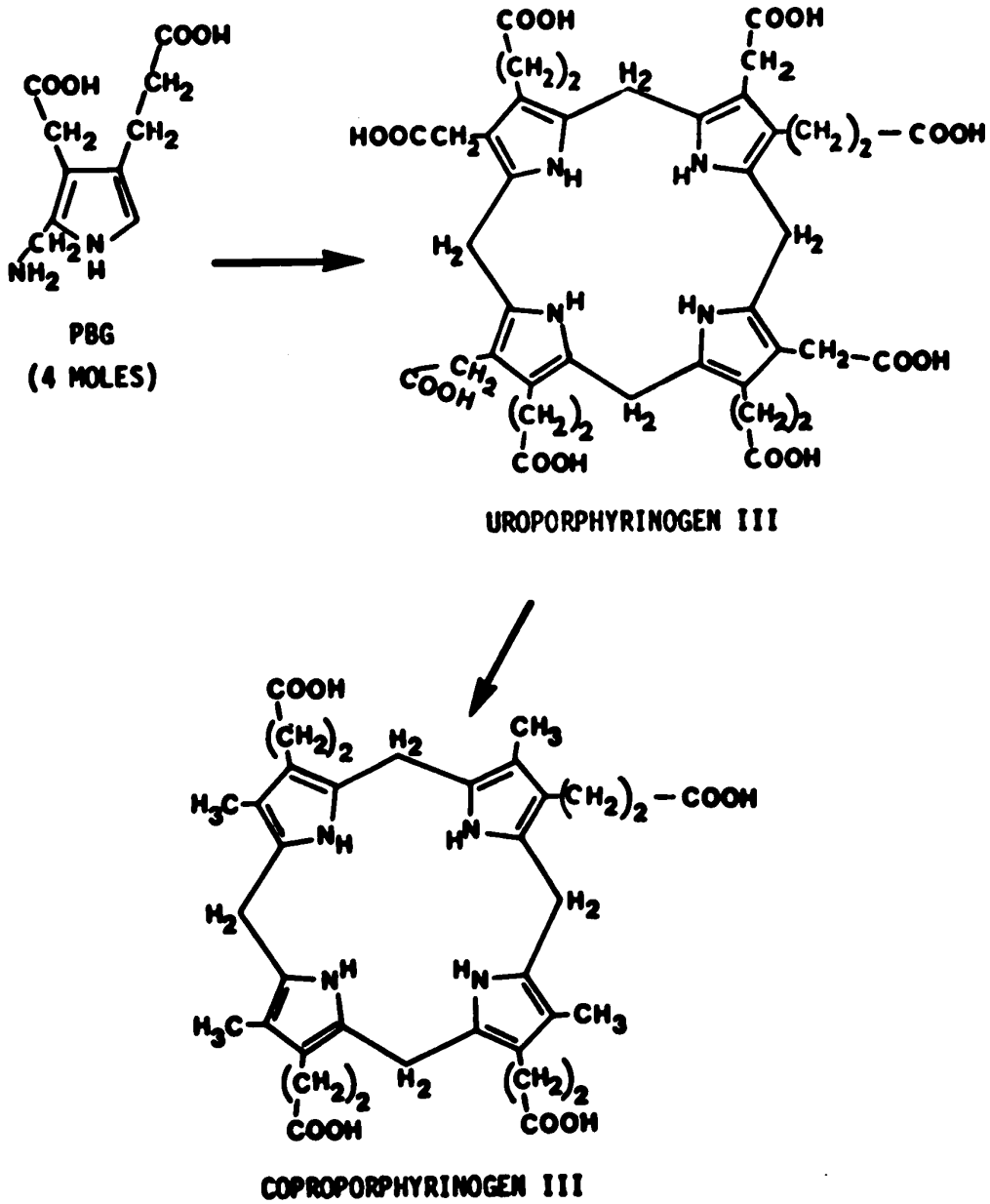


Fig. 6. Heme Biosynthesis: Conversion of Porphobilinogen (PBG) to Coproporphyrinogen III.

The enzymes necessary for the final steps in the biosynthetic pathway viz., the conversion of coproporphyrinogen III to heme, are found in the mitochondria. The conversion of coproporphyrinogen III to protoporphyrin IX (Fig. 7) involves the decarboxylation of two propionic acid side chains to vinyl groups (positions 2 & 4), and the oxidative removal of six hydrogen atoms from protoporphyrinogen. Coproporphyrinogen III is an excellent substrate for coproporphyrinogen oxidase. The type I isomer, on the other hand will not serve as a substrate (Sano & Granick, 1961). The insertion of iron into protoporphyrin to form heme (Fig. 7) is mediated by the enzyme ferrochelatase. This enzyme requires a reducing environment for activity (Labbe & Hubbard, 1960), and is associated with the inner mitochondrial membrane (McKay et al., 1969).

C. Distribution of Heme

Hemes are widely distributed throughout the animal and plant kingdoms and play a crucial role in cell metabolism.

i. Hemoglobin and Myoglobin

In vertebrate tissue hemoglobin is present in far greater quantities than other hemoproteins. For example, in man, on a weight basis the hemoglobin : myoglobin ; cytochrome c ratio is 1169 : 41 : 1 (Drabkin, 1951). Hemoglobin and myoglobin, which contain protoheme as prosthetic groups, function as oxygen carriers because of their ability to combine reversibly with oxygen. The iron must be maintained in the ferrous form for activity.

ii. Cytochromes

The cytochromes are the most widely distributed of the hemo-

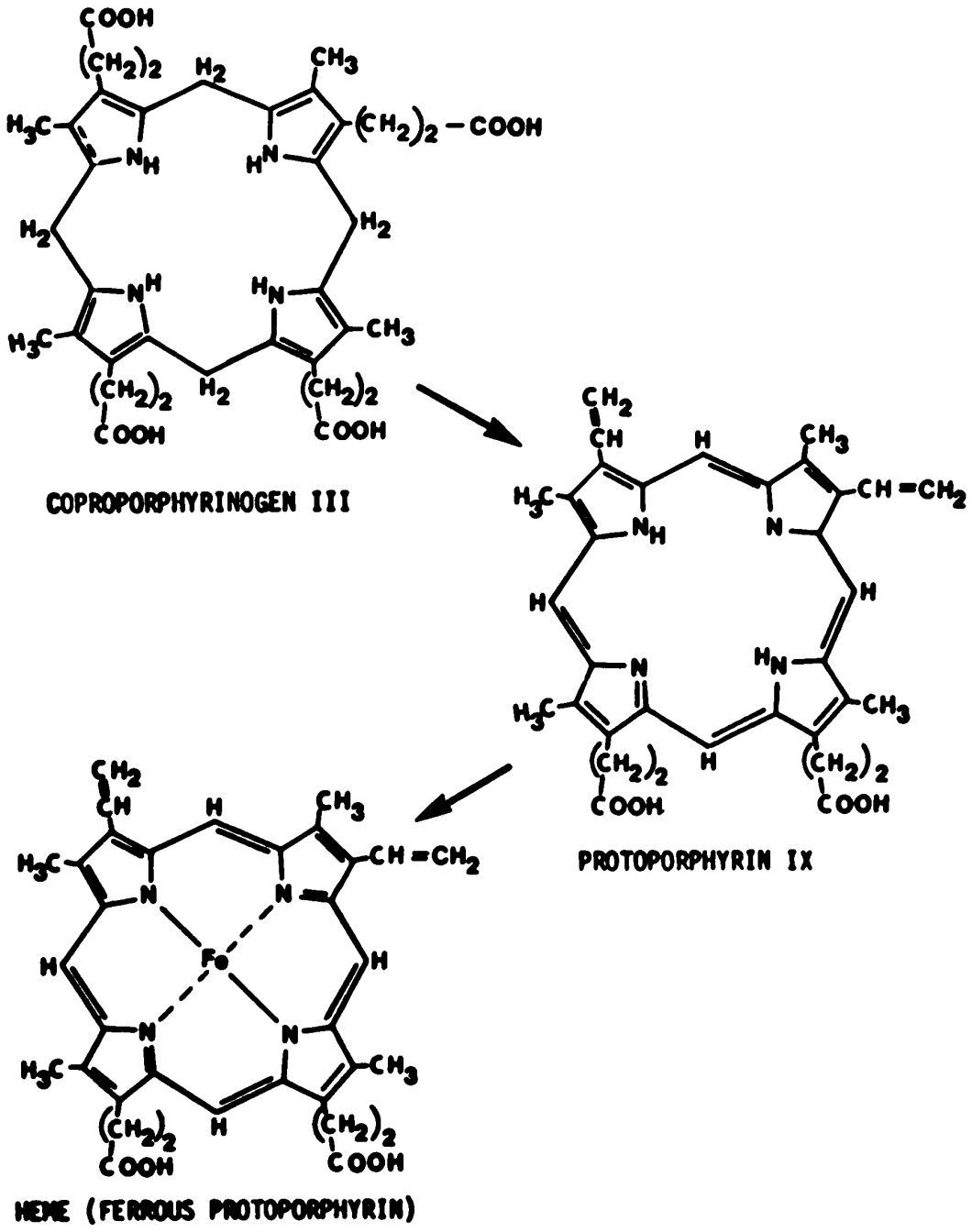


Fig. 7. Heme Biosynthesis: Conversion of Coproporphyrinogen III to Heme.

proteins (Lascelles, 1964). The precise function of a number of the cytochromes is still unknown, but they all appear to act as electron carriers by virtue of a reversible valency change of their heme iron. The cytochromes may be divided into three classes according to the structure of the heme group and its attachment to the apoprotein.

1. Cytochromes a. Cytochromes with a formyl side chain in the heme group.
2. Cytochromes b. Cytochromes with a protoheme group.
3. Cytochromes c. Cytochromes in which the protoheme group is covalently linked to the protein.

Cytochromes are identified by numerical subscripts attached to the class letter, for example cytochrome b_5. The b indicates that it has protoheme as its prosthetic group, and the 5 indicates the chronological order of its discovery in its class. Liver and heart are particularly rich sources of cytochromes (Drabkin, 1951).

iii. Catalase and Peroxidase

The catalases and peroxidases are enzymes which contain ferriheme as their prosthetic groups. Catalases are found chiefly in kidney, liver and erythrocytes. Catalase decomposes hydrogen peroxide as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Though peroxidase is mainly a plant enzyme, its presence has been demonstrated in thyroid and salivary tissue of the rat (Alexander, 1960). Peroxidases catalyze the oxidation of various substrates by hydrogen peroxide as follows:

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O$$

iv. Tryptophan Pyrrolase

Tryptophan pyrrolase is a liver ferroheme which catalyzes the oxidation of L-tryptophan to formylkynurenine (Tanaka & Knox, 1959). The reaction involves direct oxygenation of tryptophan with molecular oxygen.

D. Human Porphyrias

The porphyrias constitute a group of diseases, mainly hereditary in origin, in which vastly increased quantities of porphyrins and/or porphyrin precursors are produced. Even though the porphyrias are rare disorders, they have been extensively studied. The isolation and identification of porphyrins and precursors from patients with these diseases has contributed significantly to our understanding of the normal pathway of porphyrin metabolism and its control. In addition the porphyrias have recently assumed historical significance. Recent re-evaluation of the medical history of George III of England reveals that the King's "madness" was probably the result of porphyria (Macalpine & Hunter, 1966). In a follow up investigation, Macalpine et al. (1968) traced the disease back to Mary Queen of Scots and her son, James I of England. Among the decendants of James the I, the following were probably porphyrics: Queen Ann, King George III, King George IV, Fredrick William I (Prussia), and Fredrick the Great (Prussia). The diagnosis of porphyria was supported by the finding of porphyria in two living descendants of the family.

Early classification of the porphyrias depended almost entirely on clinical features. Schmid et al. (1954) suggested that the porphyrias should be divided into 2 main groups: (i) hepatic, in which the liver is the principal site of abnormal porphyrin formation, and (ii) erythropoietic,

in which excessive porphyrin formation occurs in the developing red blood cells of the bone marrow. The hepatic porphyrias are in turn divided into 3 major sub-groups and the erythropoietic porphyrias into 2 sub-groups (Tschudy, 1965). These will be discussed below.

1. Hepatic Porphyrias

Acute intermittent porphyria (Swedish Type, AIP) is characterized chemically by the excretion of excessive amounts of the porphyrin precursors, PBG and ALA in the urine during acute attacks. PBG is also frequently demonstrable in the urine during periods of remission. The clinical symptoms (abdominal pain, vomiting, constipation, paralysis, mental aberrations) have been explained on the basis of demyelination, which occurs in the peripheral and central nervous systems (Goldberg, 1959). Photosensitivity, which is common in other forms of porphyria does not occur in acute intermittent porphyria. This is not surprising in view of the fact that porphyrins which are the photosensitizing agents in other forms are not overproduced in AIP. The administration of barbiturates and other drugs frequently cause the onset of acute attacks in those genetically disposed to the porphyrias. The attacks precipitated by drugs are reported to be more severe than those arising in the absence of drug administration (Goldberg, 1959). Other known precipitating factors are infections, menstruation and pregnancy. The possibility that a steroid or steroid metabolite may be associated with the acute attack has been considered. The mode of hereditary transmission of acute intermittent porphyria is as an autosomal dominant. The disease is found more frequently in females than males due possibly to secondary precipitating factors (Goldberg & Rimington, 1962).

Variegate porphyria (mixed porphyria, South African porphyria) is inherited as a Mendelian dominant characteristic and is not sex-linked. The disease may manifest itself either in acute attacks similar to those observed in acute intermittent porphyria, or in chronic cutaneous lesions which are aggravated by sunlight, or in both. Cutaneous manifestations are usually more pronounced in men while acute attacks are more common in women (Dean & Barnes, 1955). As in acute intermittent porphyria, ALA and PBG are excreted in the urine during acute attacks. In variegate porphyria, in contrast to acute intermittent porphyria, fecal porphyrin excretion is always elevated, both during acute attacks and during remission. Sufferers from variegate porphyria are also sensitive to the aggravating effects of barbiturates and other drugs (Goldberg & Rimington, 1962).

Hereditary coproporphyria is transmitted as an autosomal dominant characteristic. Acute attacks of this disease are similar to those of acute intermittent porphyria and photosensitivity has also been reported (Goldberg et al., 1967).

Symptomatic porphyrias consist of a group of porphyrias in which no hereditary link has been established. Symptomatic porphyrias are characterized chemically by marked increase in urinary porphyrin excretion while fecal porphyrin is normal or only slightly elevated. ALA and PBG are not usually excreted in increased amounts. An acquired form of porphyria has been described in southeastern Turkey (Schmid, 1960). Many people in the affected region were consuming seed wheat treated with the fungicide, hexachlorobenzene, which was shown in animal experiments to be a potent porphyria-inducing agent (Ockner & Schmid, 1961).

ii. Erythropoietic Porphyrias

Congenital erythropoietic porphyria is characterized by marked photosensitivity and increased excretion of uro- and copro- porphyrins, predominantly of the abnormal type I isomer. It is a rare recessive disease which occurs with equal frequency in both sexes (Goldberg & Rimington, 1962).

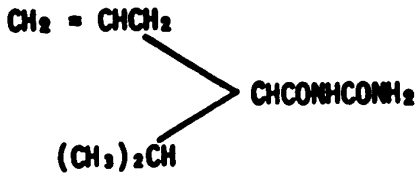
Erythropoietic protoporphyria is characterized clinically by mild photosensitivity and chemically by increases in protoporphyrin levels in erythrocytes, plasma and feces. There may be a disturbance of porphyrin metabolism of the liver as well as the reticulocytes in this disease (Tschudy 1965).

E. Experimental Porphyria

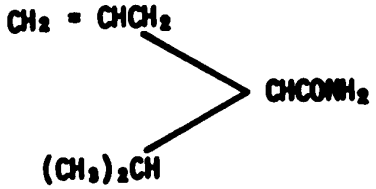
i. Porphyria-Inducing Compounds

It is well established that attacks of acute porphyria are frequently associated with drug administration. The phenomenon was first reported by Stokvis (1889) who associated the use of sulphonal (dimethylsulphone-dimethylmethane) with the acute attack. Subsequent administration of this drug to rabbits and dogs resulted in disordered porphyrin metabolism (Stokvis, 1895). These disorders of porphyrin metabolism produced in experimental animals without the genetic defect are referred to as experimental porphyrias.

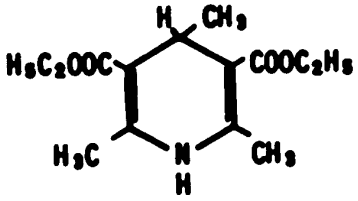
The report of a fatal porphyria in a patient treated with large amounts of the hypnotic drug Sedormid (Fig. 8; Duesberg, 1932) led Schmid and Schwartz (1952) to administer this drug to normal rabbits. This resulted in the accumulation of porphyrins and PBG in the livers of



Sedormid



Allylisopropylacetamide (AIA)



3,5-Diethoxycarbonyl - 1,4-dihydro-2,4,6-trimethylpyridine (DDC)

Fig. 8. Structures of Sedormid; Allylisopropyacetamide (AIA); and 3,5Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl-pyridine (DDC).

these animals, while the porphyrin content of erythrocytes and bone marrow was normal. Sedormid thus produced a condition which seemed promising as a model of the disturbance of porphyrin metabolism observed in human hepatic porphyria. However Sedormid produced a marked hypnotic effect in addition to the disturbance in porphyrin metabolism. For this reason allylisopropylacetamide (AIA; Fig. 8), a closely related compound which possesses the same potency as Sedormid for producing porphyria in experimental animals, but is almost devoid of hypnotic properties, has displaced Sedormid as a porphyria-inducing drug (Goldberg & Rimington, 1955).

Solomon and Figge (1959) fed 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC; Fig. 8) to mice in an attempt to stain the mucus of the forestomach and noticed that the livers and gall bladders fluoresced red when examined under ultraviolet light. The fluorescence could not be attributed to DDC, which appears as a blue fluorescent substance under ultraviolet light. Solomon and Figge showed that the red fluorescence was due to the accumulation of porphyrins. DDC like AIA has subsequently been used extensively to study porphyrin metabolism in experimental animals.

Granick (1963) described a simple and elegant procedure for demonstrating porphyria-inducing activity of chemicals. Chemicals were added to cultures of chick embryo liver cells, and the porphyrins formed observed under a fluorescence microscope. Among the numerous compounds (Granick, 1964, 1965, 1966) which he found to be active by this procedure were sex steroids. Because of the physiological origin of the steroids and their possible relationship to the pathogenesis of hepatic porphyria in man, a more detailed screening of steroids was carried out. After screening over 120 steroids in cell culture, a group of naturally occurring steroid metabolites was found to have a very high degree of activity (Granick &

Kappas, 1967). All of the active steroids were found to have in common the 5β-H configuration, and the 5α-H epimers of these active compounds were found to have only slight activity. These 2 series are distinguished by the orientation of the hydrogen atom at C5 (Fig. 9). The result is that the 5β-H compounds possess a highly anglulated A:B ring junction (A:B cis), while the 5α-H epimers have a planar A:B junction (A:B trans). In addition to their effect on liver cells the 5β-H steroids have been shown to stimulate hemoglobin synthesis in cultured embryonic erythroid cells of the chick blastoderm (Levere, 1967). This is in contrast to findings with AIA and DDC, which have no effect on this preparation.

In addition to the compounds already mentioned, a wide variety of compounds have been shown to cause disturbances of porphyrin metabolism in experimental animals and in liver cell cultures (De Matteis, 1967). While the disturbances of porphyrin metabolism produced by drugs in experimental animals bear a strong resemblance to the biochemical picture in the human hepatic porphyrias, the clinical manifestations of the human diseases are not present in the drug-treated animals. For this reason caution must be used in extrapolating from the experimental porphyrias to the human diseases.

ii. Mechanism of Induction

Granick and Urata (1963) could not detect ALA-synthetase activity in mitochondria prepared from the livers of normal guinea pigs, but if the animals were pretreated with DDC this enzyme was readily measured. No marked increases in the activities of the other enzymes of the porphyrin biosynthetic chain were observed; hence the production of ALA seemed to be the rate limiting step in the pathway. When mitochondria from normal

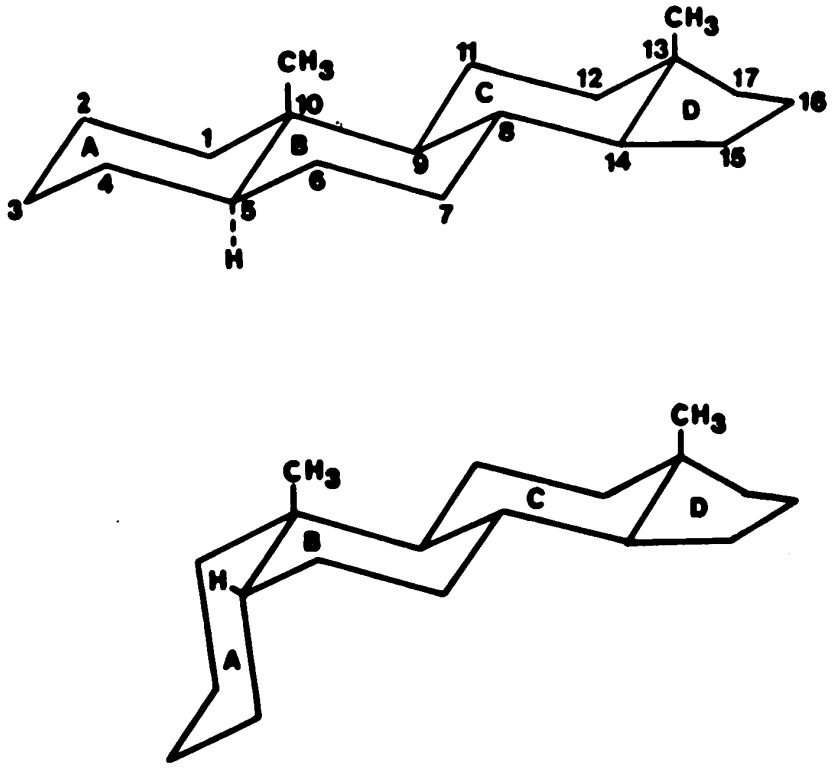


Fig. 9. Stereochemical Representation of the Basic Nuclear Structures Characteristic of 5α-H (A:B-trans-upper diagram) and 5β-H (A:B-cis-lower diagram) Steroid Metabolites.

guinea pig liver were incubated with DDC for 4 to 6 hours, no enhancement of ALA-synthetase activity was found, indicating that the drug does not activate pre-existing enzyme.

In subsequent reports Granick, (1963, 1966) using chick embryo liver cell culture showed that drug-induced porphyrin biosynthesis was blocked by the addition of inhibitors of protein synthesis, such as puromycin and actinomycin D. In these experiments Granick assumed that porphyrin accumulation could be used as a measure of ALA-synthetase activity. On the basis of these findings Granick concluded that the porphyria-inducing drugs act by causing an increased synthesis of ALA-synthetase, the first enzyme in the porphyrin biosynthetic pathway. It is interesting to note that liver ALA-synthetase was subsequently reported to be elevated in patients suffering from acute intermittent porphyria (Tschudy et al., 1965) and variegate porphyria (Dowdle et al., 1967), thus further increasing the degree of parallelism between the disturbances in porphyrin metabolism of the acute porphyrias, and those induced by drugs in experimental animals.

Granick (1966) reported that the increased synthesis of ALA-synthetase brought about by chemicals could be blocked by heme (end product repression), but using isolated mitochondria he could find no evidence for inhibition of the enzyme by heme (end product inhibition). This differs from the situation in the photosynthetic bacterium Rhodopseudomonas spheriodes, in which it was found that heme not only inhibits synthesis of new ALA-synthetase (Lascelles, 1960) but also directly inhibits the action of pre-existing enzyme (Burnham & Lascelles, 1963). Recently however a partially purified preparation of ALA-synthetase was prepared from the livers of AIA-treated rats and shown to be inhibited by heme (Scholnick

et al., 1969). This enzyme was found in the soluble fraction of the cells and is believed to represent ALA-synthetase which has been synthesized on the rough endoplasmic reticulum and is in transit to the mitochondria. The difference between these results and those of Granick may reflect the inability of added heme to penetrate mitochondria and interact with the enzyme. Since the initial and final steps in the normal biosynthesis of heme take place within the mitochondria no such barrier to feed-back inhibition of ALA-synthetase would exist in vivo. Therefore end product inhibition of ALA-synthetase cannot be ruled out as a physiologically important mechanism for the regulation of porphyrin biosynthesis.

Granick (1966) has proposed a scheme based upon regulation of the porphyrin biosynthetic pathway by end product repression to explain drug-induced experimental porphyria. In this scheme (Fig. 10), adapted from the ideas of Monod and Jacob (1961), heme biosynthesis is controlled by a repressor which can inactivate an operator gene, and thus prevent the coding of DNA for ALA-synthetase on the structural genes. This repressor is postulated to be composed of a protein, the apo-repressor, and a co-repressor which is the end-product heme. Porphyria-inducing drugs could then compete with heme for a site on the apo-repressor and prevent the repressor from acting, thus causing ALA-synthetase to be synthesized.

iii. Species, Sex and Developmental Differences

Since many of the drugs which are used to induce experimental porphyria in animals were recognized after they had precipitated attacks of acute porphyria in humans, it is only natural that attempts should have been made to reverse the order, that is to use experimental animals to predict which drugs might precipitate attacks of porphyria in humans carrying

the genetic lesion. Granick (1964) cautioned against the administration of a number of drugs to porphyric patients and their relatives, because he had found these drugs to induce the accumulation of an excess of porphyrins in cultures of chick embryo livers. Racz and Marks (1969) showed that several drugs which were active porphyria-inducing compounds in chick embryo liver cell culture or in the intact chick embryo were completely inactive in increasing porphyrin levels in the livers of intact mice. These observations are of particular interest since these same drugs have been associated with attacks of acute porphyria in humans (Cowger & Labbe, 1965) and suggest that the chicken might be useful as a model species for predicting responses to drugs in human porphyria.

Almost all the previous studies with chickens have been done with the embryo. To ascertain whether adult chickens responded in the same way as the embryo to porphyria-inducing drugs, a comparison of the effects of drugs in the embryo and adult chicken has been carried out and is reported in Chapter II of this thesis.

Recently Woods and Dixon (1970a) have reported that newborn rabbits are refractory to hepatic ALA-synthetase induction by DDC. Similarly Song et al. (1968) reported that newborn rats are refractory to hepatic ALA-synthetase induction by DDC and AIA. The inducibility of hepatic ALA-synthetase was reported to increase with the age of the animals. For this reason it was of interest to compare the drug-responses observed in the intact chick embryo and the newborn chick, with drug-responses in adult chickens. This study is reported in Chapter II of this thesis.

Considerable variability has been noted in the amount of porphyrin biosynthesis induced by drugs in individual rabbits. This has led

to the classification of rabbits as good and poor porphyrin excretors. (Goldberg & Rimington, 1962). In preliminary experiments we have found a similar variability in response to porphyria-inducing drugs in chickens. Our experiments indicated that this variability might represent sex-differences. For this reason an investigation reported in Chapter II of this thesis was carried out to see if this explanation was correct.

F. Experimental Porphyria, Cytochrome P-450 and Drug Metabolism

The main site of biotransformation of most drugs and other foreign chemicals is the liver. Brodie et al. (1955) showed that the liver enzyme system responsible for the oxidation of foreign compounds is associated with the endoplasmic reticulum and requires both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen for activity. The fact that the enzymes have these requirements enables one to classify them as mixed function oxidases. Mason (1957) defines a mixed function oxidase as an enzyme that catalyzes the consumption of one molecule of oxygen per molecule of substrate (designated as XH below) with one oxygen atom appearing in the product and the other undergoing two-equivalent reduction as illustrated below.

$$XH + O_2 + 2e \rightarrow XOH + O^{--} (H_2O)$$

This mechanism is supported by experiments of Posner et al. (1961) utilizing $^{18}O_2$ and $H_2{}^{18}O$, in which they have shown that it is atmospheric oxygen and not the oxygen of water that is incorporated during the hydroxylation of acetanilide.

The terminal oxidase of this system is believed to be a cytochrome whose presence in liver endoplasmic reticulum can be detected by treating

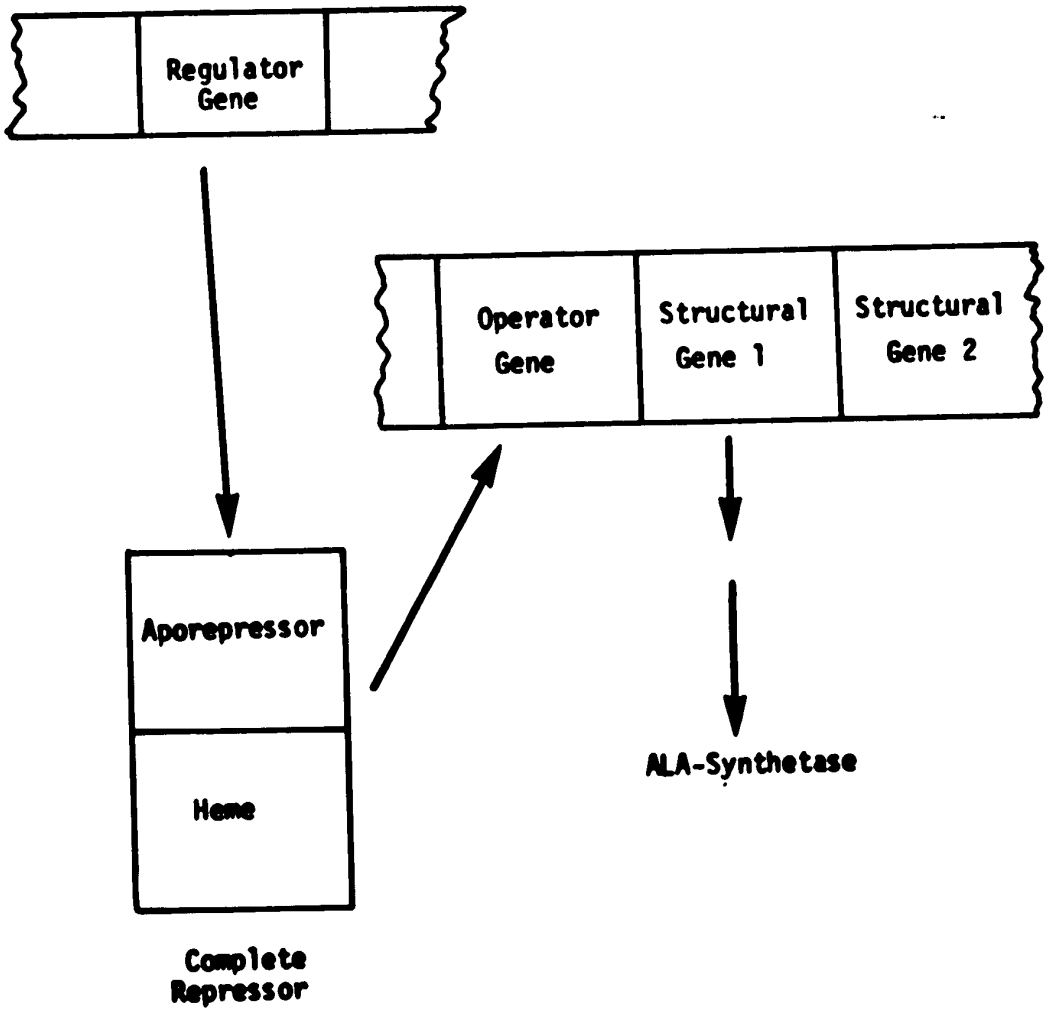
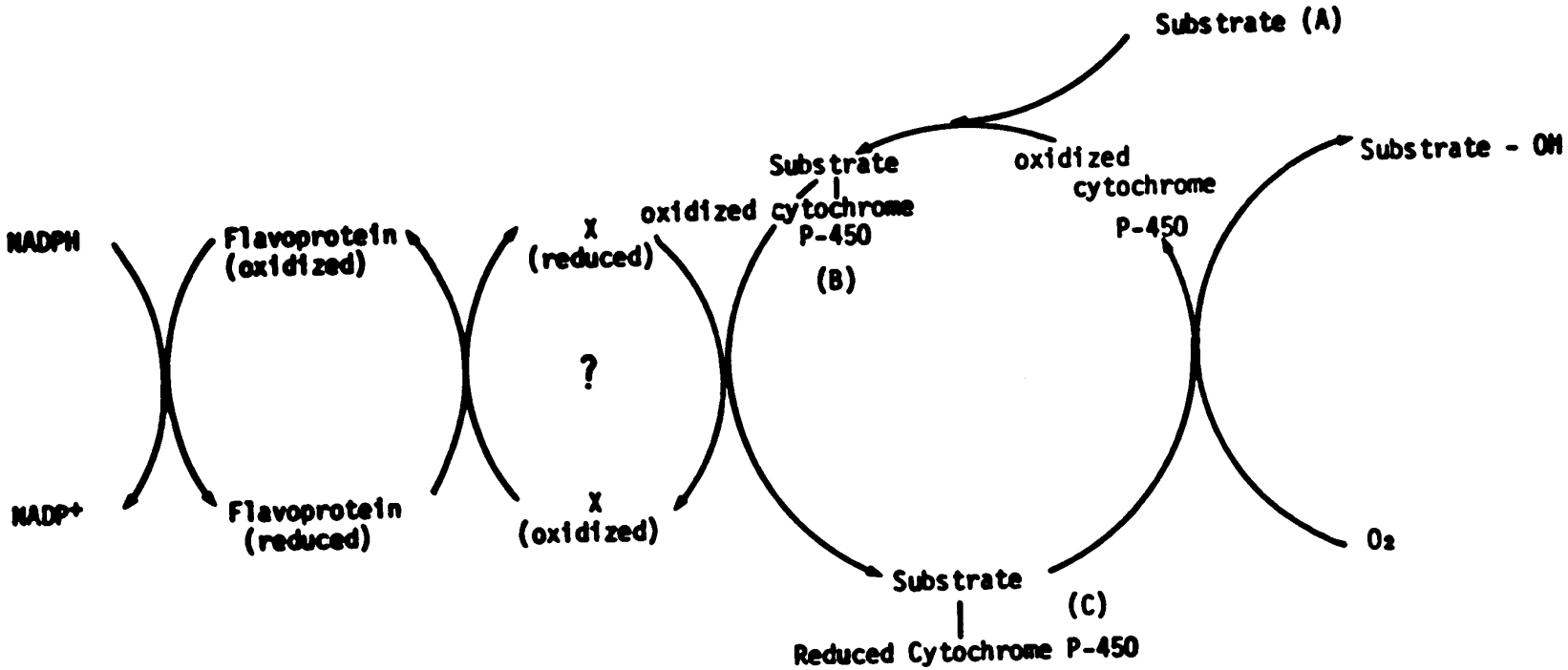


Fig. 10. Granick's Scheme for the Regulation of Heme Synthesis by End Product Repression.

a reduced microsomal suspension with carbon monoxide (Klingenberg, 1958; Garfinkel, 1958). Because the carbon monoxide spectrum of this cytochrome exhibits a maximum at 450 mμ it has been designated cytochrome P-450. Since the carbon monoxide spectrum of reduced microsomes exhibits no peaks other than that at 450 mμ the cytochrome could not be assigned to the a, b, or c category. However when microsomes were treated with snake venom or deoxycholate, cytochrome P-450 was converted to a solubilized form called cytochrome P-420 and the reduced minus oxidized spectrum of cytochrome P-420 has α and β peaks at 560 mμ and 530 mμ respectively, which are typical of a b type cytochrome (Omura & Sato, 1964).

Gillete and Gram (1969) have proposed that the following sequence of events take place in the oxidation of substrate by the mixed function oxidases of the hepatic endoplasmic reticulum (Fig. 11). First the substrate (A) combines with the oxidized form of cytochrome P-450 to form a complex (B). This complex is then reduced via NADPH, NADPH Cytochrome C reductase and possibly another intermediate. The reduced complex (C) then combines with molecular oxygen to form an O_2-cytochrome P-450-substrate complex which decomposes to oxidized substrate and the oxidized form of the cytochrome.

A great variety of drugs, steroid hormones, insecticides and other chemical compounds have been shown to cause an increase in the rate of metabolism of substrates and a parallel increase in the amount of cytochrome P-450 in the hepatic endoplasmic reticulum (Mannering, 1968). Many of the types of compounds which have been reported to cause induction of cytochrome P-450 are also effective in producing experimental porphyria, as for instance, barbiturates, steroid hormones and chlorinated insecticides. (De Matteis, 1967). In addition to this, induction of cytochrome



Flavoprotein = NADPH Cytochrome C Reductase

Fig. 11. Proposed Scheme for the Oxidation of Drugs by the Mixed Function Oxidases of the Hepatic Endoplasmic Reticulum.

P-450 and induction of experimental porphyria seem to have other factors in common. Thus both are induced by a wide variety of chemical structures and highly lipid soluble compounds (De Matteis, 1967) and both are more easily induced in fasted than well fed animals (De Matteis, 1967). The induction of each is commonly associated with an increased excretion of ascorbic acid (De Matteis, 1964). Both systems are unresponsive to inducing agents in fetal and newborn mammals (Hart et al., 1962; Song et al., 1968), and the induction of both is suppressed by the administration of hemin (Marver, 1969).

Granick (1966) has postulated the operation of a detoxifying mechanism in drug-induced experimental porphyria. In this scheme (Fig. 12) the drug induces the synthesis of ALA-synthetase, resulting in increased heme synthesis. This heme is then used as the prosthetic group of a cytochrome P-450. Induction of ALA-synthetase would thus aid in the elimination of the inducing chemical.

It would therefore follow from this hypothesis that all drugs capable of inducing experimental porphyria should cause an increase in the amount of cytochrome P-450. With this hypothesis in mind we have tested porphyria-inducing drugs to determine their effects on microsomal drug metabolism and cytochrome P-450. The results of these studies are reported in Chapters III and IV.

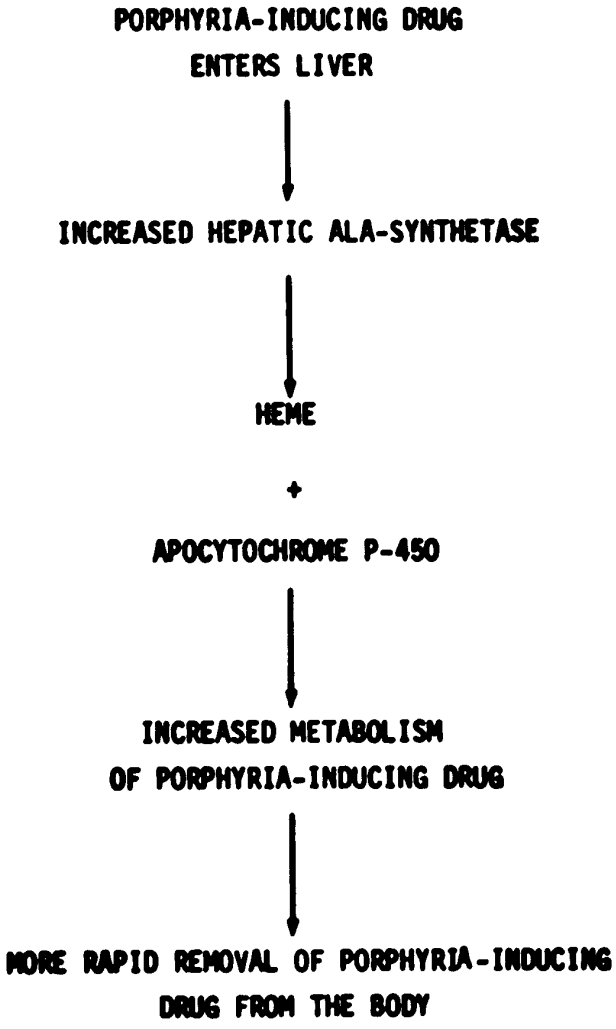


Fig. 12. Granick's Postulated "Detoxifying" Mechanism of Drug-Induced Experimental Porphyria.

CHAPTER II SPECIES, SEX AND DEVELOPMENTAL DIFFERENCES IN THE GENERATION OF EXPERIMENTAL PORPHYRIA

Introduction

Experimental porphyria has been induced in a number of species following drug administration. AIA and DDC are the most widely employed drugs for this purpose, and both are active in most species tested. A number of clinically useful drugs have been associated with provocation and aggravation of the human disease. Many of the drugs which aggravate human porphyria do not cause experimental porphyria when tested in most common laboratory animals. For instance, Goldberg (1954, 1969) found that of nine barbiturates implicated in precipitating these diseases only three caused a marked increase in urinary porphyrin excretion when administered to rabbits, while the remaining six caused little or no increase. A number of drugs which precipitate human porphyria cause accumulation of porphyrins in chick embryo liver cells grown in culture (Granick, 1964, 1965, 1966) and also in the livers of intact chick embryos (Racz & Marks, 1969). However when these drugs were administered to mice they caused no significant increase in liver porphyrin levels (Racz & Marks, 1969).

These results suggest that the chick embryo is a sensitive test animal for the investigation of drug-induced experimental porphyria, and it was therefore our intention to use the chick embryo in our studies. However, the small amount of liver available from the embryo was a disadvantage and it was of interest to ascertain whether the sensitivity to porphyria-inducing drugs extended to adult chickens. For this reason we have administered a number of porphyria-inducing drugs to 5 to 7 week old chickens and measured liver porphyrin levels.

In view of the marked sensitivity of the chick embryo to a wide variety of porphyria-inducing drugs, it is interesting that mammals tested

at birth are refractory to induction by even the most potent inducers. Song et al. (1968) reported that ALA-synthetase activity could not be increased in rats by the administration of AIA or DDC. The ability of these animals to respond to these drugs gradually increased beginning 10 to 15 days after birth and reaching a peak at about the time the rats were weaned. Woods and Dixon (1970a) reported that in the newborn rabbit, ALA-synthetase is also refractory to induction with DDC. A marked difference in the levels of hepatic ALA-synthetase activity has been reported to exist between adult and perinatal animals. In rat, rabbit and guinea pig embryos the levels of hepatic ALA-synthetase are very high, but rapidly revert to lower adult levels at the time of birth or shortly thereafter (Woods & Dixon, 1970b). To determine if similar developmental differences occur in the chicken, we have determined basal and drug-induced levels of ALA-synthetase in perinatal and adult animals.

Goldberg and Rimington (1962) noted that rabbits which had been treated with AIA or Sedormid could be grouped into two categories according to the amount of porphyrin they excreted. Animals which excreted above 6 mg of uroporphyrin daily were called "good excretors" while animals which excreted below 2 mg of uroporphyrin daily were called "poor excretors". In our experiments with chickens we have also noted a tendency of the liver porphyrin levels to fall into two separate groups. In preliminary experiments in which DDC was given to chickens it appeared that this variability in response might represent a sex difference. For this reason we have carried out a further investigation of the response of chickens of both sexes to induction with DDC.

Experimental

i. Sources of Compounds

DDC was purchased from Eastman Organic Chemicals, Rochester, N. Y. Glutethimide was supplied by Dr. H. Keberle, Forschungslaboratorien, der Ciba Aktiengesellschaft, Pharmazeutische Abteilung, Basel; methsuximide by Parke, Davis & Co., Ann Arbor, Michigan; secobarbital sodium by Eli Lilly & Co., Indianapolis, Indiana; methyprylon by Hoffman La Roche, Montreal; and mephenytoin by Sandoz Pharmaceuticals, Dorval, Quebec. 5β-pregnan-3α-17α-diol 11, 20-dione and 5β-androstan-3,17-dione were purchased from the Sigma Chemical Co., St. Louis, Mo.

ii. Experimental Animals

Fertilized eggs used were of a white Leghorn strain obtained from the University of Alberta Farm or Archers Poultry Farm, Brighton, Ontario. Incubation was at 38^{o} at a relative humidity of 68%. The eggs were automatically rotated through an angle of 90^{o} hourly throughout the period of incubation. The age of the embryo was taken as the number of days from onset of incubation.

Chickens used were either commercial hybrids purchased from Lillydale Farms, Edmonton, or were raised from eggs hatched from our incubator. They were fed Co-op chick starter daily and water was available ad lib. Chickens were 5 to 7 weeks old at the time they were utilized.

Mice used were adult white females, weighing 20 to 30 g, obtained from Taconic Farms, Germantown, N. Y. They were maintained on Purina laboratory chow and water ad lib.

iii. Calibration of Fluorescence Curve

Coproporphyrin I was employed as a fluorescence standard. A stock solution of coproporphyrin I was prepared from its tetramethyl ester by the method of Talman (1958) as modified by Schwartz and described below. An accurately weighed quantity of coproporphyrin I tetramethyl-ester (approx. 0.5 mg) was dissolved in 1 ml of 7.5 N HCL and allowed to hydrolyze overnight. This solution was then diluted to a volume of 100 ml with 1 N HCL to make a stock solution. Dilutions with 1 N HCL were made from this stock solution to produce concentrations in the range of 0.1 to 10 μg/100 ml. The calibration curves obtained using these solutions are shown in Figs. 13 a and b. All fluorometric measurements were made with a Turner Model 110 Fluorometer with a 405 mμ band pass primary filter and a Wratten No. 25 (595 mμ) sharp cut secondary filter.

iv. Extraction and Measurement of Liver Porphyrins

The procedure outlined below is that of Schwartz et al. (1960) as modified by Racz and Marks (1969). A liver sample of approximately 400 mg was weighed and homogenized with 5 ml of ethyl acetate:glacial acetic acid (4:1) in a Potter-Elvehjem homogenizer with a glass pestle. Next the homogenate was centrifuged, and the supernatant was decanted into a separatory funnel. The extraction procedure was repeated twice and the resulting supernatants were added to the separatory funnel. Ten ml of sodium acetate solution (3%) and one drop of 0.1% iodine in ethanol were then added to the separatory funnel containing the combined ethyl acetate-acetic acid extract. After shaking, the aqueous phase was removed and discarded. The organic layer was then washed with another 10 ml of sodium acetate solution and the aqueous layer was again discarded. The organic

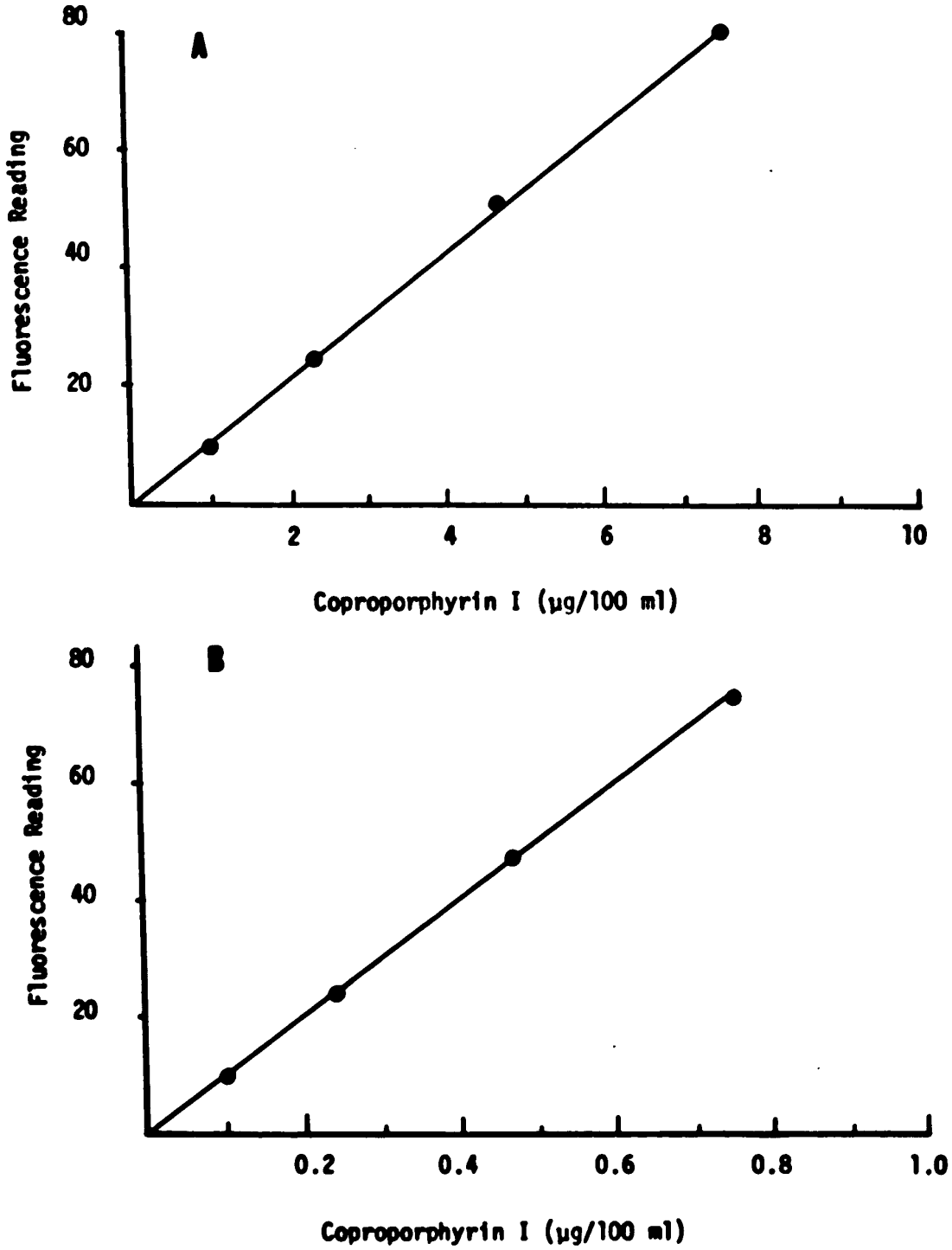


Fig. 13. Porphyrin Standard Calibration Curves; Instrument Sensitivity 3X (A) and Instrument Sensitivity 30X (B).

phase was then extracted 3 times with separate 10 ml portions of 3 N HCL to remove the porphyrins (coproporphyrin and/or protoporphyrin). The combined acid extract was diluted to a volume of 100 ml with water and the porphyrin content determined fluorometrically using 1 N HCL as the reagent blank. Using this method Racz (1970) was able to recover 97% of the coproporphyrin I added to chick embryo liver.

v. Measurement of ALA-Synthetase Activity

a. Preparation of Reagents and Resin

Acetate buffer of pH 4.6 was made by adding 57 ml of glacial acetic acid to 136 g of sodium acetate trihydrate and diluting to one liter with water (Mauzerall & Granick, 1956).

Modified Ehrlich's reagent was made by dissolving one gram of p-dimethylaminobenzaldehyde (DMAB) in approximately 30 ml of glacial acetic acid, adding 8 ml of 70% perchloric acid, and diluting to a final volume of 50 ml with glacial acetic acid (Mauzerall & Granick, 1956).

Dowex-1 acetate was prepared by washing a column of Dowex-1 chloride X8 (200-400 mesh) with 20 bed volumes of 3M-sodium acetate and then batch washing with 50 bed volumes of distilled water (Marver et al., 1966b).

b. Generation of ALA

ALA was generated by the method of Marver et al. (1966a). In this method ALA is generated in liver homogenates with added glycine and endogenously generated succinyl Co-A. The conversion of ALA to PBG is almost completely blocked by the addition of EDTA.

Livers were homogenized in 3 volumes of 0.9% sodium chloride solution containing 0.5 mM EDTA and 10 mM Tris, pH 7.4 in a Potter-

Elvehjem homogenizer with a teflon pestle. The incubation mixture contained 0.5 ml of homogenate, 200 μ moles of glycine, 20 μ moles of EDTA, and 150 μ moles of Tris-HCL buffer at a final pH of 7.2 and in a total volume of 2.0 ml. This mixture was incubated in a 25 ml Erlenmeyer flask, since it has been reported that a flask of this size provides the optimum surface area for aeriation for a volume of 2 ml (Marver et al. 1966). Incubation was for one hour in air at 37^{o}, and the reaction was terminated by the addition of 0.5 ml of 25% trichloracetic acid. The contents of six flasks were pooled and centrifuged. A 10 ml aliquot of supernatant was removed, titrated with sodium hydroxide to a pH range of 4.5 to 6.0, and buffered with an equal volume of sodium acetate buffer, pH 4.6.

c. Measurement of ALA

The quantity of ALA generated in the previous step was measured by condensing it with acetylacetone to form a pyrrole (ALA pyrrole), and measuring the red coloured complex formed between the ALA pyrrole and DMAB (Mauzerall & Granick, 1956) by a spectophotometric method (Fig. 14). However aminoacetone generated in the incubation system from acetyl-CoA and glycine also condenses with acetylacetone to form a pyrrole (aminoacetone pyrrole) which interferes with the determination of ALA (Fig. 14). Moreover some of the ALA might be converted to PBG during incubation and this substance by combining with DMAB (Fig. 14) could interfere with the estimation of ALA. Since in preliminary experiments it was found that insignificant amounts of PBG were present, it was not routinely separated from the mixture. On the other hand aminoacetone was found in sufficient quantity to warrant separation of aminoacetone pyrrole from ALA pyrrole.

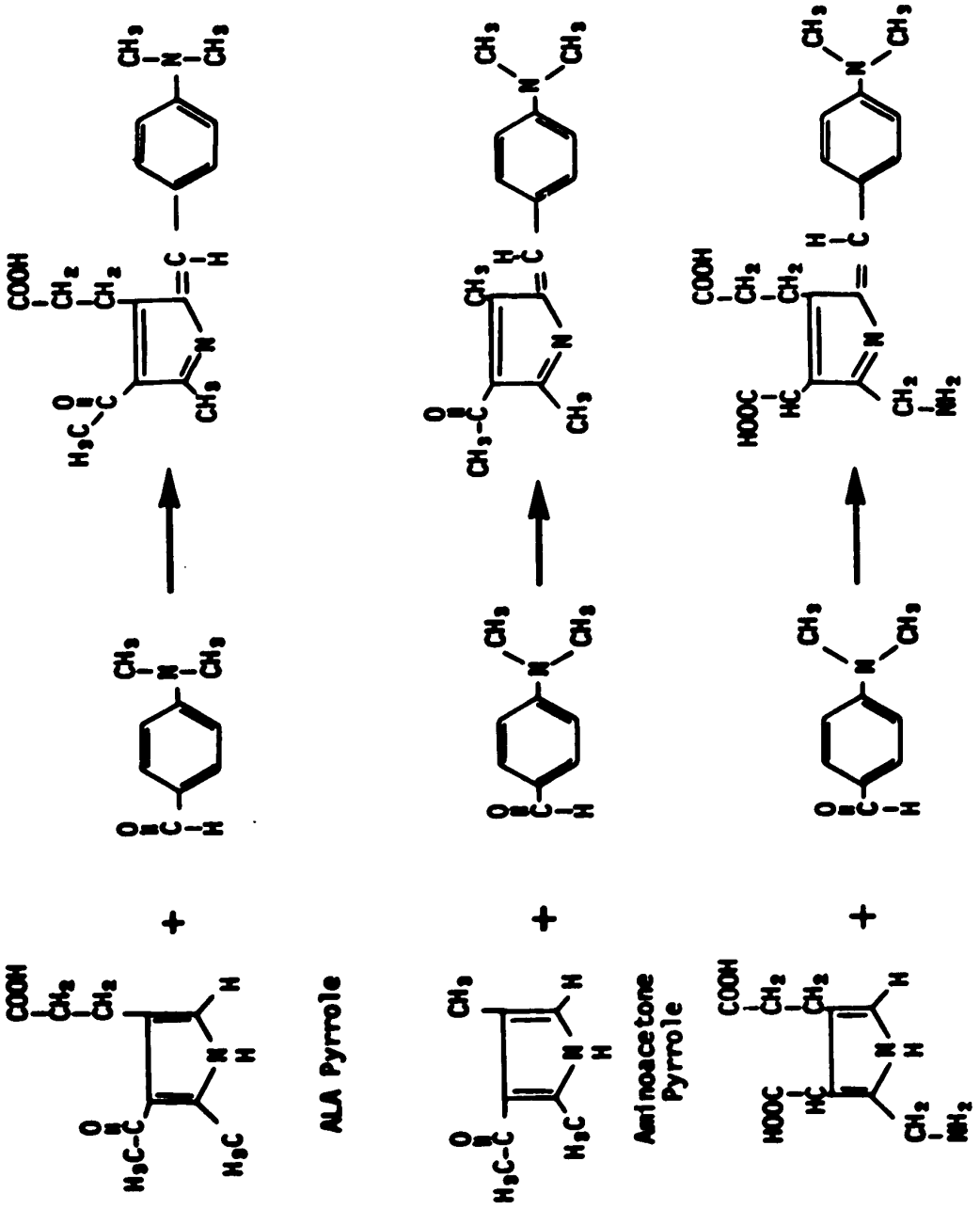


Fig. 14. Reaction of ALA Pyrrole, Aminoacetone Pyrrole and PBG with DMAB.

In order to convert ALA to a pyrrole, 0.5 ml of acetylacetone was added to the buffered TCA supernatants (20 ml) in a 50 ml loosely stoppered flask. The mixture was then heated at 100^{o} for 15 minutes. This procedure also converted the aminoacetone present in the TCA supernatant to a pyrrole and the two were separated by the method described by Marver et al. (1966b) as follows: The solution containing ALA- and aminoacetone-pyrrole was placed on a Dowex-1 acetate column (1 x 7 cm). The aminoacetone-pyrrole was eluted with 10 ml of n-butanol containing 0.01 M ammonium hydroxide and the column was washed with 10 ml of 1 M acetic acid. Finally the ALA-pyrrole was eluted with 10 ml of glacial acetic acid : water : methanol (8:1:2) (Ebert et al., 1969).

The amount of ALA-pyrrole was measured by the method of Mauzerall and Granick (1956). The eluate containing the ALA-pyrrole was mixed with an equal volume of modified Ehrlich's reagent. After waiting twenty minutes for color development, the O. D. of the solution was read in a Unicam SP 600 spectrophotometer at a wavelength of 554 mμ. An equal volume of glacial acetic acid : water : methanol (8:1:2) and modified Ehrlich's reagent was used as a solvent blank. Known amounts of ALA carried through the above procedure gave an ϵ of 5.7×10^{4}. This value is identical with the value obtained by Ebert et al. utilizing the same procedure. If the Dowex column was omitted from the above procedure an ϵ of 6.7×10^{4} was obtained, indicating that approximately 15% of the ALA-pyrrole is lost in passing through the column. An ϵ of 5.7×10^{4} was used to calculate the amount of ALA present in unknown solutions.

vi. Administration of Drugs to Chickens

The compounds to be tested, with the exception of the 5β-ster-

oids, were dissolved in propylene glycol. The 5 β-steroids were dissolved in 10% N, N'-dimethylacetamide in propylene glycol. These solutions (1.5 ml) were injected into the right side of the peritoneal cavity of 5 to 7 week old chickens. Twenty-four hours after drug administration the animals were sacrificed and a liver sample taken for porphyrin estimation.

vii. Administration of Drugs to Chick Embryos

A small hole was made in the egg shell above the air sack. The drug dissolved in the appropriate solvent was then injected through this hole and the underlying membrane into the fluids surrounding the embryo. The injection was made by means of a sterile 1 inch 22 guage needle attached to a 2 ml micrometer syringe. The hole in the shell was sealed with a drop of molten paraffin and the eggs were returned to the incubator.

viii. Induction of Porphyrin Accumulation in Chick Embryo Livers

The 5 β-steroids (10 mg) dissolved in 10% N, N'-dimethylacetamide in propylene glycol (0.2 ml) were injected into the fluids surrounding 16 day old chick embryos and the embryos were returned to the incubator. The embryos were sacrificed 24 hours after drug administration and the livers removed for porphyrin estimation.

ix. Induction of ALA-Synthetase in Chick Embryo Liver

5 β-pregnan-3α-17α-diol 11,20-dione (10 mg) dissolved in 10% N, N'-dimethylacetamide in propylene glycol (0.2 ml) was injected into the fluids surrounding 16 day old chick embryos and the embryos were returned to the incubator. The embryos were sacrificed 12 hours after drug administration and the livers were removed for determination of ALA-synthetase

activity. In order to provide sufficient tissue the livers of three embryos were pooled for each determination.

x. Induction of ALA-Synthetase in Livers of Mice

The drug dissolved in corn oil (0.2 ml) was injected intraperitoneally into mice. Twelve hours following drug administration the animals were sacrificed and a sample of liver (approx. 1.5 g) was removed for estimation of ALA-synthetase activity.

xi. Induction of ALA-Synthetase in Livers of Chickens

DDC (250 mg/Kg) dissolved in propylene glycol was injected intraperitoneally into 3 day old and 5 to 7 week old chickens. The younger chickens received 0.3 ml of solution and the older approximately 1.5 ml of solution. The animals were sacrificed 12 hours after drug administration and a liver sample (1.5 g) was taken for estimation of ALA-synthetase activity.

xii. Effect of Sex on ALA-Synthetase Induction and Porphyrin Accumulation

Male and female chickens each received DDC (250 mg/Kg) dissolved in propylene glycol (1.5 ml) by intraperitoneal injection. The animals were sacrificed 12 hours following DDC administration. A liver sample of approximately 1.5 g was taken from each animal for measurement of ALA-synthetase activity and a liver sample of approximately 0.4 g was taken for porphyrin estimation.

Results and Discussion

Our first objective was to determine if drugs that cause porphyrin

accumulation in chick embryos do the same in 5 to 7 week old chickens. For this reason the following drugs which were previously shown to induce porphyrin accumulation in the chick embryo (Racz & Marks, 1969) viz., DDC, Glutethimide, Methsuximide, Secobarbital, Methyprylon and Mephenytoin were selected for study. The results in Table I show that these compounds are also active in 5 to 7 week old chickens. DDC was found to be the most potent of the drugs tested. This is in agreement with results in chick embryo where DDC produced an accumulation of liver porphyrins greater than any other compound tested (Racz & Marks, 1969). It is of interest that DDC was the only compound in this series that exhibited activity in mice (Racz & Marks, 1969).

On the other hand when the 5β-steroids, 5β-pregnan-3α-17α-diol 11, 20-dione and 5β-androstan-3,17-dione, were administered to adult chickens, they produced little or no increase in hepatic porphyrins (Table I). This seemed to reflect a difference in response to these compounds between embryonic and 5 to 7 week old chickens, since Kappas et al. (1968) have found that hepatic ALA-synthetase activity was greatly increased in the chick embryo following administration of these 5 β-steroids. However a definite conclusion could not be drawn on the basis of this evidence for the following reason: Kappas et al. had used ALA-synthetase induction as an indicator of drug activity. On the other hand we measured porphyrin accumulation and assumed that this provided a measure of ALA-synthetase activity and hence of the ability of drugs to induce increased levels of this enzyme. This assumption was based on previous studies of Granick that led him to conclude that it was a reasonable assumption that porphyrin accumulation could be equated with the synthesis of ALA-synthetase (Granick,

TABLE I. PORPHYRIN ACCUMULATION IN THE LIVERS OF CHICKENS FOLLOWING DRUG ADMINISTRATION

Compound	Dose (mg/Kg)	Liver Porphyrin* (µg/g liver)		No. of Animals
Untreated	-	0.13	(0.09 - 0.17)	5
Solvent Control A (propylene glycol)		0.14	(0.11 - 0.19)	6
Solvent Control (10 % N,N'- dimethyl-acetamide in propyl-ene glycol)		0.18	(0.13 - 0.26)	5
DDC	50	53.0	(1.1 - 107.4)	5
Glutethimide	160	11.4	(0.15 - 21.2)	5
Methsuximide	150	28.6	(.93 - 86.4)	5
Secobarbital Sodium	50	0.11	(0.08 - 0.16)	5
	100†	18.0	(12.4 - 23.7)	2
Methyprylon	400	5.4	(0.12 - 18.8)	5
Mephenytoin	200	17.2	(0.37 - 30.6)	5
5β-Pregnan-3α-17α-Diol 11,20-Dione	100	0.15	(0.08 - 0.27)	5
	400	0.33	(0.13 - 1.00)	10
5β-Androstan-3-17-Dione	100	0.14	(0.07 - 0.21)	5
	200	2.2	(0.23 - 10.1)	5

* Expressed as µg coproporphyrin I/g wet weight liver. The extreme values are given in parentheses.

† Only two of five animals survived this dose.

1966). To clarify the difference between our results in adult chickens and the results of Kappas et al. in embryos we next measured hepatic porphyrin levels in chick embryos following administration of the above two 5 β-steroids. These compounds caused only small increases in hepatic porphyrin levels in chick embryos (Table II), showing that the responses of chick embryos and adult chickens to these steroids are similar.

Thus the 5 β-steroids caused a marked elevation of ALA-synthetase levels in chick embryos (Kappas et al. 1968), while our results show that the increased enzyme activity is not accompanied by a parallel porphyrin accumulation. If these observations are correct then the assumption that porphyrin accumulation can be equated with the synthesis of ALA-synthetase may be incorrect. However the possibility remained that our inability to induce porphyrin accumulation in chick embryo livers reflected a difference in experimental technique or in the responsiveness of the embryos employed, between our laboratory and that of Kappas et al. (1968). This seemed possible in view of the fact that 5 β-steroids are among the most potent drugs in causing porphyrin accumulation in chick embryo liver cells grown in culture (Granick & Kappas, 1967).

In order to clarify this point we administered 5β-pregnan-3α-17α-diol 11,20-dione to chick embryos and measured hepatic ALA-synthetase levels rather than porphyrin accumulation. This compound was shown to be an active inducer of ALA-synthetase, causing a 35 fold rise in the level of the enzyme (Table III). This rise was comparable in magnitude to the rise in ALA-synthetase level reported by Kappas et al. (1968) who obtained a 40 fold increase. From the above data it seems clear that administration of 5 β-steroids causes large increases in hepatic ALA-synthetase activity with little concomitant rise in hepatic porphyrin content. Such a large

TABLE II. EFFECT OF STEROIDS ON PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER*

Treatment	Porphyrins (µg/g liver)	Mean
Control (solvent only)†	0.26 0.17 0.17 0.12 0.29	0.20
5β-Pregnan-3α17α-Diol 11,20-Dione (10 mg/egg)	0.69 1.6 0.97 1.1 0.50	1.0
5βAndrostan-3,17-Dione (10 mg/egg)	1.5 0.28 0.51 0.51 0.37	0.6

* Livers were removed 24 hours after steroid administration.

† The steroids were dissolved in propylene glycol containing 10% N,N'-dimethylacetamide (0.2 ml).

TABLE III. INDUCTION OF ALA-SYNTHETASE IN CHICK EMBRYO LIVER BY A 5β STEROID

Treatment	ALA-Synthetase† (mμmoles/g/hr)	Mean ± 1 S.E.M.
5β-Pregnan-3α-17α-Diol 11,20-Dione (10 mg/egg)	642 321 440 785 1107 643	656 ± 112
Control (solvent only)*		19 ± 1.2

* The steroid was solubilized in propylene glycol containing 10% N,N'-dimethylacetamide (10 mg/0.2 ml). The control value is the mean of six determinations.

† Embryos received the steroid 12 hours prior to sacrifice. Three livers were pooled for each determination.

rise in ALA-synthetase with a small increase in porphyrin levels has not been previously reported and was of special interest because current procedures suggested for screening drugs for porphyria-inducing activity are based upon measurement of porphyrin accumulation (Granick, 1964; Racz & Marks, 1969) and not ALA-synthetase activity. This point will be further discussed in chapter IV.

With this example in mind it occurred to us that there might be other circumstances under which hepatic ALA-synthetase is induced without causing appreciable porphyrin accumulation. For instance such a separation of ALA-synthetase induction from porphyrin accumulation might explain the failure of Racz and Marks (1969) to show any porphyria-inducing activity of drugs administered daily for 14 days to mice, although these drugs were quite active in chick embryos. Since Racz and Marks measured only porphyrin accumulation it was possible that these drugs caused an increase in ALA-synthetase activity in both species, but porphyrins were formed only in chick embryo liver and not in mice.

In order to test this hypothesis we administered three of these drugs to mice in the same dose as used by Racz and Marks, and measured ALA-synthetase activity rather than porphyrin accumulation. It was found (Table IV) that two of the drugs, glutethimide and methyprylon, caused a significant increase in ALA-synthetase activity over control values within 12 hours of drug administration, but the third, methsuximide did not. Thus the direct measurement of ALA-synthetase activity is a much more sensitive index of drug activity than the indirect measurement of the enzyme by means of porphyrin accumulation. It is worth stressing that porphyrins were not detected in the livers following 14 days of consecutive drug administration while ALA-synthetase levels were elevated 12 hours after the

TABLE IV. INDUCTION OF ALA-SYNTHETASE IN MICE*

Treatment	Dose (mg/kg)	ALA-Synthetase Activity † mμmole ALA/g/hr.
Control (solvent only)	--	74 ± 5
Glutethimide	200	143 ± 10
Methyprylon	600	140 ± 14
Methsuximide	150	81 ± 8

* The drugs (dissolved in 0.2 ml of corn oil) were injected intraperitoneally. The animals were sacrificed 12 hours later and approximately 1.5 grams of liver was removed for ALA-synthetase determination.

† Values are expressed as means ± 1 S.E.M.

first drug administration.

It is interesting to consider why these drugs do not cause porphyrin accumulation despite the fact that they have the ability to cause elevated ALA-synthetase levels. In considering this problem it should be born in mind that porphyrinogens, and not porphyrins, are intermediates in the biosynthesis of heme. Porphyrins are formed in the liver through the irreversible oxidation of porphyrinogens, and are normally found in minute quantities. Since ALA-synthetase is the rate limiting enzyme in the heme biosynthetic pathway, an increase in its activity would increase the amount of heme produced. Only if ALA-synthetase is increased to such an extent that other enzymes in the heme biosynthetic pathway become limiting would porphyrinogens and their oxidation products, porphyrins, accumulate. From the above it can be seen that not only the activity of ALA-synthetase, but the activities of the other enzymes necessary for the conversion of uroporphyrinogen to heme will determine the quantity and type of porphyrins which will accumulate following drug administration. These facts would provide two possible explanations as to why these drugs cause porphyrin accumulation in chick embryo, but not in mice, while elevating ALA-synthetase in both.

First of all the mouse may have a greater capacity for converting the PBG formed as a result of increased ALA-synthetase activity to heme. If this were the case porphyrinogens would not accumulate to be oxidized to porphyrins. Since neither heme production nor the levels of other enzymes involved in heme biosynthesis were measured, it is difficult to assess the importance of this mechanism.

Secondly the drugs may cause a much smaller increase in ALA-synthe-

tase activity in the mouse than the chick embryo. This could come about in two ways: 1. The mechanism controlling levels of ALA-synthetase might be much less sensitive to drugs in the mouse than in the chick embryo. 2. The amount of drug reaching the site of action might be much greater in the chick embryo than in the mouse due to differences in distribution, metabolism and excretion of the drug.

The inability of the drugs to cause a sufficiently large increase in ALA-synthetase would seem likely to provide at least a partial explanation for the lack of porphyrin accumulation. Though two of the drugs tested in mice did cause significant increases in ALA-synthetase, the levels were still somewhat less than doubled. In contrast DDC, which did cause porphyrin accumulation in mouse liver (Racz & Marks, 1969), caused a 9 fold increase in ALA-synthetase activity when administered to mice (Wada et al., 1968).

In recent years increasing interest has been focussed on the levels of drug-metabolizing enzymes in the liver of the newborn and the fetus at different stages of development. The reason for this is the realization that the fetus and newborn are often extremely sensitive to many drugs due to a deficiency in these enzymes. Moreover experiments with interesting therapeutic implications have been conducted to attempt to increase the levels of these enzymes (Conney, 1967). Since the level of ALA-synthetase might be a controlling factor in the level of cytochrome P-450 which in turn is critical for the metabolism of many drugs, the levels of this enzyme and its inducibility at different stages of development are of considerable interest.

In our next series of experiments we investigated the levels and

inducibility of ALA-synthetase in chickens at different stages of development. It was found that control levels of ALA-synthetase (Table V) in 17 day old chick embryos were only 1/3 those of adult chickens. Levels in newborn chicks were found to be intermediate between adult and embryonic levels. This contrasts sharply to changes in ALA-synthetase activity with development in rats, rabbits and guinea pigs reported by Woods and Dixon (1970b). They found levels at ALA-synthetase were much greater in embryos than adults of these three species. For example, in rabbit embryos one week before delivery ALA-synthetase was approximately 35 times higher than adult levels. In this species ALA-synthetase levels declined rapidly and reached normal adult levels at birth or shortly thereafter.

Dixon and Woods (1970b) have speculated that the "physiologic" jaundice which occurs in most newborn animals may be related to elevated hepatic ALA-synthetase levels. Since jaundice also occurs in embryonic and newborn chickens (Needham, 1963), at a time when we have shown ALA-synthetase activity is lower than adult levels it would seem unlikely that this suggestion is correct.

When DDC was administered to newborn and adult chickens (Table V), large increases in ALA-synthetase activity were measured. The induction in newborn and adult chickens caused by the same dose of DDC (250 mg/Kg) were of similar magnitude. In contrast it has been reported that newborn rabbits are much less responsive than adult rabbits to induction with the same dose of DDC (250 mg/Kg) (Woods & Dixon, 1970a) and newborn rats are completely refractory to AIA and DDC administration (Song et al., 1968).

In the experiment just described, in which DDC was administered to adult chickens, it was noted that from a group of six, three livers ob-

TABLE V. INDUCABILITY OF ALA-SYNTHETASE* IN CHICKENS

Treatment	Embryo	Newborn	Adult
None	16 ± 0.7	37 ± 3	49 ± 3
Control (solvent only)†	-	38 ± 5	54 ± 3
DDC 250 mg/kg	-	795 ± 312	627 ± 187

* ALA-synthetase activity is expressed as mμmoles ALA/g/hr. All values are the means of six determinations ± 1 S.E.M.

† Propylene glycol was used to dissolve the DDC. 0.2 ml of propylene glycol were administered to newborn chickens while 1.4 to 1.6 ml were administered to adult chickens.

tained from female chickens had higher ALA-synthetase activity than the three from male chickens. On this basis it appeared likely that the sex of the animal influenced the drug response, though the number of animals was too small to be certain. In order to determine if the sex of the chicken was in fact important, we administered the same dose of DDC (250 mg/Kg) to a larger group of chickens (6 male; 6 female). The results in Table VI indicate that there was no difference between sexes in this group. The higher ALA-synthetase levels of females of the earlier group was probably coincidental.

TABLE VI. EFFECT OF SEX ON ALA-SYNTHETASE INDUCTION AND PORPHYRIN ACCUMULATION IN 6 WEEK OLD CHICKENS*

Sex	Treatment	Porphyrin (µg/g Liver)	ALA-Synthetase (mµmoles/g/hr)	ALA-Synthetase (mean)
F	DDC 250 mg/kg	0.40	30	
F	"	30.4	452	
F	"	2.3	262	207
F	"	1.03	36	
F	"	3.6	214	
F	"	2.8	250	
M	"	2.8	202	
M	"	0.27	30	
M	"	46.3	571	223
M	"	2.9	167	
M	"	1.5	95	
M	"	31.0	274	

* Six week old chickens were injected intraperitoneally 12 hours prior to sacrifice, and liver samples removed from each for porphyrin estimation and determination of ALA-synthetase activity.

CHAPTER III THE EFFECT OF PORPHYRIA-INDUCING DRUGS ON N-DEMETHYLASE ACTIVITY OF CHICK EMBRYO LIVER

Introduction

The administration of phenobarbital and a variety of other drugs to experimental animals leads to an increase in liver microsomal drug-oxidizing activity which is paralleled by an increase in the amount of microsomal cytochrome P-450 (Remner & Mohr, 1965; Orrenius & Ernster, 1964; Conney, 1967). This cytochrome plays an important role in drug oxidation, and it has been suggested that the increased drug-oxidizing activity depends mainly on increased synthesis of this cytochrome (Remner & Mohr, 1965). Since phenobarbital and a variety of other drugs induce the formation of ALA-synthetase in liver mitochondria with a concomitant increase in porphyrin synthesis, Granick (1966) suggested the following sequence of events in response to a porphyria inducing drug. Derepression of ALA-synthetase in mitochondria leads to increased porphyrin and heme formation. The heme is utilized for the prosthetic group of cytochrome P-450 so that an increased amount of cytochrome is available and hence an increased level of drug oxidizing activity. The object of the experiments reported in this chapter was to test the validity of this hypothesis.

From this hypothesis is would follow that the presence of a drug-oxidizing system would be a prerequisite for the induction of porphyria in an experimental animal. In view of the fact that Granick's studies were carried out in chick embryo liver cells, which were responsive to porphyria-inducing drugs from the time the embryo was 9 days old, our first experiments were directed to demonstrating the presence of a drug-oxidizing system in these livers at this stage of development. In studies with several mammalian species the fetal livers have been shown to be lacking in the ability to oxidize drugs (Fouts & Adamson, 1959; Brodie &

Maickel, 1961; Dallner, et al. 1966). In contrast, Brodie and Maickel (1961) have reported that drug-oxidation can be demonstrated in the chick embryo liver after 16 days of development. The chick embryo has a special problem in disposing of lipid soluble substances since it is confined in a relatively impermeable shell. Brodie and Maickel (1961) have theorized that the drug-oxidizing system is present in the chick embryo in order to metabolize lipid-soluble waste material such as skatoles and indoles to water-soluble derivatives, which can be stored until hatching. On the other hand mammalian fetuses would have no need for this system since this function could be performed by the maternal liver.

It would also follow from the above hypothesis of Granick that administration of porphyria-inducing drugs should cause an increase in drug-oxidizing activity in chick embryo liver. It has been reported that drug-oxidizing activity cannot be stimulated in rabbit liver before the last four days of fetal life (Hart et al., 1962), but no information was available regarding the inducibility of this activity in the chick embryo. Our second series of experiments was directed to determining whether this drug-oxidizing activity could be induced in chick embryo with porphyria-inducing drugs.

In our third series of experiments we sought to determine the effect of hemin (ferriheme chloride) on the induction of hepatic drug metabolizing enzymes by porphyria-inducing drugs. Granick (1966) has shown that hemin prevents the induction of porphyria by these drugs and suggested that it acted as a co-repressor and prevented ALA-synthetase induction. If porphyria-inducing drugs cause increased drug-oxidizing activity by increasing the supply of heme for cytochrome P-450 formation, then the following consequences should follow from the administration of hemin together with a porphyria-inducing drug: (1) porphyria induction would be prevented and

(2) an increase in drug-oxidizing activity should occur since the quantity of hemin administered should provide heme for synthesis of cytochrome P-450. Hemin was used rather than heme because of the following considerations: Burnham and Lascelles (1963) have shown that hemin and heme are essentially equal in their ability to inhibit ALA-synthetase. For this reason and because of the technical difficulties involved in keeping heme in the reduced state, hemin was used.

In our fourth series of experiments, a positive correlation was sought between the ability of DDC and several of its analogues to induce an increased level of drug-oxidizing activity in liver and their porphyria-inducing activity. As an approximate index of the level of drug-oxidizing activity in the liver, the oxidative N-demethylation of aminopyrine was measured.

Experimental

i. Source of Compounds

DDC analogues employed in this study were synthesized in our laboratory by U. K. Terner and G. L. Bubbar (Marks et al. 1965).

ii. Determination of Aminopyrine N-demethylase Activity

In the demethylation of aminopyrine to 4-aminoantipyrine by liver microsomes two molecules of formaldehyde are formed (Fig. 15). The measurement of formaldehyde formed during incubation thus provides a means of measuring the rate of aminopyrine N-demethylation. The formaldehyde formed was first trapped as the semicarbazone by the addition of semicarbazide to the incubation mixture. It was then converted to 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 16) by reaction with acetylacetone

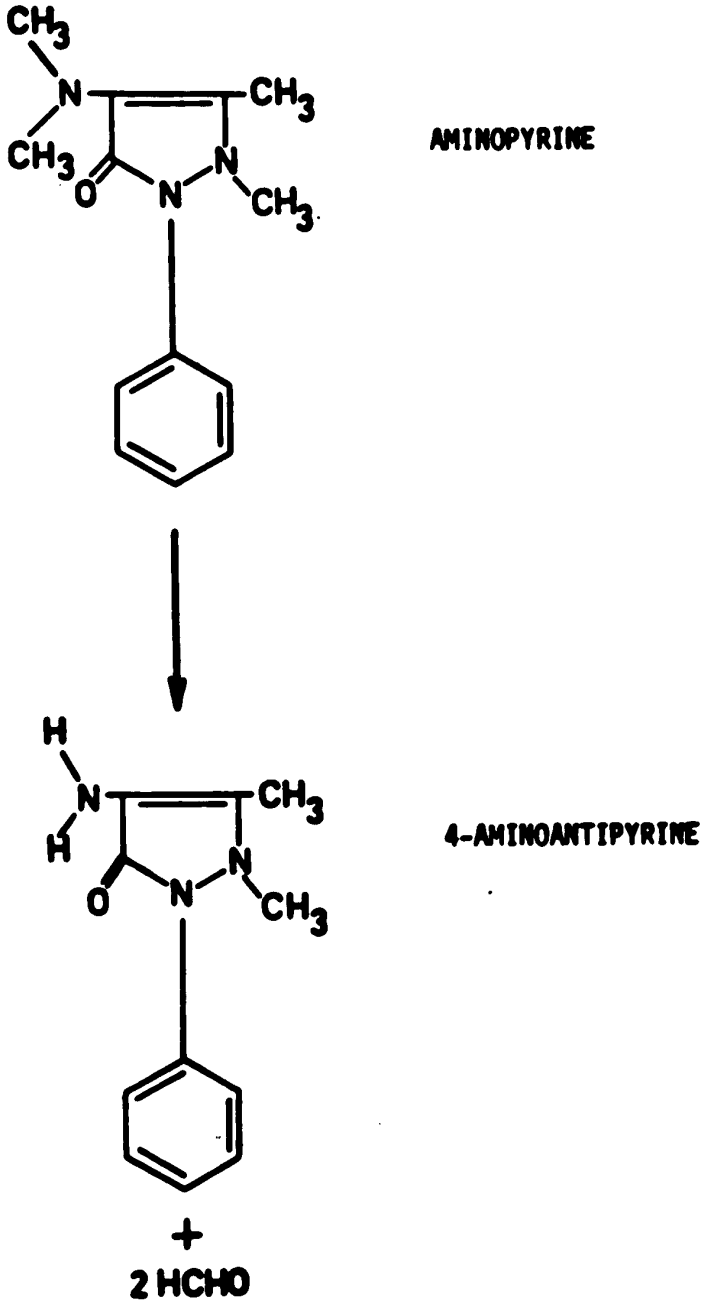


Fig. 15. The N-demethylation of Aminopyrine to 4-aminoantipyrine.

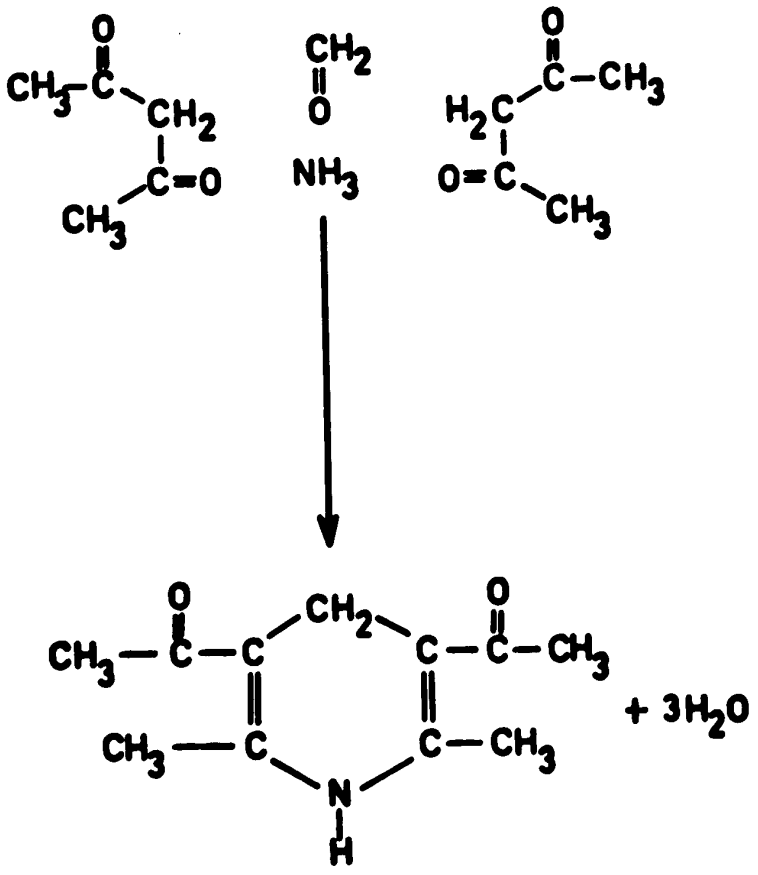


Fig. 16. Measurement of Formaldehyde by the Formation of 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine.

in the presence of an excess of ammonium salt (Nash, 1953). The amount of 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine was measured spectrophotometrically by its absorption at 415 mμ.

a. Preparation of Reagents

Nash reagent was prepared on the day of use by mixing 0.1 ml of acetyl acetone with 25 ml of a 30% ammonium acetate solution (Cochin & Axelrod, 1959).

Buffered potassium chloride solution was prepared by combining 900 ml of potassium chloride solution (1.15%) with 100 ml of potassium phosphate buffer (0.1 M), pH 7.4.

b. Preparation of 9,000 g Supernatant Fraction

The embryos were killed by decapitation and the livers immediately removed and homogenized in the cold with a Potter-Elvehjem apparatus with a teflon pestle in 5 volumes of buffered potassium chloride solution. The homogenate was centrifuged at 9,000 g for 20 minutes at 4^{0} to remove unbroken cells, nuclei and mitochondria. Taking care not to disturb the fatty material which is present on the surface or the pellet, the 9,000 g supernatant fraction, which contains the microsomes, was removed by means of a Pasteur pipet.

c. Incubation of Liver 9,000 g Supernatant Fraction With Aminopyrine

The incubation mixture used was as follows: the 9,000 g supernatant fraction obtained from 0.4 g of liver was incubated with 5.0 μ moles aminopyrine, 0.6 μ moles NADP, 6.0 μ moles glucode 6-phosphate, 50 μ moles nicotinamide, 45 μ moles semicarbazide hydrochloride, 25 μ moles $MgCl_2$ and 3 ml of 0.1 M potassium phosphate buffer, pH 7.4 in a total volume

of 6 ml. Incubation was for 1 hour at 37° in air. NADP and glucose 6-phosphate were added in order to generate NADPH by a reaction catalyzed by glucose 6-phosphate dehydrogenase found in the 9,000 g supernatant. Nicotimamide prevents the destruction of NADP by NADPase present in the 9,000 g supernatant. $MgCl_2$ was added to provide magnesium ion, which is essential for glucose 6-phosphate dehydrogenase activity.

d. Measurement of Formaldehyde Formed

The amount of formaldehyde formed during incubation was measured by the method of Nash (1953) as modified by Cochin and Axelrod (1959). Incubation was terminated by the addition of 2 ml of zinc sulphate solution (20%) followed by 2 ml of a saturated solution of barium hydroxide. This mixture was centrifuged and a 5 ml aliquot of clear supernatant was removed. Two ml of Nash reagent were added to this 5 ml aliquot, and the solution was placed in a water bath at 60° for 30 minutes. Upon cooling the absorbance at 415 mμ was measured against a blank prepared by the procedure outlined above, but omitting aminopyrine from the incubated mixture. The amount of formaldehyde was determined from a standard curve (Fig. 17) prepared by determining the absorbance of known amounts of formaldehyde by this procedure.

iii. Determination of Microsomal Protein Content

a. Preparation of Reagents

Folin reagent was prepared by diluting 5 ml of Folin and Ciocalteu Phenol Reagent (British Drug Houses) with 50 ml of distilled water.

Copper reagent was prepared by combining 1 ml of copper sulphate solution (1%) and 1 ml of sodium potassium tartrate solution (2%)

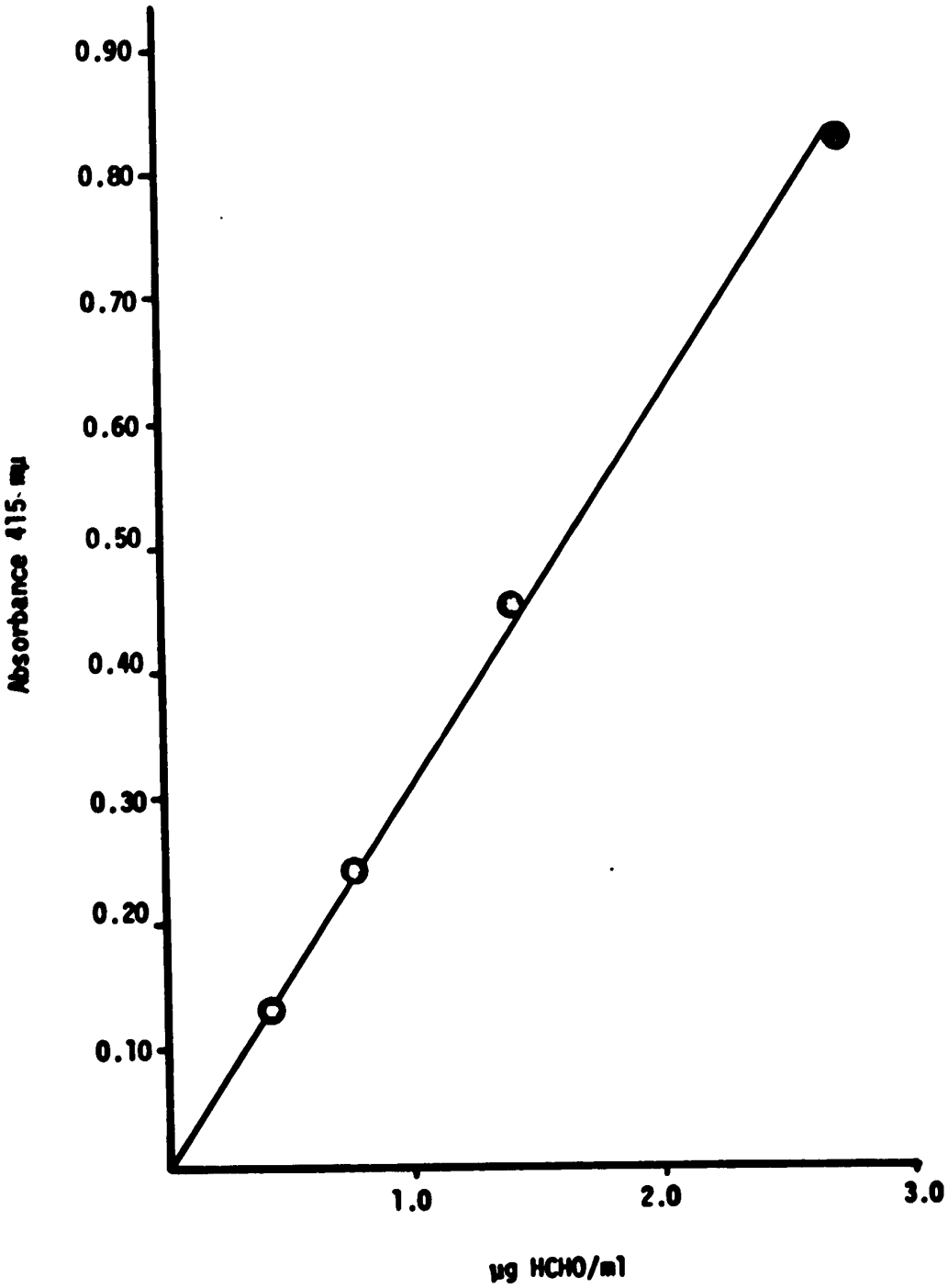


Fig. 17. Standard Calibration Curve for Determining the Amount of Formaldehyde Present in Unknown Solutions.

with 20 ml of sodium carbonate (10%) in 0.5 M NaOH.

b. Isolation of Microsomes

A 1 ml aliquot of 9,000 g supernatant fraction prepared as described above was routinely removed for determination of microsomal protein. The sample was diluted to a volume of 6 ml with buffered potassium chloride solution, placed in a centrifuge tube and centrifuged at 100,000 g at 4^{0} for a period of 1 hour. The supernatant fluid was discarded and the microsomal pellet was resuspended in distilled water using a Potter-Elvehjem homogenizer with teflon pestle. The resuspended microsomes were diluted to a final volume of 50 ml and 1 ml samples removed for protein determination.

c. Measurement of Microsomal Protein

The method outlined below is that of Lowry et al. (1951) as modified by Miller (1959). Copper reagent (1 ml) was added to the microsomal sample (1 ml) prepared as described above. The resulting solution was mixed and allowed to stand for 10 minutes at room temperature. Folin reagent (3 ml) was added and the mixing was repeated. The solution was heated in a water bath at 50^{0} for 10 minutes. The absorbance of the blue colored complex formed under these conditions was measured at 540 mμ. The amount of protein present was estimated from a standard curve (Fig. 18) prepared by carrying known amounts of bovine serum albumin through the above procedure.

iv. Administration of Drugs to Chick Embryos

Phenobarbital sodium and AIA dissolved in water (0.1 ml) were injected through the membrane of the air sack into the fluids surrounding

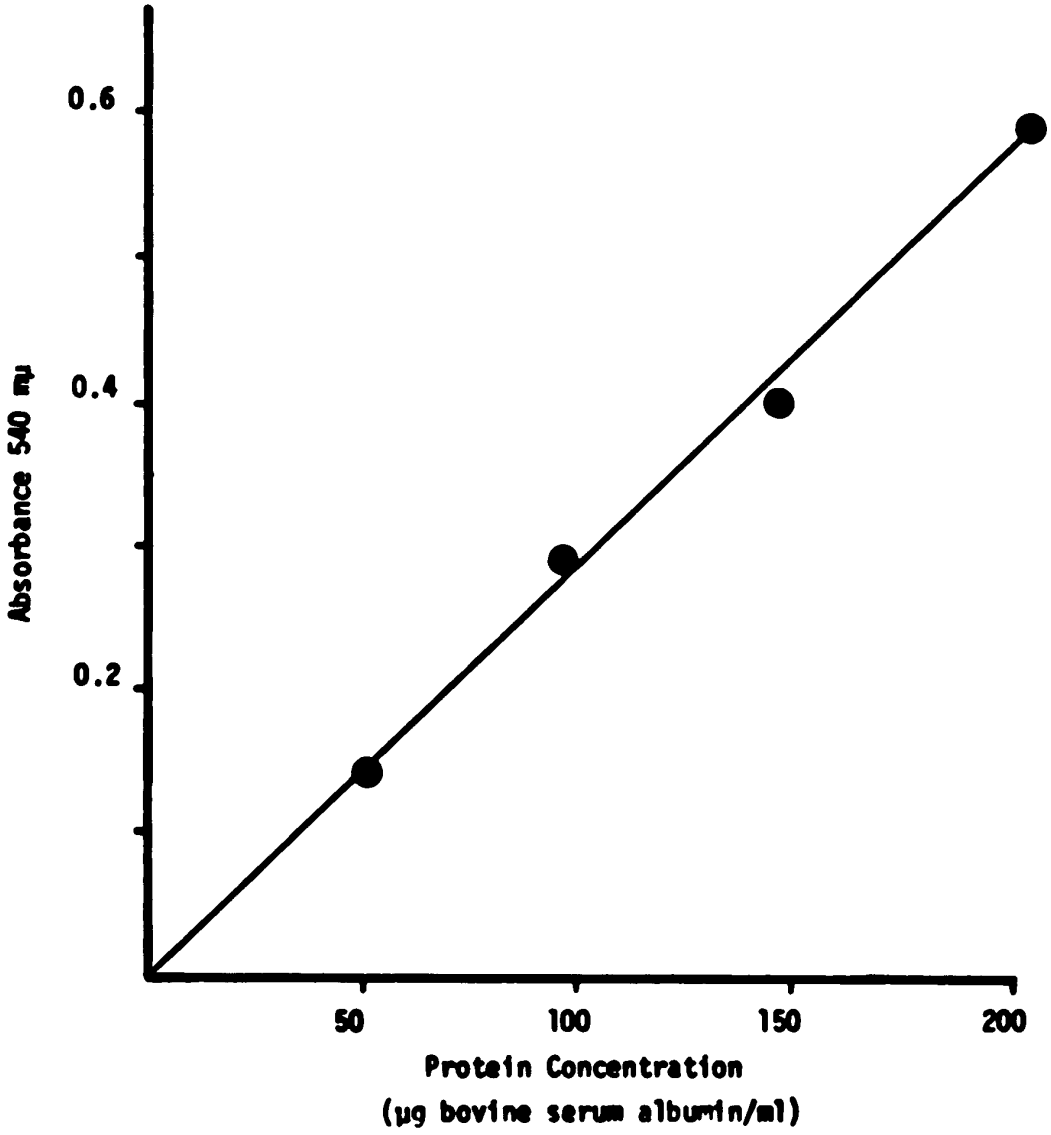


Fig. 18. Protein Calibration Curve.

the embryo as described in chapter II. DDC and its analogues were dissolved in 95% ethanol (0.01 ml) and injected in the same manner. Drugs were injected daily on days 15, 16 and 17 of development. The embryos were sacrificed on day 18 of development and the livers removed for determination of aminopyrine N-demethylase activity. Controls were carried out in which the solvent alone was injected.

v. Addition of DDC and Analogues to the Incubation Mixture

DDC or its analogues, dissolved in 95% ethanol (0.01 ml) were added to the incubation mixture (6 ml) just prior to incubation. Incubation and assay of aminopyrine N-demethylase activity were then carried out in the usual manner. Controls were carried out in which the solvent alone was added.

vi. Effect of Hemin on DDC-Induced Liver Porphyrin Accumulation

DDC or DDC and hemin dissolved in dimethylsulfoxide (0.1 ml) were injected into the fluids surrounding 16 day old chick embryos, and the embryos were returned to the incubator. The embryos were sacrificed 24 hours after drug administration and the livers were removed for porphyrin estimation.

Results and Discussion

The results in Fig. 19 show the presence of considerable amounts of N-demethylase activity, expressed per gram of liver, as early as the twelfth day of development. Moreover, the amount of N-demethylase activity increases steadily with the age of the embryo and continues to increase after hatching. When N-demethylase activity is expressed per mg microsomal

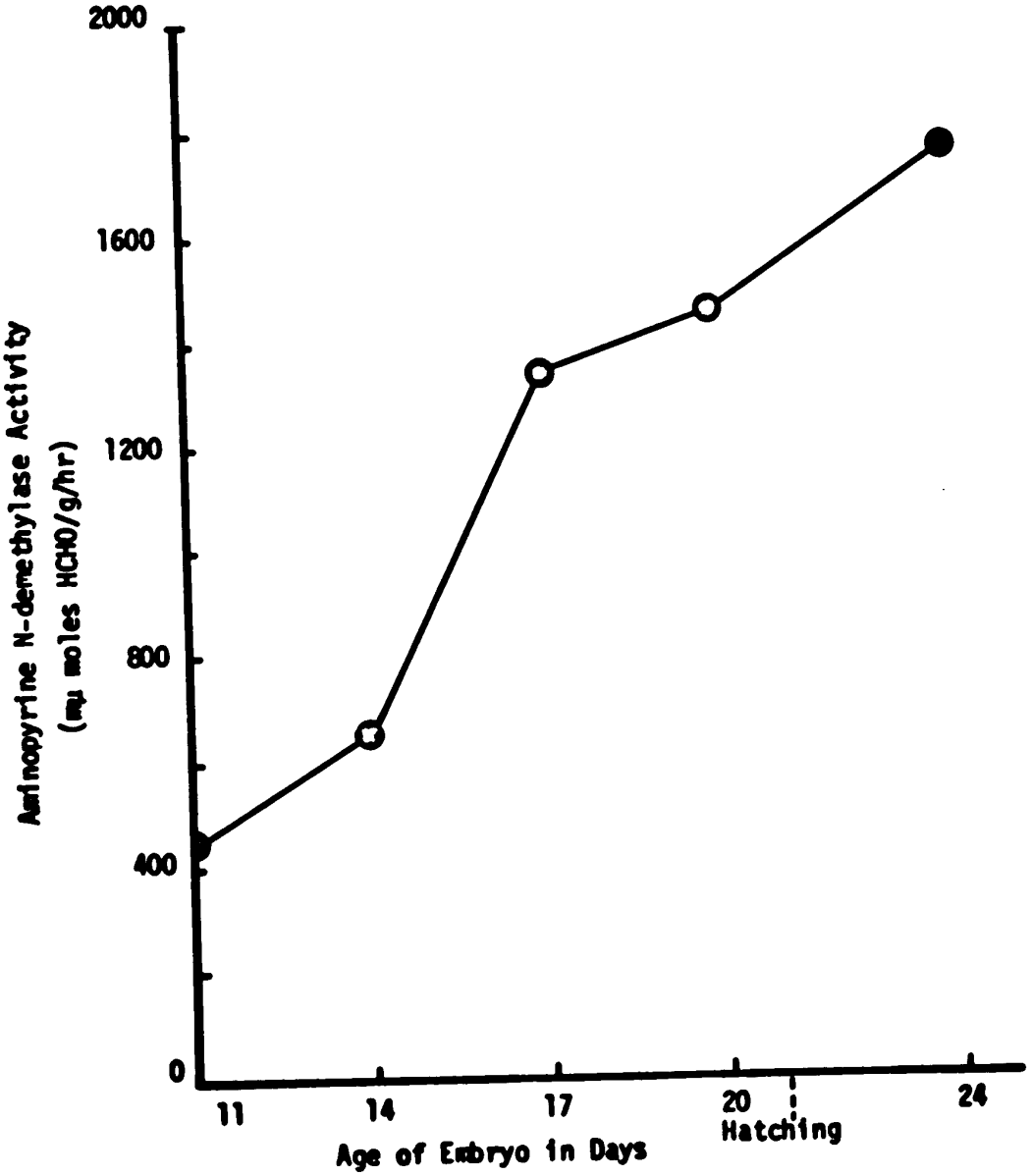


Fig. 19. Changes in N-demethylase Activity at Various Stages of Development of Chick-Embryo Liver Expressed per Gram Liver (Wet Weight). The Averages of the N-demethylase Activity Observed in at Least Three Separate Experiments are Plotted at Each Time Period.

protein (Fig. 20) a similar pattern is seen. However when the results are expressed in this manner, there is a decrease in activity between the 18th and 21st days of development, indicating that N-demethylase activity does not keep pace with the increase in microsomal protein content at this stage of development. Soyka (1969) have reported that the highest value of aminopyrine N-demethylase activity reached in rat liver at different stages of fetal and adult development is 950 mμ moles/g liver. It is of interest that this value is lower than that reached in chick embryo liver at 17 days of development (Fig. 19). Strittmatter and Umberger (1969), who studied the level of N-demethylation of mephobarbital, have reported that activity is present in the 15 day old embryo and increases with age thereafter. Granick had demonstrated porphyria-inducing activity in 9 day old embryos. It was impractical to measure N-demethylase activity at this stage of development so that our first measurements were carried out with 11 day old embryos. The demonstration of the presence of drug-oxidizing activity in 11 day old chick embryo livers is consistent with Granick's interpretation of the sequence of events in response to a porphyria-inducing drug.

Having established the presence of drug-oxidizing activity in the chick embryo liver, our next series of experiments was directed to determining if this enzyme activity could be increased by administration of porphyria-inducing drugs. Phenobarbital was chosen for the first experiments because of its well established ability to stimulate an increase in hepatic drug-oxidizing activity in several species (Conney, 1967). It has also been established that phenobarbital is capable of inducing porphyrin accumulation in chick embryo livers (Racz & Marks, 1969). In Table VII it can be seen that pretreatment with phenobarbital for 3 days causes an

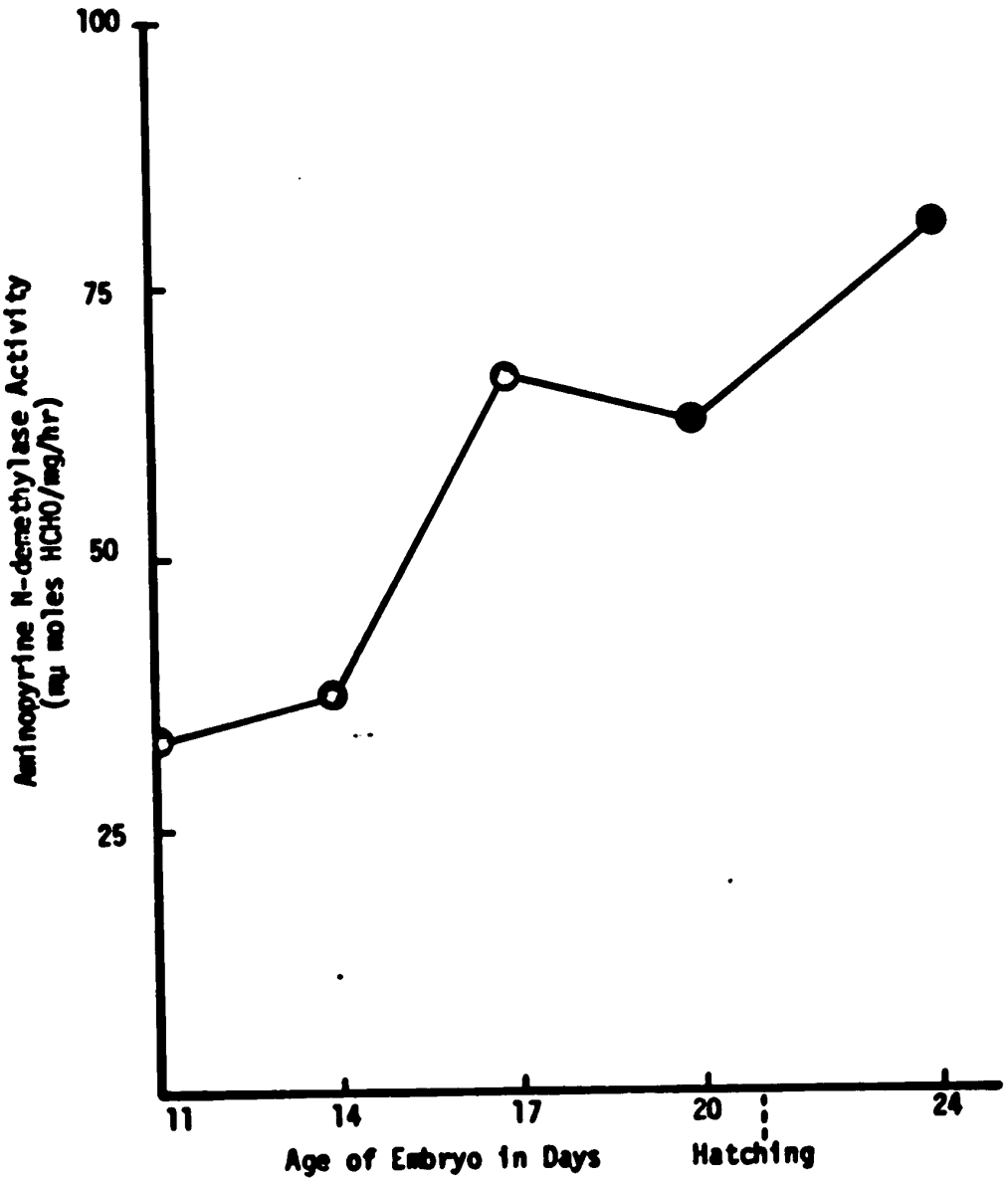


Fig. 20. Changes in N-demethylase Activity at Various Stages of Development of Chick-Embryo Liver Expressed per mg Microsomal Protein. The Average of the N-demethylase Activity Observed in at Least Three Separate Experiments are Plotted at Each Time Period.

TABLE VII. EFFECT OF PHENOBARBITAL AND AIA ON N-DEMETHYLASE ACTIVITY OF CHICK EMBRYO LIVER*

Compound injected in water (0.1 ml) once daily for 3 days	Daily dose (mg/egg)	Liver N-demethylase activity (mμmoles formaldehyde/g liver)	% Control
Control	-	1232	-
Phenobarbital sodium	5	1824	148
Control	-	1465	-
AIA	0.1	885	61
AIA	1.0	2650	180

* The embryos were injected on days 14, 15 and 16 of development and the the enzyme activity was measured on day 17. Controls received the solvent alone. Livers of at least six embryos were pooled for each determination and the above data represent the average of two experiments.

increase in N-demethylase activity. Strittmatter and Umberger (1969) have found that phenobarbital also causes an increase in mephobarbital N-demethylase activity of 18 day old chick embryo liver in animals which were pretreated on days 15 and 17. The potent porphyria-inducing drug AIA, behaved similarly to phenobarbital and caused an increased level of N-demethylase activity (Table VII) when administered in a dose of 1 mg per egg for three days. These observations are in agreement with Granick's interpretation of the sequence of events in response to a porphyria-inducing drug. On the other hand when AIA (1 mg/egg) was administered in a single dose a decrease in aminopyrine N-demethylase activity after six hours was observed (control 860 mμ moles HCHO/g liver/hr, observed value 420 mμ moles/g liver/hr).

A smaller dose of AIA (0.1 mg/egg) caused a decreased level of N-demethylase activity (Table VII) after three days. It is not known whether a dose as small as 0.1 mg per egg will cause porphyrin accumulation in chick embryo liver, while a dose of 1 mg per egg is known to be active (Racz, 1970). The significance of these observations in relation to Granick's hypothesis was unclear at this stage of investigation.

It has been established that hemin administration greatly reduces the porphyrin accumulation caused by AIA or DDC in chick embryo liver cells grown in culture (Granick, 1966; Schneck, 1969). Prior to determining the effect of hemin on the increase in hepatic drug-oxidizing activity caused by porphyria-inducing drugs, it was first necessary to determine the effect of hemin on drug-induced hepatic porphyrin accumulation in the chick embryo. For this reason the relationship between the dose of DDC administered and porphyrin accumulation was determined in the chick embryo liver (Figs. 21, 22, 23). The effect of various doses of hemin on the DDC-induced porphyrin

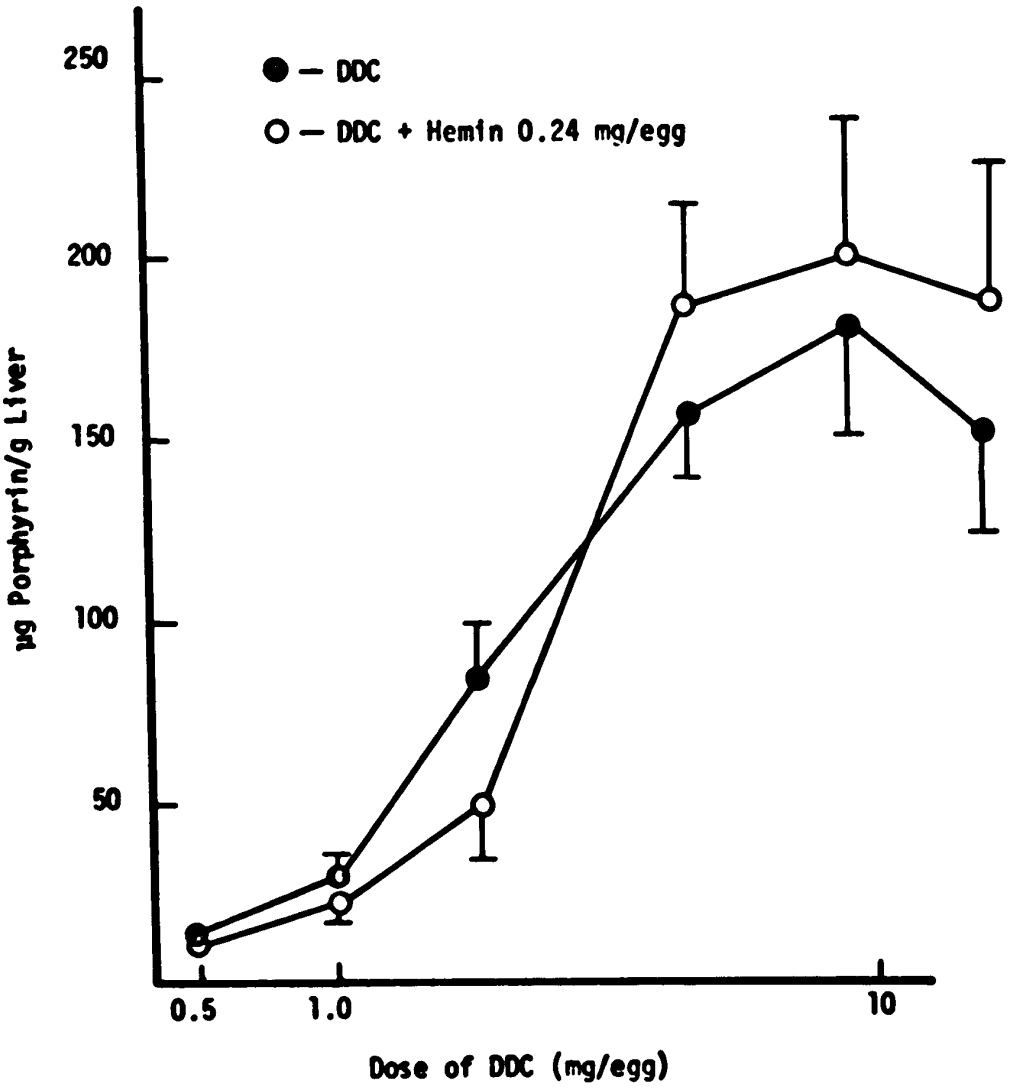


Fig. 21. Effect of 0.24 mg of Hemin per Egg on the Accumulation of Porphyrins in Chick-Embryo Liver Caused by DDC. Each Point Represents the Mean of at Least Five Determinations. The Vertical Bars Represent 1 S.E.M.

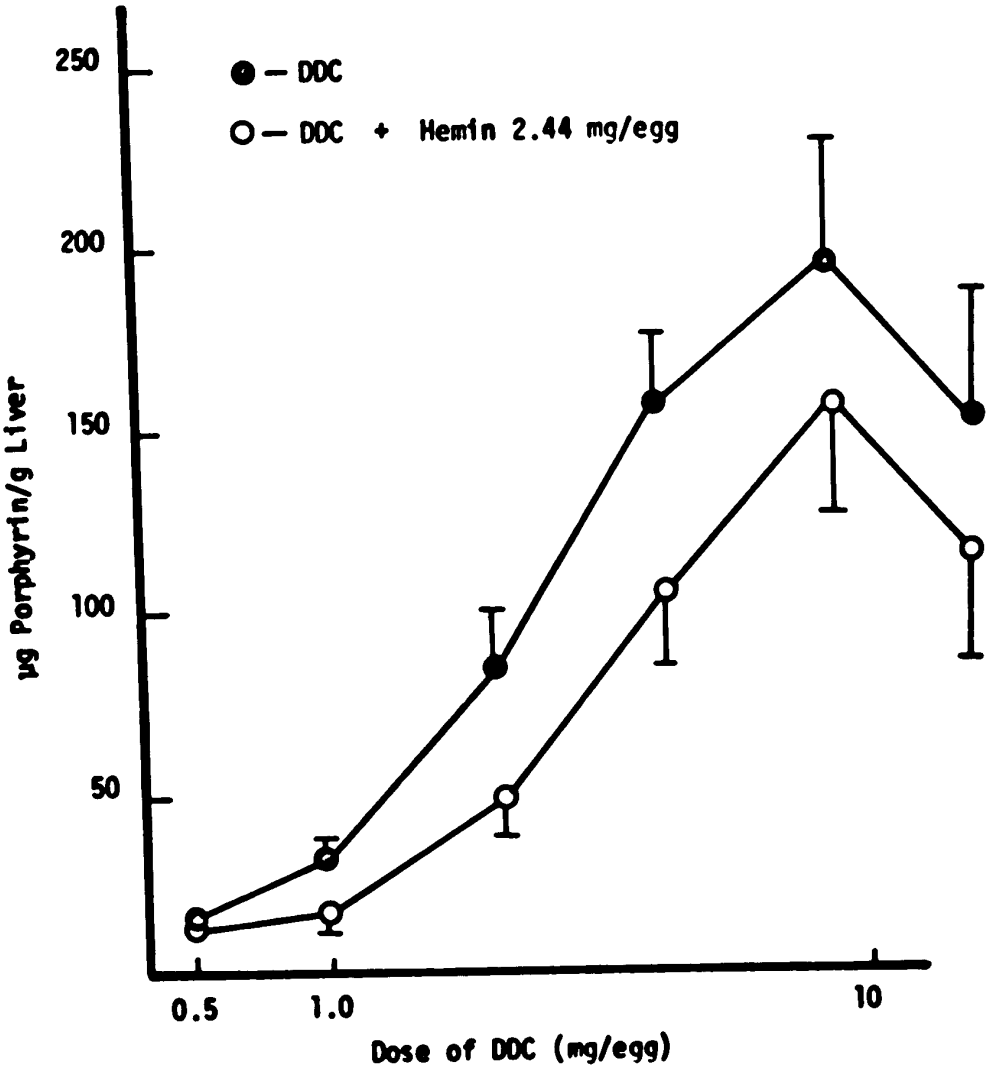


Fig. 22. Effect of 2.44 mg of Hemin per Egg on the Accumulation of Porphyrins in Chick-Embryo Liver Caused by DDC. Each Point Represents the Mean of at Least Five Determinations. The Vertical Bars Represent 1 S.E.M.

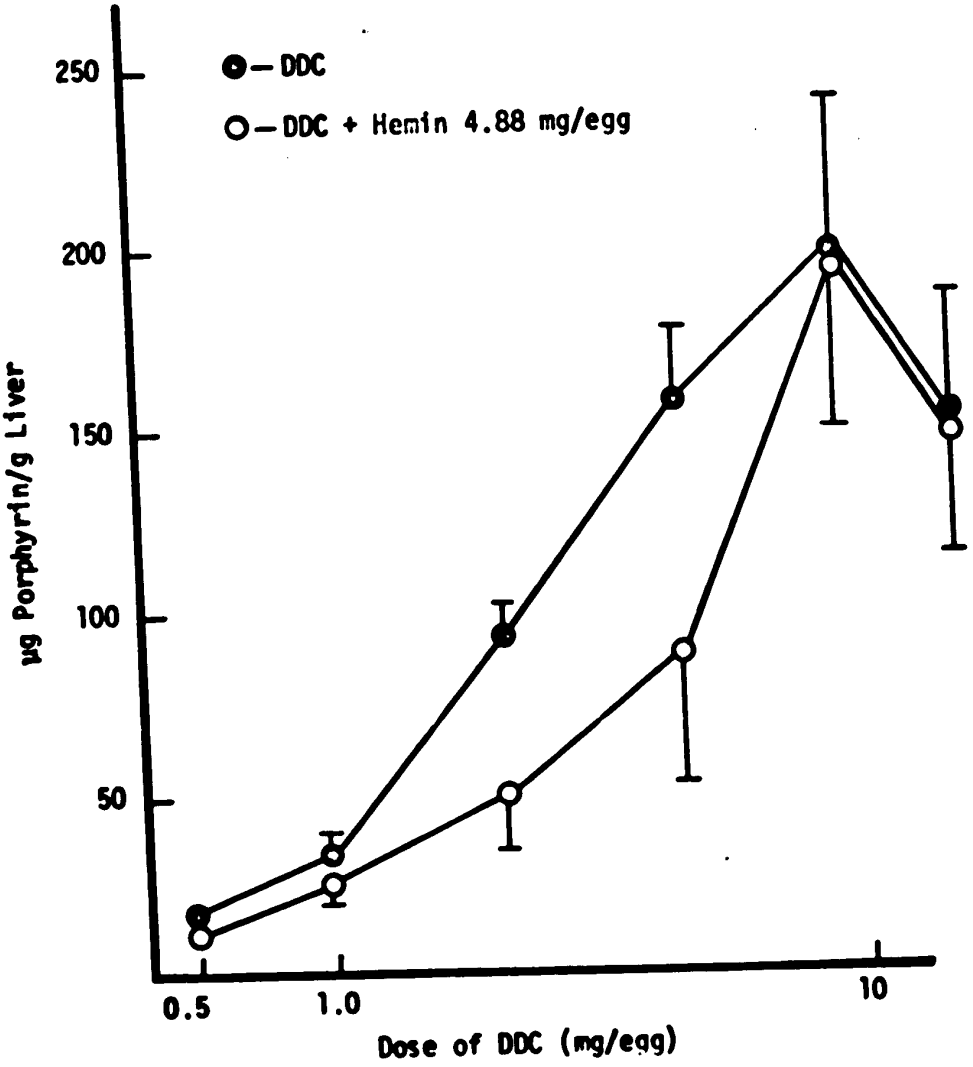
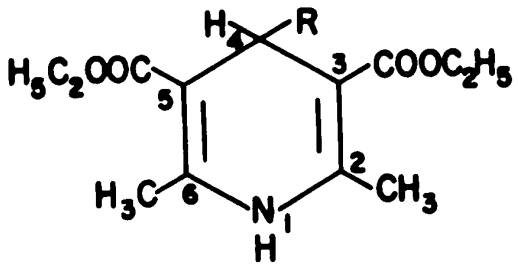


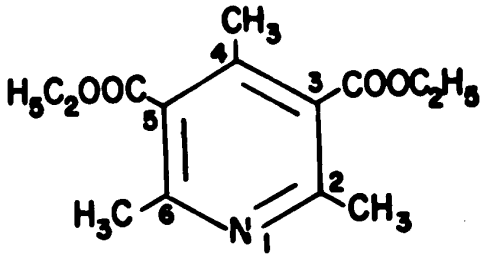
Fig. 23. Effect of 4.88 mg of Hemin per Egg on the Accumulation of Porphyrins in Chick-Embryo Liver Caused by DDC. Each Point Represents the Mean of at Least Five Determinations. The Vertical Bars Represent 1 S.E.M.

accumulation was then determined (Figs. 21, 22, 23). The administration of various doses of hemin produced little or no diminution in DDC-induced porphyrin accumulation (Figs. 21, 22, 23). One possible explanation of this unexpected result is suggested by a recent observation of Scholnick et al. (1969). These workers found that albumin prevented the inhibition of partially purified ALA-synthetase by hemin and suggested that albumin acted by binding the hemin. If this is the case the abundance of albumin found in the fluids surrounding the chick embryo might explain the difference between the activity of hemin when injected into the egg, and its activity when administered to cells in culture. Because we were unable to show a definite effect of hemin on drug-induced porphyrin accumulation in chick embryo livers we abandoned any further studies with this compound. Recently it has been reported (Marver, 1969) that hematin (ferriheme hydroxide) inhibited the phenobarbital-stimulated increase in hepatic drug-oxidizing activity when administered to rats. This is contrary to the results anticipated if Granick's hypothesis is correct. In addition to preventing increases in the levels of various components of the hepatic drug-oxidizing system, hematin administration prevented the increases in microsomal phospholipid and protein normally seen following phenobarbital administration. Such a non-specific effect is difficult to explain on the basis of hematin exerting its effect exclusively on the heme biosynthetic pathway. This has led Marver (1969) to speculate that heme may act by preventing the porphyria-inducing drug from reaching its site of action.

In our next series of experiments, a positive correlation was sought between the porphyria-inducing activity of DDC and several of its analogues (Fig. 24) and their ability to induce increased N-demethylase activity in chick embryo liver. The results (Table VIII) show that contrary to what



(a)



(e)

(a), R = H

(b); R = CH_3

(c); R = CH_2CH_3

(d); R = $CH_2CH_2CH_3$

Fig. 24. Chemical Structure of: (a) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine; (b) 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; (c) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; (d) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine; (e) 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine.

TABLE VIII. EFFECTS OF DDC AND ANALOGUES ON LIVER N-DEMETHYLASE ACTIVITY AFTER INJECTION INTO THE FLUIDS SURROUNDING THE CHICK EMBRYO*

Compound injected in 95% ethanol (0.01 ml) daily for 3 days	Liver N-demethylase activity (mμmoles formaldehyde/g liver)	% Control	Porphyria-inducing activity† (μg porphyrin/g liver)
Control	785	-	0.35 (0.32-0.38)
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 24a)‡	830	108	
Control	1280		0.70 (0.37-0.94)
3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine (Fig. 24e)	1240	97	
Control	1270		136 (72-208)
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (Fig. 24b)	307	24	
Control	1515		Data not available¶
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (Fig. 24d)	125	8	
Control	1435		217 (201-247)
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 24c)	125	9	

* The embryos were injected on days 14, 15 and 16 of development with 500 μg drug/day, and enzyme activity was measured on day 17. Livers of at least six embryos were pooled for each determination. The above

data represent the average of two experiments, with the exception of DDC (Fig. 24b) which represents the average of three experiments.

† Drugs (4 mg) dissolved in dimethylsulfoxide were injected into the fluids surrounding the embryo. After incubation for 24 hours, the porphyrins were extracted from the liver and estimated flurometrically. The data in this column are the averages of at least four determinations. The extreme values are given in parentheses. (Data of Racz & Marks, 1969).

‡ This compound was dissolved in 0.02ml of warm 95% ethanol due to its low solubility.

¶ This compound was found to be toxic at the 4 mg dose level. However, data from cell culture studies (Marks, et al. 1965) show this compound to have activity of a similar magnitude to 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 24c).

was anticipated, the administration of DDC and two of its analogues resulted in decreased N-demethylase activity, (Table VIII; 24b, c and d) while N-demethylase activity was not significantly changed by the remaining two compounds (Table VIII; 24a and e). Moreover, those drugs causing the greatest decrease in N-demethylase activity were the most potent in inducing porphyrin accumulation in the liver when injected into the fluids surrounding the chick embryo (Table VIII). It is of interest that Wada et al. (1968) have recently demonstrated that administration of DDC to mice results in decreased levels of aminoazo dye N-demethylase activity in liver microsomes. Since the administration of DDC and its analogues resulted in decreased N-demethylase activity instead of the anticipated increase, it is clear that the sequence of events suggested by Granick (1966) to occur in response to porphyria-inducing drugs does not apply to these compounds.

In the above experiments the levels of N-demethylase were observed to be decreased 3 days after the beginning of drug administration. It was of interest to determine whether the drugs used required a long period of time to produce their inhibitory effects or whether they directly inhibited the enzyme. For this reason the following series of experiments were carried out: DDC and its analogues were added to incubation mixtures containing the 9,000 g supernatant fraction prepared from the livers of untreated chick embryos. This resulted in loss of N-demethylase activity (Table IX). Moreover, those drugs causing the most significant decreases in N-demethylase activity were the most potent in inducing porphyria accumulation in chick embryo liver cells grown on coverslips. We have chosen to compare the effect of DDC and its analogues on the N-demethylase

TABLE IX. EFFECT OF DDC AND ANALOGUES ON N-DEMETHYLASE ACTIVITY OF COMBINED MICROSOMAL AND SOLUBLE FRACTION*

Compound added in 95% ethanol (0.01 ml) †	Dose/ incubation tube (µg/6 ml)	N-demethylase activity (mµmoles HCHO/g liver)	% Control	Porphyria-inducing activity‡
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 24a)	Control	1391		
	50	1325	96	1
	Control	1350		
	500	975	72	
3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine (Fig. 24e)	Control	1391		
	50	1167	84	2
	Control	1580		
	500	466	30	
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Fig. 24b)	Control	1391		
	50	825	59	3
	Control	1580		
	500	275	14	
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-propylpyridine (Fig. 24d)	Control	1391		
	50	184	14	4
	Control	1580		
	500	75	4	
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (Fig. 24c)	Control	1391		
	50	109	8	5
	Control	1580		
	500	66	4	

* The drugs were added to the incubation mixture just prior to incubation. Data represent the average of two experiments at each dose level.

† 0.1 ml of 95% ethanol was required to dissolve 500 µg of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (Fig. 24a).

‡ Compounds were ranked according to their threshold dose for inducing porphyrin accumulation in cell culture (Marks, et al. 1965). The most potent was ranked 5; the least potent was ranked 1, etc.

activity of the 9,000 g supernatant fraction (Table IX) with the porphyria-inducing activity of the drugs in chick embryo liver cells grown in culture rather than with the results in the intact chick embryo. This procedure appeared reasonable, since porphyria-inducing activity observed in the intact chick embryo may be complicated by dynamic factors such as absorption, distribution, metabolism and excretion.

We further examined the inhibition of N-demethylase activity by determining if the time the 9,000 g supernatant was in contact with the drugs had any bearing on the degree of inhibition produced. When the complete incubation mixture (containing the 9,000 g supernatant and the NADPH-generating system) was pre-incubated for 30 minutes with AIA or DDC a greater inhibition of N-demethylase activity resulted then if the incubation mixture was not pre-incubated with the drug (Table X). The NADPH-generating system was not required during the pre-incubation period for the enhanced drug effect (Table XI) and it was therefore inferred that the formation of an oxidative metabolite of AIA or DDC was not responsible for the increased drug effect during the pre-incubation period.

TABLE X. EFFECT OF PRE-INCUBATION ON THE INHIBITION OF N-DEMETHYLASE ACTIVITY CAUSED BY AIA AND DDC

Compound Added*	Dose (µg/6 ml)	Aminopyrine N-demethylase Activity† Not Pre-incubated (mµmole HCHO/ g/30 min)	(% Control)	Pre-incubated (mµmole HCHO/ g/30 min)	(% Control)
Control (solvent only)	-	733	100	783	100
AIA	100	650	89	483	62
AIA	1000	467	64	283	36
DDC	50	433	59	200	26
DDC	500	250	34	67	8

* Compounds were added dissolved in 5 µl of ethanol 95%.

† The entire incubation mixture (described under experimental, 1.c), minus aminopyrine, was pre-incubated with the drug for 30 minutes at 37°.

TABLE XI. THE EFFECT OF OMISSION OF THE NADPH GENERATING SYSTEM DURING THE PRE-INCUBATION PERIOD

Compound Added*	Dose (µg/6 ml)	Aminopyrine N-demethylase Activity			
		Pre-incubated With NADPH Generating System† (mµmole HCHO/ g/30 min)	(% Control)	Pre-incubated Without NADPH Generating System‡ (mµmole HCHO/ g/30 min)	(% Control)
Control (solvent only)	-	588	100	600	100
AIA	1000	184	32	217	36
DDC	500	67	11	50	8

* Compounds were added dissolved in 5 µl of ethanol 95%.

† The entire incubation mixture (described under experimental, i c), minus aminopyrine was pre-incubated with the drug for 30 minutes at 37°.

‡ Conditions were the same as described above except NADP and glucose-6-phosphate were omitted from the incubation mixture during the pre-incubation period.

CHAPTER IV FURTHER INVESTIGATIONS INTO THE POSSIBLE ROLE OF THE HEPATIC DRUG-OXIDIZING SYSTEM IN DRUG-INDUCED EXPERIMENTAL PORPHYRIA

Introduction

In the previous chapter we have shown that administration of DDC to chick embryos causes a fall in hepatic aminopyrine N-demethylase activity. In addition it was found that the ability of DDC and several of its analogues to cause a decrease in aminopyrine N-demethylase activity correlated well with their ability to cause experimental porphyria. When AIA (1 mg/egg) was administered for nine hours a decrease in N-demethylase activity was observed, whereas when given daily for three days it caused an increase in aminopyrine N-demethylase activity (Table VII). This observation is not surprising since it is well known that the administration of a number of drugs have a biphasic effect on hepatic drug-oxidizing activity (Conney, 1967), inhibiting activity for several hours after administration and increasing activity thereafter. It is interesting to note that of nine compounds cited by Conney (1967) as causing a biphasic effect on drug-oxidizing activity six have been tested for porphyria-inducing activity; viz., glutethimide, nikethamide, tolbutamide, trimethadione mesantoin and β-diethylaminoethyl diphenylpropyl acetate (SKF 525-A), and all six were found to be active (Granick, 1966; De Matteis, 1967, 1971). The compounds which have not been reported as having been tested for porphyria-inducing activity are: chlorcyclizine, phenaglycadol and N-methyl-3-piperidyl diphenyl carbamate (MPDC). These results indicate that a decrease in hepatic drug-oxidizing activity may be a common factor associated with induction of experimental porphyria by drugs.

The question thus arises as to how a decrease in drug-oxidizing activity may be linked to the induction of experimental porphyria. The following explanation appeared plausible: Porphyria-inducing drugs lead

in some unknown manner to breakdown of the heme moiety of cytochrome P-450 leading to decreased levels of this cytochrome. Heme is removed from the intracellular pool for the formation of new cytochrome P-450 leading to de-repression of ALA-synthetase and therefore, to experimental porphyria. The decreased levels of cytochrome P-450 would be reflected in decreased drug-oxidizing activity.

In order to evaluate these ideas our next series of experiments were directed to measuring the levels of hepatic aminopyrine N-demethylase, cytochrome P-450, ALA-synthetase and porphyrins at various intervals following administration of AIA or DDC. If a decrease in the level of cytochrome P-450 was essential for the induction of ALA-synthetase, it was considered necessary to demonstrate a decrease in the level of this cytochrome prior to an increase in the level of ALA-synthetase. Moreover the changes in the level of cytochrome P-450 should parallel changes in the levels of N-demethylase activity.

If porphyria-inducing drugs produce an elevation in ALA-synthetase activity by causing a decrease in the intracellular heme pool as described above, their administration might be expected to result in decreases in the levels of other heme containing enzymes by causing a deficiency of heme available for enzyme synthesis. In agreement with this idea, AIA and DDC have been reported to cause a fall in hepatic catalase activity (De Mattais & Prior, 1962). In order to determine if the levels of other heme containing enzymes are also decreased in experimental porphyria, we have measured the levels of cytochrome b_5 at various times following the administration of AIA or DDC. Cytochrome b_5 is associated with the hepatic endoplasmic reticulum where it functions as part of an electron transport chain

utilizing NADH as its major source of reducing equivalents (Sato et al., 1969).

In Chapter II we have shown that the measurement of hepatic porphyrin content is not always an accurate index of ALA-synthetase activity. For this reason ALA-synthetase activity was measured directly and compared with the levels of accumulated porphyrin.

Experimental

I. Isolation of Chick Embryo Liver Microsomes

The method used is essentially that used by Omura and Sato (1964). Three embryos were killed by decapitation and the livers immediately removed and immersed four times in separate cold buffered potassium chloride solutions, pH 7.4. Buffered potassium chloride solution was prepared by combining 900 ml of potassium chloride solution (1.15%) with 100 ml of potassium phosphate buffer (0.1 M), pH 7.4. This procedure was necessary to minimize hemoglobin contamination, as perfusion of chick embryo livers was found to be impractical. The livers were finely chopped with a razor blade and homogenized in the cold with 5 volumes of buffered potassium chloride solution, pH 7.4 in a Potter-Elvehjem apparatus equipped with a teflon pestle. The homogenate was centrifuged for 20 minutes at 9,000 x g and 4^{0} to remove unbroken cells, nuclei and mitochondria. The 9,000 g supernatant fraction, containing the microsomes was removed by means of a Pasteur Pipet taking care not to disturb the fatty material present on the surface of the supernatant or the pellet. The microsomes were then sedimented by centrifugation at 106,000 x g for 1 hour at 4^{0} in a Beckman model L2-65B ultracentrifuge. The supernatant was discarded and

the microsomal pellet was resuspended in buffered potassium chloride solution, pH 7.4 by approximately 5 gentle hand strokes with a Potter-Elvehjem apparatus. The microsomes were again centrifuged at 106,000 g for one hour. The supernatant was discarded and the microsomal pellet was resuspended in 8 ml of 0.1 M potassium phosphate buffer. A pH 7.0 phosphate buffer was used for determination of cytochrome P-450 and b_5, while a pH 7.4 phosphate buffer was used for determination of NADPH oxidase activity. The resulting microsomal suspensions were routinely stored frozen overnight.

II. Measurement of Microsomal Spectra

The difference spectra of microsomal preparations were measured by means of a Unicam SP 800 spectrotophotometer equipped with a scale expansion accessory and a slave recorder. The cells containing the microsomal suspension were placed in the sample position closest to the detector in order to minimize the light scattering effect of the microsomal suspension. Spectra were measured at room temperature in cuvettes of 1 cm optical path length.

III. Measurement of Cyrochrome P-450

The method outlined below is essentially that of Omura and Sato (1964). The microsomal suspension, prepared as described above, was placed in both the reference and sample cuvettes, and the baseline was determined by scanning from 550 mμ to 400 mμ. After determining the baseline, nitrogen was gently bubbled into the sample cuvette for 30 seconds. A few milligrams of sodium dithionite were added and then carbon monoxide was bubbled into the sample cuvette for 30 seconds. The cuvette was then

securely stoppered. The reference cuvette was treated with a few milligrams of sodium dithionite. The difference spectra was recorded from 500 mμ to 400 mμ. The quantity of cytochrome P-450 was calculated using the absorbance difference between 450 mμ and 500 mμ and a molar extinction coefficient of 9.1×10^4 (Omura & Sato, 1964).

IV. Measurement of Cytochrome b_5

The microsomal suspension prepared as described above was placed in both the reference and sample cuvettes and the baseline determined. A few milligrams of sodium dithionite was added to the sample cuvette and the spectrum was recorded from 500 mμ to 400 mμ. Cytochrome b_5 content was determined from the absorbance difference between 423 mμ and 500 mμ in the reduced minus oxidized difference spectra using a molar extinction coefficient of 1.7×10^5 (Stittmatter & Velick, 1956).

V. Measurement of NADPH Oxidase

The rate of oxidation of NADPH was determined spectrophotometrically by measuring the rate of disappearance of the absorption peak at 340 mμ as described by Gillette et al. (1957). Three ml of a microsomal suspension prepared as described above were added to both the sample and reference cuvettes together with 100 μmoles of nicotinamide. 250 mμ moles of NADPH were added to the sample cuvette and the absorbance at 340 mμ was recorded for a period of 11 minutes at room temperature. The absorbance measured at the end of one minute minus the absorbance at the end of 11 minutes was used to represent the rate of oxidation of NADPH. This rate was found to be constant over the time period measured.

VI. Determination of Anisole O-demethylase Activity

The O-demethylation of anisole (methoxybenzene) was estimated by measuring the amount of formaldehyde formed as described in Chapter II for the determination of aminopyrine N-demethylase activity, except that 5 μmoles of anisole were added to the incubation mixture in place of the aminopyrine.

VII. Administration of Drugs to Chick Embryos

AIA (1 mg/egg) or DDC (2 mg/egg) dissolved in 95% ethanol (0.02 ml) were injected into the fluids surrounding the embryos at various times from one to 48 hours prior to sacrifice. Embryos were always sacrificed when 17 days old. The livers were immediately removed for determination of ALA-synthetase activity, porphyrin levels, aminopyrine N-demethylase activity and the levels of cytochrome b_5 and P-450, as previously described. Controls were carried out in which the solvent alone was injected.

VIII. Substitution of NADPH for the NADPH Generating System

The incubation mixture containing the 9,000 g supernatant fraction was prepared as previously described under determination of aminopyrine N-demethylase activity (Chapter III) except that 1.0 μmole of NADPH was substituted for NADP and glucode-6 phosphate. Incubation was for 30 minutes and an additional 1.0 μmole of NADPH was added at five minute intervals throughout the incubation period.

IX. Addition of AIA, DDC and Aminopyrine to Cuvettes

AIA (1 mg) or DDC (50 μg) dissolved in 95% ethanol (5 μl) were

added to sample and reference cuvettes prepared as described above for the measurement of NADPH oxidase activity. Appropriate solvent controls were carried out.

Results and Discussion

In Fig. 25 it can be seen that ALA-synthetase activity of chick embryo liver began to rise sharply from one to three hours following the administration of DDC (2 mg/egg). The level of this enzyme reached a peak value of 26 times the control level approximately 12 hours after drug administration and declined slowly thereafter. The ALA-synthetase level 36 hours after drug administration was still 12 times higher than control levels. When the amount of porphyrin accumulation in chick embryo livers was examined following DDC administration, it was found (Fig. 25) that for the first six hours following drug administration only small amounts of porphyrin accumulated. The fact that ALA-synthetase activity begins to increase rapidly one to three hours after drug administration, while a large increase in hepatic porphyrin levels does not occur until six to twelve hours after drug administration can probably be explained on the basis of the time required for levels of heme intermediates to build up to a point where they exceed the capacity of other enzymes in the heme biosynthetic pathway to handle them. Following this initial lag period liver porphyrin levels rose rapidly and were still rising 24 hours after drug administration (Fig. 25). When the amount of porphyrin accumulating in the liver was expressed as porphyrin accumulating per hour for the various time periods (Fig. 26) it was found that the rate of porphyrin accumulation paralleled ALA-synthetase activity (Fig. 25). These results would seem to

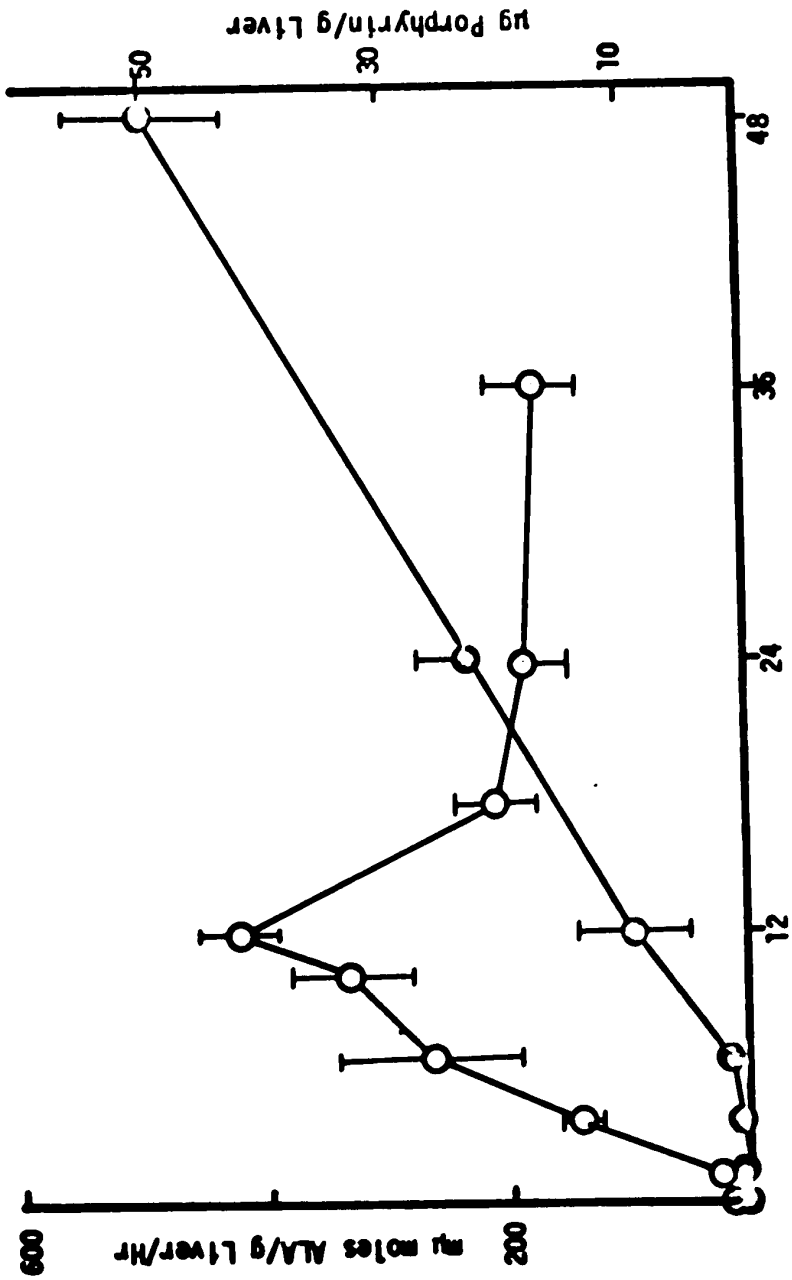


Figure 25. Time Course of ALA-Synthetase Induction (O—O) and Porphyrin Accumulation (O—O) in Chick-Embryo Liver Following the Administration of DDC (2 mg/egg). Each Point Represents the Average of at Least Six Determinations. The Verticle Bars Represent 1 S.E.M.

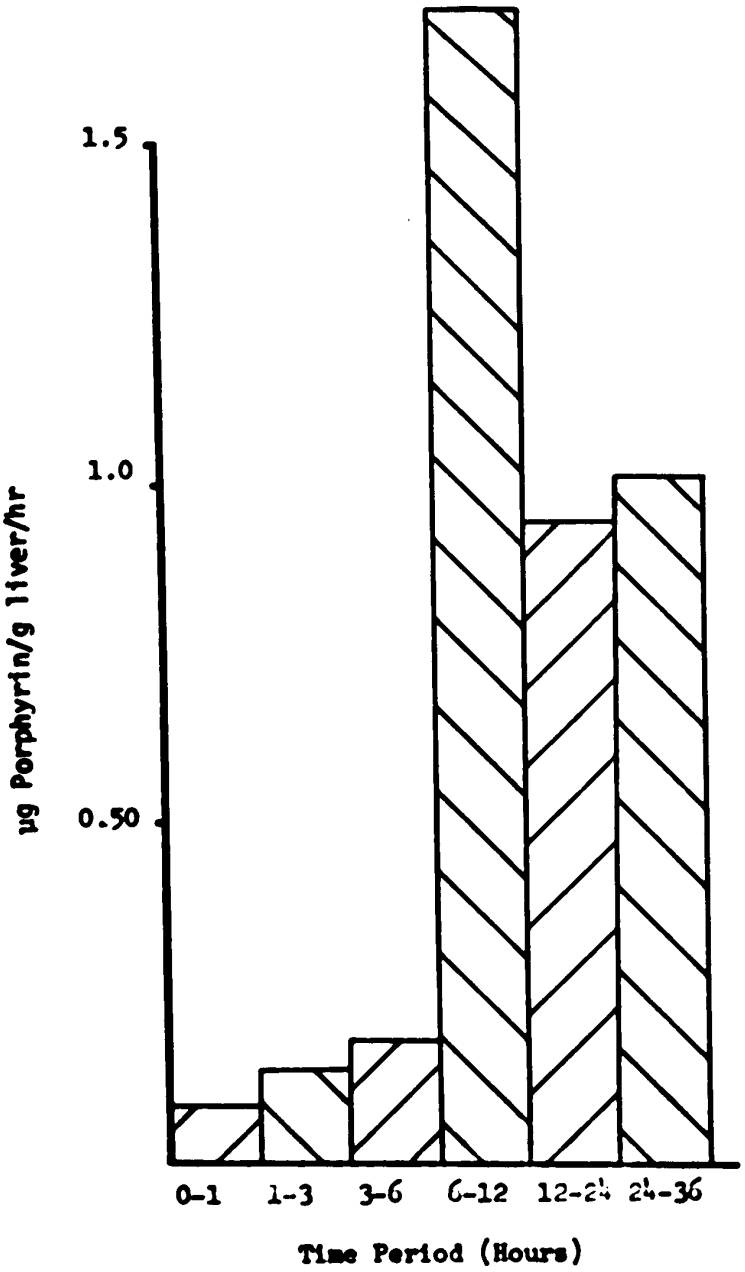


Fig. 26. Average Hourly Rates of Hepatic Porphyrin Accumulation for Various Intervals Following DDC Administration (2 mg/egg).

indicate that the rate of porphyrin accumulation following DDC administration provides a reasonable index of ALA-synthetase activity.

When hepatic aminopyrine N-demethylase activity was examined following the administration of 2 mg of DDC (Fig. 27) it was found to have fallen precipitously to approximately 25% of control activity as early as one hour after drug administration, and it remained at this level throughout the duration of the experiment (48 hours). This was clearly an event which preceded the induction of ALA-synthetase. If aminopyrine N-demethylase activity was accurately reflecting changes in the level of cytochrome P-450 the above observations would lend support to our working hypothesis viz., that ALA-synthetase induction was caused by a decrease in the level of intracellular heme, which was in turn brought about by destruction of the heme group of cytochrome P-450. However, upon direct examination of the effect of DDC administration (2 mg/egg) on the level of hepatic cytochrome P-450 levels this was found not to be the case. From Table XII it can be seen that no change in cytochrome P-450 level (expressed per gm liver) preceded the change in ALA-synthetase activity. When cytochrome P-450 levels are expressed per milligram of microsomal protein only a slight change in level was detected in the first 6 hour period during which ALA-synthetase levels rose markedly. These observations do not support the hypothesis we have suggested.

Changes in the level of cytochrome b_5 with time following DDC administration (2 mg/egg) (XIII) were found to closely parallel changes in cytochrome P-450 levels in both magnitude and time course.

When ALA-synthetase activity was measured at various intervals following the administration of AIA (1 mg/egg) the response (Fig. 28) was

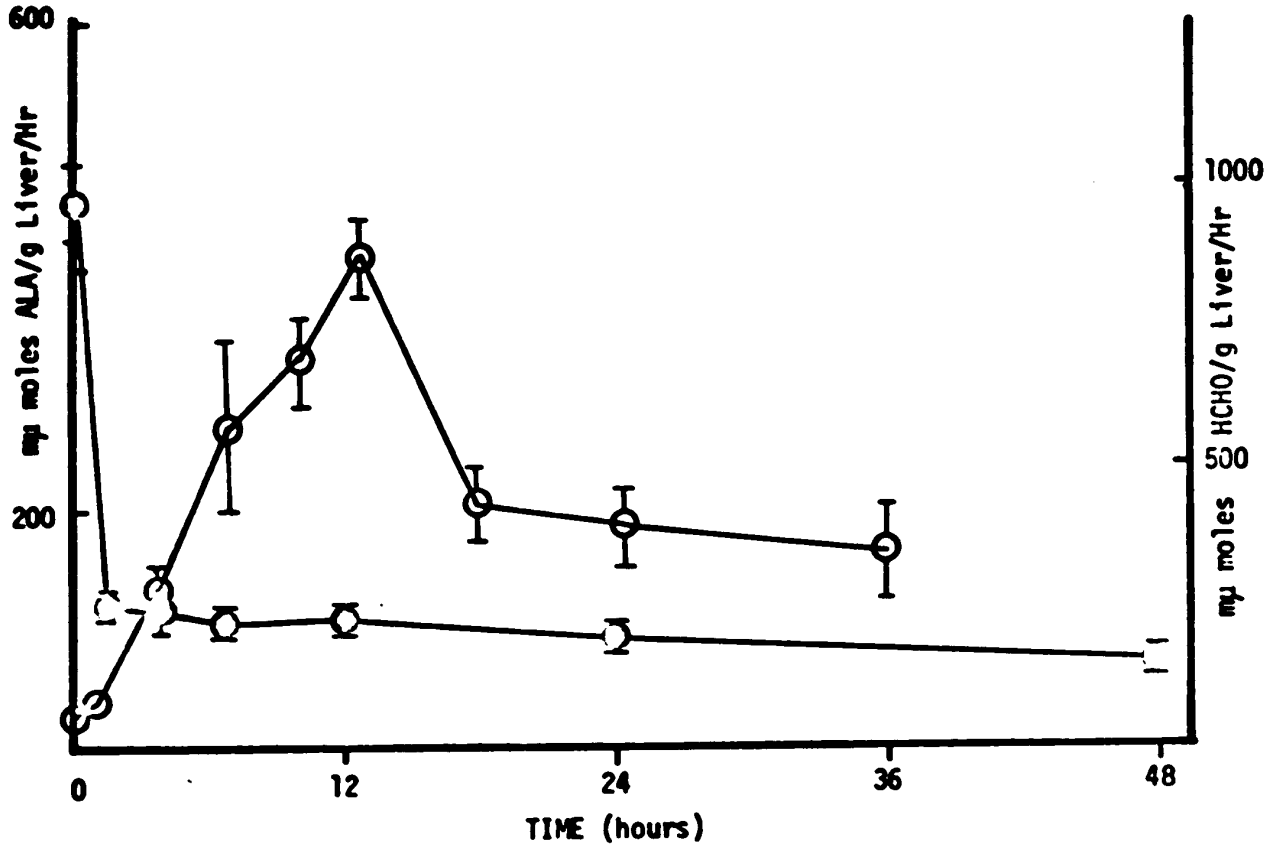


Fig. 27. Time Course of ALA-synthetase Induction (O——O) and Aminopyrine N-demethylase Inhibition (●——●) in Chick Embryo Liver Following the Administration of DDC (2 mg/egg). Each Point Represents the Average of at Least Six Determinations. The Verticle Bar Represents 1 S.E.M.

TABLE XII. LEVELS OF CYTOCHROME P-450 IN CHICK EMBRYO LIVER FOLLOWING ADMINISTRATION OF DDC

Time After Administration (hours)	No. of Determinations†	Cytochrome P-450 Levels* (mμmoles/g liver)	(mμmoles/mg Protein
0	9	2.6 ± 0.1	0.33 ± 0.02
1	6	2.7 ± 0.1	0.31 ± 0.02
6	6	2.5 ± 0.3	0.28 ± 0.02
12	6	2.5 ± 0.3	0.24 ± 0.02
24	6	2.5 ± 0.2	0.22 ± 0.01
48	6	1.8 ± 0.1	0.21 ± 0.01

* Values are given as means ± S.E.M.

† The livers of three chick embryos were pooled for each determination.

TABLE XIII. LEVELS OF CYTOCHROME b_5 IN CHICK EMBRYO LIVER FOLLOWING ADMINISTRATION OF DDC

Time After Administration (hours)	No. of Determinations†	Cytochrome b_5 Levels* (mμmoles/g liver)	(mμmoles/mg protein)
0	9	2.0 ± 0.2	0.25 ± 0.02
1	6	2.2 ± 0.2	0.24 ± 0.01
6	6	2.0 ± 0.2	0.22 ± 0.01
12	6	2.1 ± 0.2	0.21 ± 0.01
24	6	2.3 ± 0.1	0.20 ± 0.01
48	6	1.6 ± 0.1	0.19 ± 0.01

* Values are given as means ± S.E.M.

† The livers of three chick embryos were pooled for each determination.

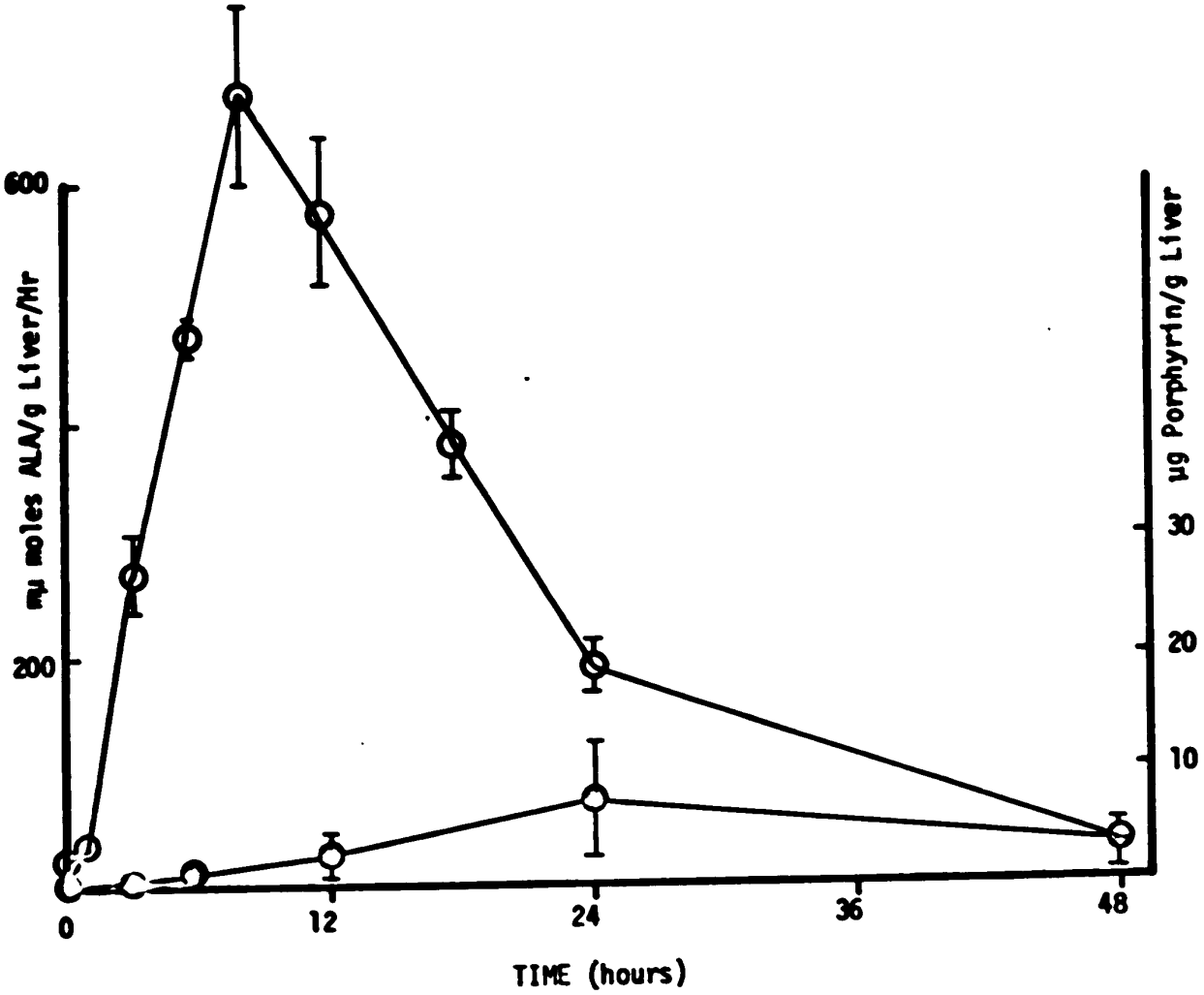


Figure 28. Time Course of ALA-synthetase Induction (○——○) and Porphyrin Accumulation (○——●) in Chick Embryo Liver Following the Administration of AIA (1 mg/egg). Each Point Represents the Average of at Least Six Determinations. The Verticle Bars Represent 1 S.E.M.

found to be similar to that produced by DDC (2 mg/egg) (Fig. 25). ALA-synthetase activity began to rise sharply from one to three hours after AIA administration and reached a peak value of 43 times the control level approximately 9 hours after drug administration. On the other hand liver porphyrin levels (Fig. 28) were found to be much lower following AIA administration (1 mg/egg) than was the case following DDC administration (Fig. 25). Moreover, the rate of hepatic porphyrin accumulation per hour for the various time periods (Fig. 29) did not correlate well with ALA-synthetase activity (Fig. 27). It is thus evident that measurement of hepatic porphyrin levels provides a poor index of ALA-synthetase activity following AIA administration.

In Fig. 30 we have graphed the levels of hepatic ALA-synthetase and porphyrins measured 12 and 24 hours respectively after the administration of DDC, AIA and 5β-pregnan-3α-17α-diol 11,20-dione. From this figure it can be seen that DDC administration produces much higher hepatic porphyrin levels while producing lower ALA-synthetase levels than the other two compounds. AIA produced porphyrin levels intermediate between those produced by DDC and 5β-pregnan-3α-17α-diol 11,20-dione. These results indicate that in the chick embryo definite conclusions about the level of ALA-synthetase cannot be drawn on the basis of hepatic porphyrin levels following drug administration.

The differences in the ratios of ALA-synthetase to porphyrin levels (Fig. 30) among the compounds tested may be indicative of different mechanisms of action. The high ratio of porphyrin to ALA-synthetase level found following DDC administration compared to the other two compounds might be explained in two ways. 1. Either more porphyrins are formed per

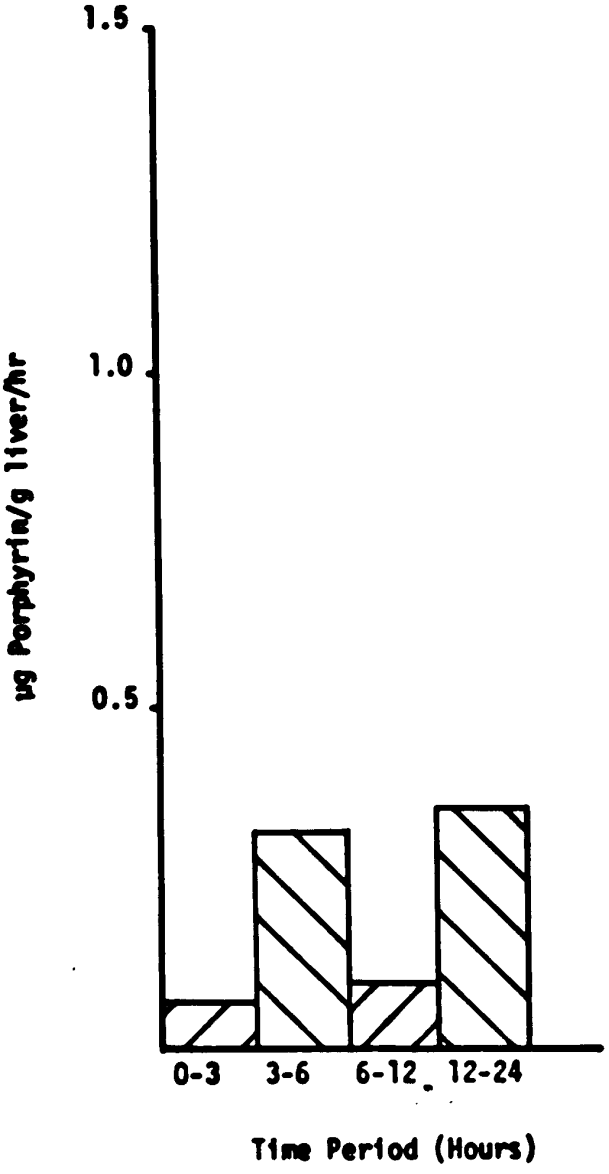


Fig. 29. Average Hourly Rates of Hepatic Porphyrin Accumulation for Various Intervals Following AIA Administration (1 mg/egg).

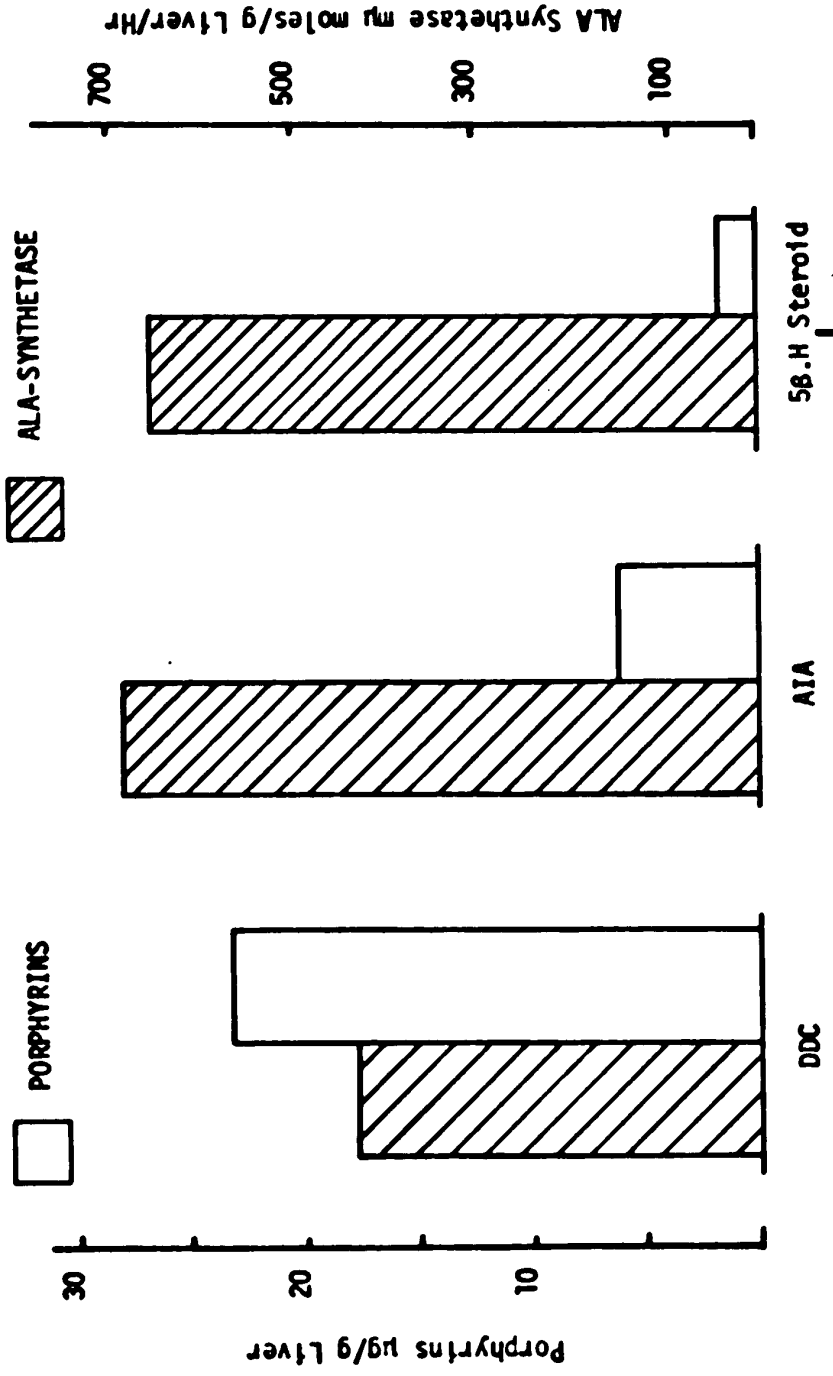


Fig. 30. Bar Graph of ALA-synthetase Levels Measured 12 Hours and Porphyrin Levels Measured 24 Hours After the Administration of DDC, AIA and a 5β-Steroid.

unit of ALA-synthetase activity following DDC administration or 2. porphyrins may be formed at the same rate per unit of ALA-synthetase activity following the administration of all three drugs, but much of the porphyrins formed might be removed from the liver in the case of AIA and 5β-pregnan-3α-17α- diol 11,20 dione. The first possibility is supported by reports (Onisawa & Labbe, 1963) that DDC, but not AIA, blocks the conversion of protoporphyrin to heme. The second possibility cannot be evaluated with the information available at present since the excretion of porphyrins by the chick embryo has not been studied.

As was the case following DDC administration, the administration of AIA (1 mg/egg) was found to produce a rapid fall in aminopyrine N-demethylase activity, which preceded the increase in the level of ALA-synthetase activity (Fig. 31). The decrease in N-demethylase activity was greatest when measured one hour after AIA administration, and the activity gradually increased thereafter until it reached a level significantly greater than the control level when measured 24 hours after AIA administration. Thus the effect of AIA on N-demethylase activity shows a time course typical of other drugs which have been classified as having a biphasic effect on drug-oxidizing activity (Conney, 1967). However, when cytochrome P-450 levels were measured following AIA administration (Table XIV), it was not possible to show a decrease in the level of this cytochrome prior to the increase in ALA-synthetase activity. Therefore with AIA as in the case of DDC a decrease in the level of cytochrome P-450 does not precede the induction of ALA-synthetase. Though there was little change in the level of cytochrome P-450 in the early time periods (1 and 3 hours), AIA caused a marked increase in the level of cytochrome P-450 as early as six hours after administration (Table XIV). The levels continued to rise and reached a

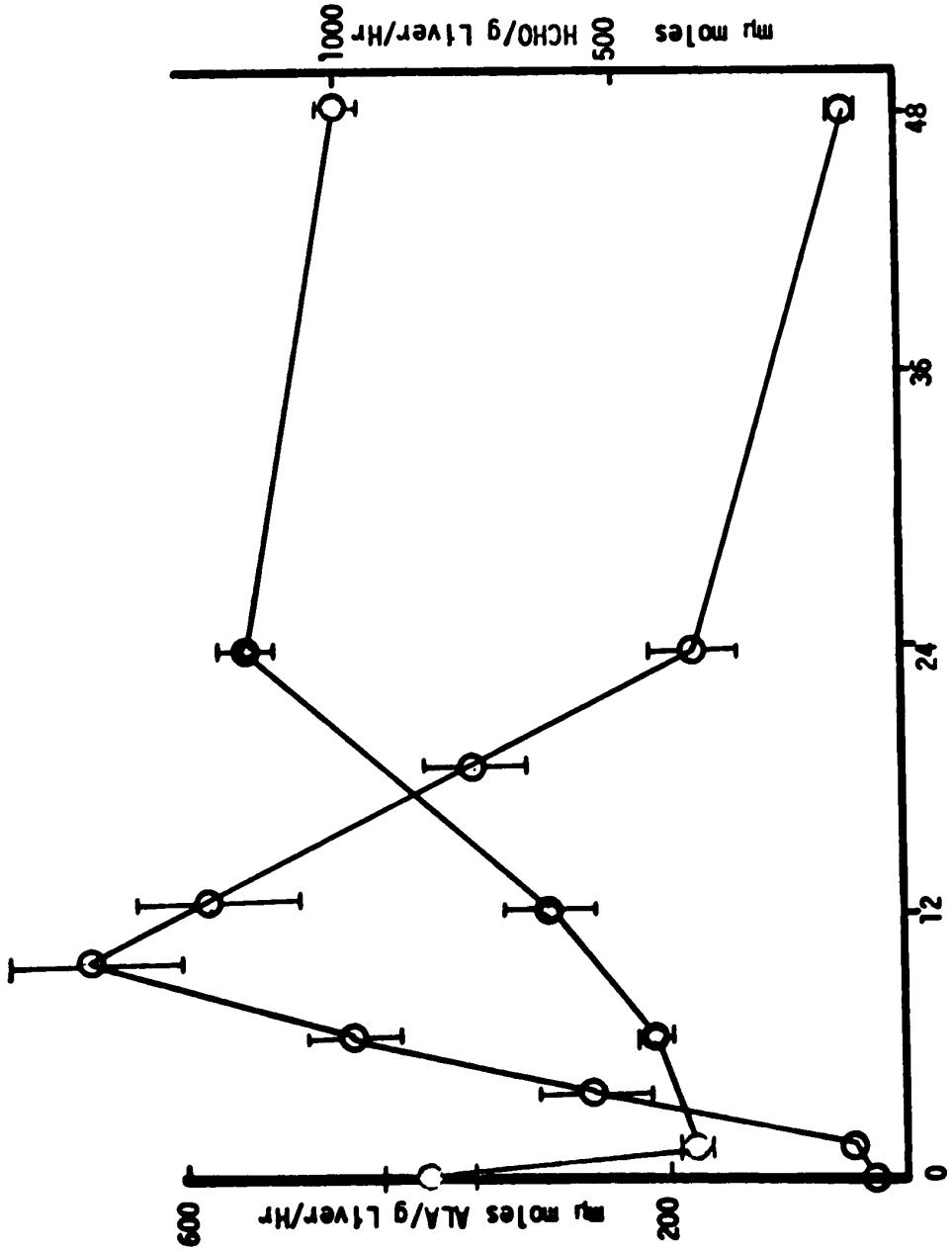


Fig. 31. Time Course of ALA-synthetase Induction(○——○)and Aminopyrine N-demethylase Inhibition(●——●)in Chick Embryo Liver Following the Administration of AIA (1 mg/egg). Each Point Represents the Average of at Least Six Determinations.

TABLE XIV. LEVELS OF CYTOCHROME P-450 IN CHICK EMBRYO LIVER FOLLOWING ADMINISTRATION OF AIA

Time After Administration (hours)	No. of Determinations†	Cytochrome P-450 Levels* (mμmoles/g liver)	(mμmoles/mg protein)
0	9	2.6 ± 0.1	0.33 ± 0.02
1	11	2.7 ± 0.1	0.34 ± 0.02
3	6	2.4 ± 0.1	0.29 ± 0.01
6	6	3.5 ± 0.5	0.61 ± 0.04
12	6	6.9 ± 0.4	0.75 ± 0.12
24	6	11.1 ± 0.9	1.18 ± 0.08
48	6	9.1 ± 0.4	0.95 ± 0.07

* Values are given as means ± S.E.M.

† The livers of three chick embryos were pooled for each determination.

peak value approximately four times greater than the control level 24 hours after drug administration.

It thus appears from the data on AIA and DDC that there is no specific pattern of cytochrome P-450 level accompanying the induction of ALA-synthetase. The possibility must therefore be considered that the effects of porphyria-inducing drugs on ALA-synthetase and on cytochrome P-450 are not associated. Our hypothesis that porphyria-inducing drugs lead to the breakdown of the heme moiety of cytochrome P-450 is thus not supported by these results.

Measurement of the amount of microsomal cytochrome b_5 activity was complicated by the appearance of a peak at 450 mμ in the reduced minus oxidized difference spectra (Fig. 32) of microsomes prepared from animals which had received AIA (1 mg/egg). This peak at 450 mμ was found in addition to the normal cytochrome b_5 peak at 423 mμ and was evident (Fig. 32) in microsomes prepared from animals which had received AIA 12, 24 and 48 hours prior to sacrifice. Normally the amount of cytochrome b_5 is measured by the height of the 423 mμ peak relative to the absorbance at 500 mμ. Due to the atypical nature of the reduced minus oxidized difference spectra estimates of the amount of cytochrome b_5 (Table XV) during these time periods are of questionable accuracy.

The presence of an absorption maximum at 450 mμ in hepatic microsomes in the absence of carbon monoxide was also observed by Gillette and Sasame (1965) when mouse liver microsomes were preincubated with SKF-525A. Since these spectral characteristics are similar to the spectrum observed when cyrochrome P-450 is combined with carbon monoxide, Gillette (1966) has suggested that SKF-525A inhibits drug metabolism by causing the pro-

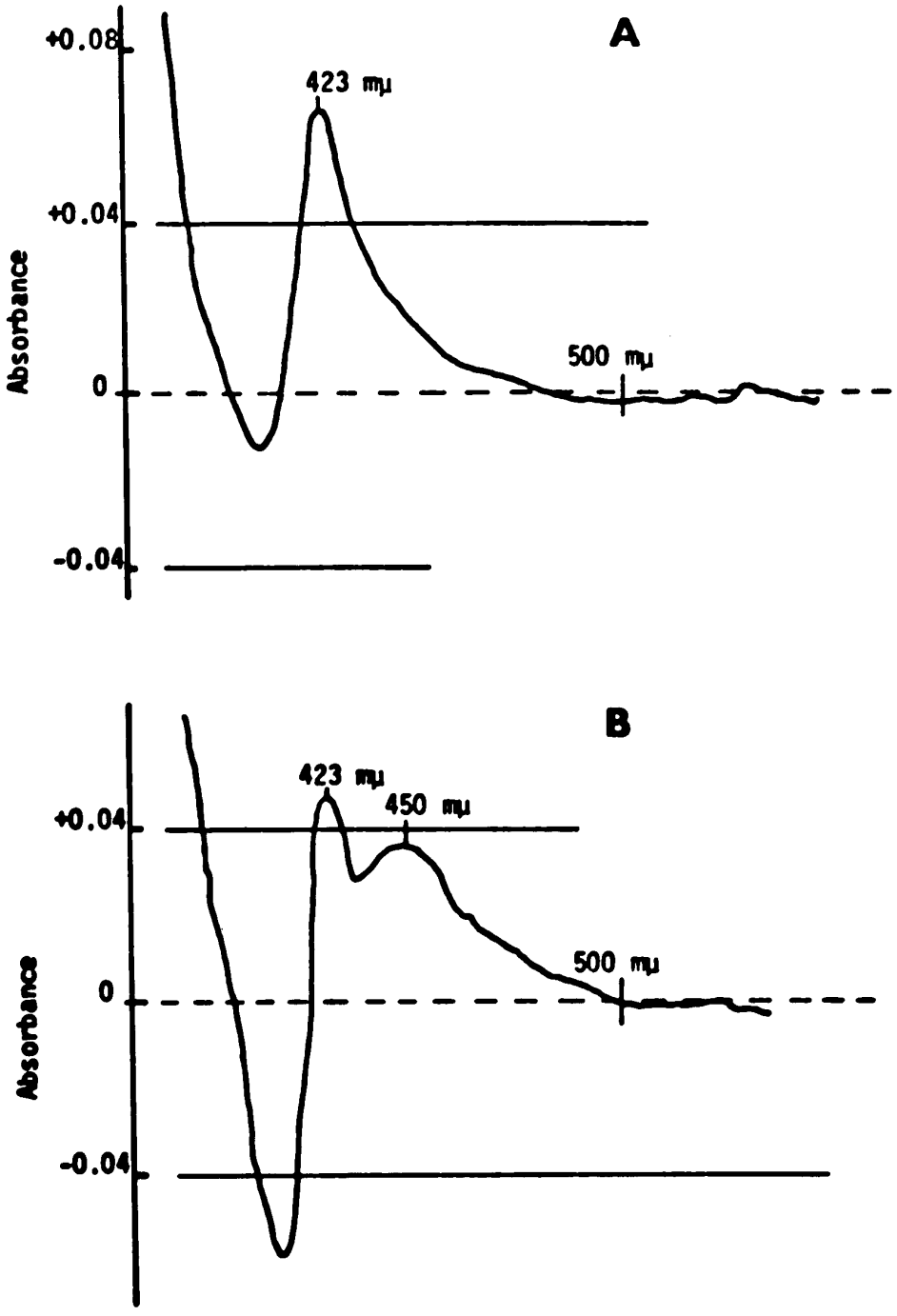


Fig. 32. Reduced Minus Oxidized Difference Spectra of Chick Embryo Liver Microsomes: A. Spectrum of Microsomes from Control Animals. B. Spectrum of Microsomes from Animals Treated with AIA (1 mg/egg) 24 Hours Prior to Sacrifice.

TABLE XV. LEVELS OF CYTOCHROME b_5 IN CHICK EMBRYO LIVER FOLLOWING ADMINISTRATION OF AIA

Time After Administration (hours)	No. of Determinations†	Cytochrome b_5 Levels* (mμmoles/g liver)	(mμmoles/mg protein)
0	9	2.0 ± 0.2	0.25 ± 0.02
1	11	2.1 ± 0.1	0.26 ± 0.02
3	6	1.8 ± 0.1	0.22 ± 0.01
6	6	1.8 ± 0.2	0.28 ± 0.02
12	6	1.6 ± 0.1	0.16 ± 0.01
24	6	2.0 ± 0.2	0.22 ± 0.02
48	6	3.6 ± 0.1	0.37 ± 0.02

* Values are given as means ± S.E.M.

† The livers of three chick embryos were pooled for each determination.

duction of carbon monoxide, which combined with cytochrome P-450. If AIA caused conversion of cytochrome P-450 to an inactive form in the manner suggested above for SKF-525A we could explain why 12 hours after AIA administration N-demethylase activity is still below control level (Fig. 31) while the level of cytochrome P-450 (Table XIV) is 2.6 times greater than control. The cytochrome P-450 measured might represent active and inactive cytochrome and the level of active cytochrome P-450 may in fact parallel that of N-demethylase activity. This idea is supported by the microsomal peak at 450 mμ in the absence of exogenous carbon monoxide (Fig. 32B). The non-parallelism between the level of N-demethylase activity and cytochrome P-450 in the earlier time periods cannot, however, be explained in this manner since no microsomal peak was observed at 450 mμ in the early periods in the reduced minus oxidized spectra.

This early period of inhibition of N-demethylase activity following the administration of AIA or DDC is of particular interest since it precedes the period in which ALA-synthetase begins to increase rapidly. Even though it does not reflect levels of cytochrome P-450, the decrease in N-demethylase levels which takes place may still in some way be linked to induction of ALA-synthetase. For this reason we have attempted to further elucidate the mechanism of inhibition of N-demethylase activity by these drugs. However, it was first of interest to determine whether the inhibitory effects of these drugs was a generalized one on the hepatic drug-oxidizing system or if it was specific for the N-demethylation of aminopyrine. We tested the effect of AIA and DDC on the O-demethylation of anisole, and it was found that both AIA and DDC are also effective inhibitors of anisole O-demethylation (Table XVI).

TABLE XVI. INHIBITION OF ANISOLE O-DEMETHYLATION BY THE ADDITION OF AIA OR DDC TO THE INCUBATION MIXTURE

Compound	Dose (μg/6 ml)	Liver O-demethylase Activity* (mμmoles HCHO/g/30 min)	(% of Control)
Control (solvent only)†	--	500	100
AIA	100	230	47
DDC	50	100	20

* Values represent the averages of duplicate determinations at each dose level.

† AIA, DDC and Anisole were dissolved in 95% ethanol (0.01 ml).

Possible ways in which AIA and DDC might interfere with the N-demethylation of aminopyrine while not lowering the level of cytochrome P-450 were suggested by the observation of Brodie et al. (1955) that the liver enzyme system responsible for the oxidation of drugs requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) for activity. A decrease in drug-oxidizing activity would result if AIA or DDC either diminished the amount of NADPH available or decreased the rate of ANDPH oxidation and hence the flow of electrons from NADPH to cytochrome P-450 (Fig. 11).

In order to test the first of these two possibilities, viz., that AIA or DDC might act by diminishing the amount of NADPH, NADPH was substituted for NADP and glucose-6-phosphate normally utilized to generate NADPH in the incubation mixture. Substitution of NADPH for the NADPH-generating system made no difference to the inhibition of aminopyrine N-demethylase activity by AIA or DDC (Table XVII). Clearly therefore AIA or DDC do not inhibit N-demethylase activity by inhibiting the generation of NADPH in the incubation mixture.

The second possibility; viz., that AIA or DDC might act by decreasing the rate at which NADPH was oxidized, was especially interesting in view of the work of Labbe et al. (Cowger et al. 1962; Cowger & Labbe, 1967; Labbe et al., 1969). These workers studied the affect of porphyria-inducing drugs on beef heart NADH oxidase activity and showed that the structural requirements for inhibition of NADH-oxidation correlated well with the structural requirements for the induction of experimental porphyria as measured by porphyrin accumulation in cell culture. These workers have suggested that porphyria represents a specific block in the terminal stage

TABLE XVII. EFFECT OF SUBSTITUTION OF NADPH FOR THE NADPH-GENERATING SYSTEM ON THE INHIBITION OF N-DEMETHYLASE ACTIVITY

Source of NADPH*	Drug Added†	N-demethylase Activity (mμmoles HCHO/g/30 min.)
NADPH-generating system	None	670
NADPH	None	680
NADPH-generating system	AIA	430
NADPH	AIA	430
NADPH-generating system	DDC	100
NADPH	DDC	100

* The NADPH-generating system consisted of 0.6 μmoles of NADP plus 6.0 μmoles of glucose-6-phosphate per 6 ml. The remainder of the incubation mixture was prepared as described previously (Chapter III, Experimental i c). When NADPH was utilized 1.0 μmoles was added per 6 ml at the start of incubation and at 5 minute intervals thereafter.

† AIA was added in a concentration of 1.0 mg/6 ml; DDC was added in a concentration of 0.5 mg/6 ml.

TABLE XVIII. EFFECTS OF AIA AND DDC ON THE RATE OF NADPH OXIDATION BY LIVER MICROSOMES*

Compound Added to Microsomes	Rate of NADPH Oxidation * (ΔO.D. at 340 mμ/10 min.)
Control (solvent only)†	0.169
AIA	0.164
DDC	0.174

* Both sample and reference cuvettes contained microsomal suspensions prepared from 133 mg of liver plus 100 μmoles of nicotinamide suspended in 0.1 M potassium phosphate buffer (pH 7.4) to a final volume of 3 ml. 250 mμmoles of NADPH were added to the sample cuvette just prior to incubation.

† AIA (1 mg) or DDC (50 μg) were dissolved in 5 μl of ethanol (95%).

♦ Values are the means of three determinations.

of oxidative metabolism - specifically the NADH oxidase. It has been suggested that certain types of respiratory defects might lead to the induction of ALA-synthetase, the rate controlling enzyme in the synthesis of the heme moiety of heme-containing enzymes (Tschudy, 1965; Cowger and Labbe, 1965). When we measured the rate of NADPH oxidation by chick embryo liver microsomes it was found to be unaffected by the addition of AIA or DDC (Table XVIII). Thus it would appear that these drugs do not inhibit aminopyrine N-demethylase by interfering with the source of electrons for cytochrome P-450. The above facts leave open the means by which AIA and DDC are inhibiting N-demethylase, but point to some form of direct interaction between these compounds or their metabolites and cytochrome P-450.

Conclusions

In summarizing our results it is convenient to consider the results obtained with DDC and with AIA separately.

3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)

We have shown that no change in cytochrome P-450 level (expressed per g liver) precedes the increase in ALA-synthetase activity following administration of DDC. Recently several groups of workers have studied the relationship between ALA-synthetase induction and cytochrome P-450 levels following administration of porphyria-inducing drugs. Wada et al. (1968) treated mice at 12 hour intervals with DDC and showed that the level of cytochrome P-450 had dropped after 12 hours at which time the level of ALA-synthetase had risen markedly. Waterfield et al. (1969) treated mice and rabbits daily for five days with DDC. At the end of this period hepatic cytochrome P-450 levels were shown to have fallen. These workers

did not measure the levels of ALA-synthetase and it is difficult to see the relevance of cytochrome P-450 levels measured five days after administration of the drug. Clearly the level of cytochrome P-450 should be measured at earlier time periods when the level of ALA-synthetase is known to be rising. Sweeney et al. (1971) injected DDC into rats and showed that after approximately two hours the levels of cytochrome P-450 had dropped at which time ALA-synthetase levels had risen. De Matteis (1971) administered DDC to rats twice a day for two days. At the end of this period he found that there was a marked increase in ALA-synthetase activity with no change in cytochrome P-450 levels.

The reason for the differences in experimental results obtained by different groups of workers might be explained by factors, such as species, differences, drug dose and schedule of drug administration. Regardless of the reasons for these differences, we believe the important point to emerge from our work and that of De Matteis (1971) is that DDC administration can cause large increases in ALA-synthetase activity without altering the levels of cytochrome P-450. From this we feel that it is unlikely that the ability of DDC to alter levels of cytochrome P-450 under various circumstances is directly related to its ability to cause an increase in ALA-synthetase activity.

Taking into consideration the results reported in this thesis and those from other laboratories the induction of ALA-synthetase which occurs following the administration of DDC might be explained as follows: The administration of DDC blocks the conversion of protoporphyrin to heme as originally reported by Onisawa and Labbe (1963) and recently confirmed by Tephly et al. (1971). This block would lead to the accumulation of proto-

porphyrin and a decrease in the amount of hepatic heme. The accumulation of protoporphyrin would explain the large amounts of porphyrins which we have observed in the liver of DDC treated animals (Figs. 25 & 30). The decrease in the amount of heme might then lead to an increase in the amount of ALA-synthetase, via de-repression. That such a decrease in the amount of hepatic heme following DDC administration does take place has been confirmed by Waterfield et al. (1969). We would like to put forward the hypothesis that the drop in levels of heme would not be sufficient to rapidly effect levels of hepatic cytochrome P-450 in the chick embryo but might effect the levels of cytochrome P-450 in species such as mice, rats and rabbits.

Allylisopropylacetamide (AIA)

The results from other laboratories with regard to the effect of AIA on the levels of hepatic cytochrome P-450 have been much more consistant despite the fact that different species have been used. Thus Wada et al. (1968), De Matteis (1970) and Sweeney (1971) have all reported that AIA exerts a biphasic effect on hepatic cytochrome P-450 levels, first causing a transient decrease followed by an increase. In our experiments we have clearly shown the increase, however we were able to show only a very small decrease at about three hours after drug administration at which time a marked rise in ALA-synthetase activity had occurred. The difference between our results and those of other workers can probably be attributed to species and dose differences. It thus appears that as was the case with DDC the level of cytochrome P-450 is not the controlling factor in the increase in ALA-synthetase activity.

With AIA there is no evidence for a block in heme synthesis as was

the case with DDC. However the following observations suggest that AIA causes increased heme breakdown: (1) Israels (1963) found that the administration to dogs of Sedormid, a close congener of AIA, caused the early bilirubin peak to appear at three hours instead of at 12 to 24 hours as in normal dogs. Since this early peak is believed to represent the breakdown of non-erythropoietic heme this could be taken as evidence for an increase in hepatic heme breakdown. (2) AIA increases the rate of breakdown of hepatic microsomal heme (Meyer & Marver, 1971; De Matteis, 1971). (3) Landow et al. (1970) fed 2 ^{14}C--glycine to rats and this radioactive substrate was incorporated into heme causing a labelling of the methyne bridges with ^{14}C. Upon breakdown of heme at least one of the methyne bridges is converted to carbon monoxide. Landow et al. (1970) demonstrated that animals treated with AIA exhaled ^{14}C-carbon monoxide at a higher rate than control animals. (4) In our experiments we have observed an absorption peak at 450 mμ in the absence of added carbon monoxide in microsomes from AIA treated chick embryo liver. An increase in the rate of heme breakdown providing a source of carbon monoxide would provide an explanation for this finding.

It is thus possible that AIA in common with DDC may cause ALA-synthetase induction by lowering the level of hepatic heme. DDC would lower heme levels by blocking its synthesis while AIA would lower it by increasing its breakdown. The lowered hepatic heme level would also serve to explain the decreases in cytochrome P-450, b_5 and catalase which have been observed in some species following their administration (Wada et al. 1968; De Matteis & Prior, 1962).

It therefore appears that future studies should be directed toward

measuring hepatic heme levels together with the rate of synthesis and breakdown of this compound following the administration of porphyria-inducing drugs.

BIBLIOGRAPHY

ALEXANDER, N.M. (1960). The mechanism of iodination reactions in thyroid glands. Fed. Proc. 19, 173.

BOGORAD, L. (1958a). The enzymatic synthesis of porphyrins from porphobilinogen. I. Uroporphyrin I. J. Biol. Chem. 233, 501.

BOGORAD, L. (1958b). The enzymatic synthesis of porphyrins from porphobilinogen. II. Uroporphyrin III. J. Biol. Chem. 233, 510.

BRODIE, B.B., AXELROD, J.A., COOPER, J.R., GAUDETTE, L., LA DU, B.N., MITOMA, C. & UDENFRIEND, S. (1955). Detoxication of drugs and other foreign compounds by liver microsomes. Science 121, 603.

BRODIE, B.B. & MAICKEL, R.P. (1961). Comparative biochemistry of drug metabolism. In Proc. First. Int. Pharmacological Meet. (ed. BORGE UVNAS). Vol. 6, p. 299. Macmillan Co., New York.

BURNHAM, B.F. & LASCELLES, J. (1963). Control of porphyrin biosynthesis through a negative-feedback mechanism. Studies with preparations of δ-aminolaevulate synthetase and δ-aminolaevulate dehydrates from Rhodopseudomonas spheroides. Biochem. J. 87, 462.

COCHIN, J. & AXELROD, J. (1959). Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J. Pharmacol. Exp. Ther. 125, 105.

CONNEY, A.H. (1967). Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19, 317.

COWGER, M.L. & LABBE, R.F. (1965). Contraindications of biological-oxidation inhibitors in the treatment of porphyria. Lancet i, 88.

COWGER, M.L. & LABBE, R.F. (1967). The inhibition of terminal oxidation by porphyrinogenic drugs. Biochem. Pharmacol. 16, 2189.

COWGER, M.L., LABBE, R.F. & MACKLER, B. (1962). Relation of barbiturate structure to DPNH oxidase inhibition. Arch. Biochem. 96, 583.

DALLNER, G., SIEKEVITZ, P. & PALADE, G.E. (1966). Biogenesis of endoplasmic reticulum membranes. II. Synthesis of constitutive microsomal enzymes in developing rat hepatocytes. J. Cell. Biol. 30, 97.

DEAN, G. & BARNES, H.D. (1955). The inheritance of porphyria. Brit. Med. J. 2, 89.

DE MATTEIS, F. (1964). Increased synthesis of L-ascorbic acid caused by drugs which induce porphyria. Biochim. Biophys. Acta. 82, 611.

DE MATTEIS, F. (1967). Disturbances of liver porphyrin metabolism caused by drugs. Pharmacol. Rev. 19, 523.

DE MATTEIS, F. (1970). Rapid loss of cytochrome P-450 and haem caused in the liver microsomes by the porphyrinogenic agent. 2-allyl-2 isopropylacetamide. FEBS Letters. 6, 343.

DE MATTEIS, F. (1971). Drugs and porphyria. In Proceedings of the International Conference on Porphyrin Metabolism and the Porphyrias. Cape Town, 1970. S. Afr. J. Lab. Clin. Med. in press.

DE MATTEIS, F. & PRIOR, B.E. (1962). Experimental hepatic porphyria caused by feeding 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl-pyridine. Biochem. J. 83, 1.

DOWDLE, E.B., MUSTARD, P. & EALES, L. (1967). δ-aminoleavulenic acid synthetase activity in normal and porphyric human livers. S. Afr. Med. J. 41, 1093.

DRABKIN, D.L. (1951). Metabolism of hemin chromoproteins. Physiol. Rev. 31, 345.

DUESBERG, R. (1932). Toxische porphyrie. Munchen. Med. Wschr. 79, 1821.

EBERT, P.S., CHIRIGOS, M.A., ELLSWORTH, P.A. & MALININ, G.I. (1969). Effect of Friend leukemia virus infection upon polycythemia and δ-aminolevulinic acid synthetase activity in murine spleen and liver. Life Sci. 8, 147.

FOUTS, J.R. & ADAMSON, R.H. (1959). Drug metabolism in the newborn rabbit. Science 129, 897.

GARFINKEL, D. (1958). Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Arch. Biochem. 77, 493.

GIBSON, K.D., NEUBERGER, A. & SCOTT, J.J. (1955). The purification and properties of δ-aminolaevulic acid dehydrase. Biochem. J. 61, 618.

GILLETTE, J.R. (1966). Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. In Advances in Pharmacology (eds. GARATTINI, S. & SHORE, P.A.). Vol. 4, p. 219. Academic Press, New York.

GILLETTE, J.R., BRODIE, B.B. & LA DU, B.N. (1957). The oxidation of drugs by liver microsomes: On the role of TPNH and oxygen. J. Pharmacol. Exp. Ther. 119, 352.

GILLETTE, J.R. & GRAM, T.E. (1969). Cytochrome P-450 reduction in liver microsomes and its relationsnip to drug metabolism. In Microsomes and Drug Oxidations (ed. GILLETTE, J.R.). p. 133. Academic Press, New York.

GILLETTE, J.R. & SASAME, H.A. (1965). Mechanisms of inhibition of nitroreductase in mouse liver microsomes by carbon monoxide (CO) and SKF 525A (β-Diethyl-aminoethyl diphenyl propylacetate). Fed. Proc. 24, 152.

GOLDBERG, A. (1954). The effect of certain barbiturates on the porphyrin metabolism of rabbits. Biochem. J. 57, 55.

GOLDBERG, A. (1959). Acute intermittent porphyria. A study of 50 cases. Quart. J. Med. 28, 183.

GOLDBERG, A. (1969). The effects of certain barbiturates on the hepatic porphyrin metabolism of rats. Biochem. Pharmacol. 19, 751.

GOLDBERG, A. & RIMINGTON, C. (1955). Experimentally produced porphyria in animals. Proc. Roy. Soc. London (Biol.) 143, 257.

GOLDBERG, A. & RIMINGTON, C. (1962). Diseases of Porphyrin Metabolism (ed. KUGLELMASS, I.N.) p. 72, p. 186. Charles C. Thomas, Springfield.

GOLDBERG, A. RIMINGTON, C. & LOCHHEAD, A.C. (1967). Hereditary coproporphyria. Lancet i, 632.

GRANICK, S. (1963). Induction of the synthesis of δ-aminolevulinic acid synthetase in liver parenchyma cells in culture by chemicals that induce acute porphyria. J. Biol. Chem. 238, 821.

GRANICK, S. (1964). A test for detection of porphyria-inducing drugs. J. Am. Med. Assoc. 190, 475.

GRANICK, S. (1965). Hepatic porphyria and drug-induced or chemical porphyria. Ann. N. Y. Acad. Sci. 123, 188.

GRANICK, S. (1966). The induction in vitro of the synthesis of δ-aminolevulinic acid synthetase in chemical porphyria: A response to certain drugs, sex hormones and foreign chemicals. J. Biol. Chem. 241, 1359.

GRANICK, S. & KAPPAS, A. (1967). Steroid control of porphyrin and heme biosynthesis: A new biological function of steroid hormone metabolites. Proc. Nat. Acad. Sci. USA 57, 1463.

GRANICK, S. & MAUZERALL, D. (1960). Metabolism of heme and chlorophyll. In Pathways of Chemical Metabolism. (ed. GREENBERG, D.). Vol. II, p. 526. Academic Press, New York.

GRANICK, S. & URATA, G. (1963). Increase in activity of δ-aminolevulinic acid synthetase in liver mitochrondria induced by feeding 3,5-dicarbethoxy-1,4-dihydrocollidine. J. Biol. Chem. 238, 821.

HART, L.G., ADAMSON, R.H., DIXON, R.L. & FOUTS, J.R. (1962). Stimulation of hepatic microsomal drug metabolism in the newborn and fetal rabbit. J. Pharmacol. Exp. Ther. 137, 103.

ISRAELS, L.G., YAMAMOTO, T., SKAIDERBERG, J. & ZIPURSKY, A. (1963). Alternate pathways of bilirubin production. In Proc. 9th Cong. Europ. Soc. Haemat., Lisbon, p. 891. S. Karger, New York.

JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J. Molec. Biol. 3, 318.

KAPPAS, A., SONG, C.S., LEVERE, R.D., SACHSON, R.A. & GRANICK, S. (1968). The induction of δ-aminolevulinic acid synthetase in vivo in chick embryo liver by natural steroids. Proc. Nat. Acad. Sci. USA 61, 509.

KIKUCHI, G., KUMAR, A., TALMAGE, P. & SHEMIN, D. (1958). The enzymatic synthesis of δ-aminolevulinic acid. J. Biol. Chem. 223, 1214.

KLINGENBERG, M. (1958). Pigments of rat liver microsomes. Arch. Biochem. 75, 376.

LABBE, R.F. & HUBBARD, N. (1960). Preparation and properties of the iron-protoporphyrin chelating enzyme. Biochim. Biophys. Acta 41, 185.

LABBE, R.F., NUTTER, J. & COWGER, M.L. (1969). Control of heme biosynthesis. Correlation of porphyrinogenesis with NADH oxidase activity. Biochem. Med. 3, 210.

LANDOW, S.A., CALLAHAN, E.W. & SCHMID, R. (1970). Catabolism of heme in vivo: Comparison of the simultaneous production of bilirubin and carbon monoxide. J. Clin. Invest. 49, 914.

LASCELLES, J. (1957). Synthesis of porphyrins by cell suspensions of Tetrahymena vorax: Effect of members of the vitamin B. group. Biochem. J. 66, 65.

LASCELLES, J. (1960). The synthesis of enzymes concerned in bacterio-chlorophyll formation in growing cultures of Rhodopseudomonas spheroides. J. Gen. Microbiol. 23, 487.

LASCELLES, J. (1964). Tetrapyrrole Biosynthesis and its Regulation. p. 3. Benjamin, Inc., New York.

LAVER, W.G., NEUBERGER, A. & SCOTT, J.J. (1959). α-Amino β-keto-acids. Part II. Rates of decarboxylation of the free acids and the behavior of derivatives on titration. J. Chem. Soc. p. 1483.

LAVERE, R.D., KAPPAS, A. & GRANICK, S. (1967). Stimulation of hemoglobin synthesis in chick blastoderms by certain 5β androstane and 5β pregnane steroids. Proc. Nat. Acad. Sci. USA 58, 985.

LEVIN, E.Y. & COLEMAN, D.L. (1967). The enzymatic conversion of porphobilinogen to uroporphyrinogen catalyzed by extracts of hematopoetic mouse spleen. J. Biol. Chem. 242, 4248.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Proteon measurement with the folin phenol reagent. J. Biol. Chem. 193, 265.

MACALPINE, I. & HUNTER, R. (1966). The "insanity" of King George III: A classic case of porphyria. Brit. Med. J. i, 65.

MACALPINE, I., HUNTER, R. & RIMINGTON, C. (1968). Porphyria in the royal houses of Stewart, Hanover and Prussia. Brit. Med. J. i, 7.

McKAY, R., DRUYAN, R., GETZ, G.S. & RABINOWITZ, M. (1969). Intramitochondrial localization of δ-aminolevulenate synthetase and ferrochelatase in rat liver. Biochem. J. 114, 455.

MANNERING, G.J. (1968). Significance of stimulation and inhibition of drug metabolism in pharmacological testing. In Selected Pharmacological Testing Methods (ed. BURGER, A.) p. 51. Edward Arnold (Publishers) Ltd., London.

MARKS, G.S., HUNTER, E.G., TERNER, U.K. & SCHNECK, D.W. (1965). Studies of the relationship between chemical structure and porphyria-inducing activity. Biochem. Pharmacol. 14, 1077.

MARVER, H.S. (1969). The role of heme in the synthesis and repression of microsomal protein. In Microsomes and Drug Oxidations. (ed. GILLETTE, J.R.). p. 495. Academic Press, New York.

MARVER, H.S., TSCHUDY, D.P., PERLROTH, M.G. & COLLINS, A. (1966a). δ-aminolevulinic acid synthetase. I. Studies in liver homogenates. J. Biol. Chem. 241, 2803.

MARVER, H.S., TSCHUDY, D.P., PERLROTH, M.G., COLLINS, A. & HUNTER, G. (1966b). The determination of aminoketones in biological fluid. Anal. Biochem. 14, 53.

MASON, H.S. (1957). Mechanism of oxygen metabolism. Advances Enzym. 19, 79.

MAUZERALL, D. & GRANICK, S. (1956). The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J. Biol. Chem. 219, 435.

MAUZERALL, D. & GRANICK, S. (1958). Porphyrin biosynthesis in erythrocytes. III. Uroporphyrinogen and its decarboxylase. J. Biol. Chem. 232, 1141.

MEYER, U.A. & MARVER, H.S. (1971). Chemically induced porphyria: Increased microsomal heme turnover after treatment with allylisopropylacetamide. Science 172, 65.

MILLER, G.L. (1959). Protein determination for large numbers of samples. Anal. Chem. 31, 964.

NASH, T. (1953). The colorometric estimation of formaldehyde by means of the Hantzech reaction. Biochem. J. 55, 416.

NEEDHAM, J. (1963). Chemical Embryology Vol. III, p. 1372. Hafner Publishing Co., New York.

NEUBERGER, A. (1961). Aspects of the metabolism of glycine and of porphyrins. Biochem. J. 78, 1.

OCKNER, R.K. & SCHMID, R. (1961). Acquired porphyria in man and rat due to hexachlorobenzene intoxication. Nature (London) 189, 499.

OMURA, T. & SATO, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370.

ONISAWA, J. & LABBE, R.F. (1963). Effects of diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate on the metabolism of porphyrins and iron. J. Biol. Chem. 238, 724.

ORRENIUS, S. & ERNSTER, L. (1964). Phenobarbital-induced synthesis of the oxidative demethylating enzymes of rat liver microsomes. Biochem. Biophys. Res. Commun. 16, 60.

POSNER, H.S., MITOMA, C., ROTHBERG, S. & UDENFRIEND, S. (1961). Enzymic hydroxylation of aromatic compounds. III. Studies on the mechanism of microsomal hydroxylation. Arch. Biochem. 94, 280.

RACZ, W.J. (1970). Drug Induced Porphyrin Biosynthesis. Ph.D. Thesis. Univ. of Alberta.

RACZ, W.J. & MARKS, G.S. (1969). Drug-induced porphyrin biosynthesis. II. Simple procedure for screening drugs for porphyria-inducing activity. Biochem. Pharmacol. 18, 2009.

REMMER, H. & MOHR, H.J. (1965). Effect of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. Ann. N. Y. Acad. Sci. 123, 79.

RIMINGTON, C. (1959). Biosynthesis of heamoglobin. Brit. Med. Bull. 15, 19.

SANO, S. & GRANICK, S. (1961). Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J. Biol. Chem. 236, 1173.

SATO, R. NISHIBAYASHI, H. & ITO, A. (1969). Characterization of two hemoproteins of liver microsomes. In Microsomes and Drug Oxidations. (ed. GILLETTE, J.R.). p. 111, Academic Press, New York.

SCHMID, R. (1960). Cutaneous porphyria in Turkey. New Eng. J. Med. 263, 397.

SCHMID, R. & SCHWARTZ, S. (1952). Experimental porphyria. III. Hepatic type produced by Sedormid. Proc. Soc. Exp. Biol. Med. 81, 685.

SCHMID, R., SCHWARTZ, S. & WATSON, C.J. (1954). Porphyrin content of bone marrow and liver in the various forms of porphyria. Arch. Intern. Med. 93, 167.

SCHNECK, D.W. (1969). Studies on the Mechanism of Porphyria. Induction by Drugs and the Nature of the Inhibition by Protohemin of Drug-induced Porphyria. Ph.D. Thesis. Univ. of Alberta.

SCHOLNICK, P.L., HAMMAKER, L.E. & MARVER, H.S. (1969). Soluble hepatic δ-aminolevulinic acid synthetase: End product inhibition of the partially purified enzyme. Proc. Nat. Acad. Sci. USA 63, 65.

SCHULMAN, M.P. & RICHERT, D.A. (1957). Heme synthesis in vitamin B6 and pantothenic acid deficiencies. J. Biol. Chem. 226, 181.

SCHWARTZ, S., BERG, M.H., BOSSENMAIER, I. & DINSMORE, H. (1960). Determination of porphyrins in biological materials. In Methods of Biochemical Analysis (ed., GLICK, D.), Vol. 8, p. 221. Interscience Publishers, New York.

SHEMIN, D. (1956). The biosynthesis of porphyrins. The Harvey Lectures (1954 - 1955), p. 258. Academic Press, New York.

SHEMIN, D. (1968). Mechanism and control of pyrrole synthesis. In Porphyrins and Related Compounds (ed. GOODWIN, T.W.). p. 75. Academic Press, London.

SOLOMON, H.M. & FIGGE, H.G. (1959). Disturbance in porphyrin metabolism caused by feeding diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3, 5-dicarboxylate. Proc. Soc. Exp. Biol. Med. 100, 583.

SONG, C.S., SINGER, J.W., LEVERE, R.D., HARRIS, D.F. & KAPPAS, A. (1968). Developmental and gestational influences on drug induction of ALA-synthetase in rat liver. J. Lab. Clin. Med. 72, 1019.

SOYKA, L.F. (1969). Determinants of hepatic aminopyrine demethylase activity. Biochem. Pharmacol. 18, 1029.

STOKVIS, B.J. (1889). Over twee zeldzame kluerstoffen in urine van zieken. Nederl. T. Geneesk. 25, 409.

STOKVIS, B.J. (1895). Zur pathogenese der hamatoporphyrinurie. Z. Klin. Med. 28, 1.

STRITTMATTER, C.F. & UMBERGER, T. (1969). Oxidative enzyme components of avian liver microsomes. Changes during embryonic development and the effects of phenobarbital administration. Biochim. Biophys. Acta 180, 18.

STRITTMATTER, P. & VALICK, S.F. (1956). The isolation and properties of microsomal cytochrome. J. Biol. Chem. 221, 253.

SWEENEY, G.D., JANIGAN, D., MAYMAN, D. & LAI, H. (1971). The experimental porphyrias - a group of distinctive metabolic lesions. In Proceedings of the International Conference on Porphyrin Metabolism and the Porphyrias. Cape Town, 1970. S. Afr. J. Lab. Clin. Med. in press.

TALMAN, E.L. (1958). Porphyrins in urine. In Standard Methods of Clinical Chemistry (ed., SELIGSON, D.) Vol. 2, p. 137. Academic Press, New York.

TANAKA, T. & KNOX, W.E. (1959). The nature and mechanism of the tryptophan pyrrolase (peroxidas-oxidase) reaction of pseudomonas and of rat liver. J. Biol. Chem. 234, 1162.

TEPHLY, T.R., HAEGAWA, E. & BARON, J. (1971). Effect of drugs on heme synthesis in the liver. Metabolism 20, 200.

TOMIO, J.M., GARCIA, R.C., SAN MARTIN DE VIALE, L.C. & GRINSTEIN, M. (1970). Porphyrin biosynthesis. vii. Porphyrinogen carboxy-lyase from avian erythrocytes. Purification and properties. Biochim. Biophys. Acta 198, 353.

TSCHUDY, D.P. (1965). Biochemical lesions in porphyria. J. Amer. Med. Assoc. 191, 718.

TSCHUDY, D.P., PERLROTH, M.G., MARVER, H.S., COLLINS, A., HUNTER, G., & RECHCIGL, M., (1965). Acute intermittent porphyria: the first "overproduction disease" localized to a specific enzyme. Proc. Nat. Acad. Sci. USA 53, 841.

VAN HEYNINGEN, S. (1971). Association and metal-activation properties of δ-aminoleavulinate dehydratase from Rhodopseudomonas spheroides. In Proceedings of the International Conference on Porphyrin Metabolism and the Porphyrias. Cape Town, 1970. S. Afr. J. Lab. Clin. Med. in press.

WADA, O., YANO, Y., URATA, G. & NAKAO, K. (1968). Behavior of hepatic microsomal cytochromes after treatment of mice with drugs known to disturb porphyrin metabolism in liver. Biochem. Pharmacol. 17, 593.

WATERFIELD, M.D., DEL FAVERO, A. & GRAY, C.H. (1969). Effect of 1,4-dihydro -3,5-dicarbethoxycollidine on hepatic microsomal haem, cytochrome b_5 and cytochrome P-450 in rabbits and mice. Biochim. Biophys. Acta 184, 470.

WILSON, E. & DOWDLE, E.B. (1971). The isolation and purification of ALA-dehydratase. In Proceedings of the International Conference on Porphyrin Metabolism and the Porphyrias. Cape Town, 1970. S. Afr. J. Lab. Clin. Med. in press.

WOODS, J.S. & DIXON, R.L. (1970a). Neonatal differences in the induction of hepatic aminolevulinic acid synthetase. Biochem. Pharmacol. 19, 1951.

WOODS, J.S. & DIXON, R.L. (1970b). Perinatal differences in delta-aminolevulinic acid synthetase activity. Life Sci. 9, 711.

ZUDERHOUDT, F.M.J., BURST, P. & HUIJING, F. (1969). Intramitochondrial localization of 5-aminoleavulinate synthetase induced in rat liver with allylisipropylacetamide. Biochim. Biophys. Acta 178, 408.