NATIONAL LIBRARY

OTTAWA

BIBLIOTHÈQUE NATIONALE

OTTAWA

CANADA

6236

NAME OF AUTHOR....WILLIAM J. RAC Z.....

TITLE OF THESIS....DRUG-INDUCED.....

...PORPHYRIN....BIOSYNTHESIS.

.....

UNIVERSITY.....of.....ALBERTA.....

DEGREE....Ph.D.....YEAR GRANTED...1970.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)~~.....~~

PERMANENT ADDRESS:

Dept. of Pharmacology
Queen's University
KINGSTON, ONT.

DATED. Dec. 17.....1969

NL-91

THE UNIVERSITY OF ALBERTA

DRUG-INDUCED PORPHYRIN BIOSYNTHESIS

by

© WILLIAM JOHN RACZ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

SPRING, 1970

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 Porphyrin Biosynthesis", submitted by William John Racz in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Gerald S. Marks
Supervisor

George B Frank

Charles W Rack

J. F. Henderson

A F Cary

Ethel S. Sonz
External Examiner

Date Dec. 15, 1969

ABSTRACT

The porphyria-inducing activity of a series of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine in which the 4-methyl substituent was replaced with other substituents was investigated in monolayer cultures of chick embryo liver cells. In the analogue containing a 4-isopropyl group, activity was retained, whereas in analogues containing a benzyl, cyclohexyl, or cyclohex-3-enyl substituents, only very weak activity was retained. Replacement of the ethoxycarbonyl groups of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine with cyano groups resulted in loss of activity. The optical antipodes of glutethimide were found to be equipotent as porphyria-inducing agents and it was concluded that neither of these antipodes could be used with safety in the treatment of porphyric patients.

A series of drugs has been tested for porphyria-inducing activity in the intact chick embryo and the results compared with those previously obtained in other animals and in the monolayer cultures of chick embryo liver cells. The results obtained in the intact chick embryo corresponded, in the majority of cases, with results obtained with the isolated liver cells. For this reason, the intact chick embryo may be used as a rapid and simple procedure for screening drugs for porphyria-inducing activity. Although many of the drugs which induced porphyrin accumulation in the chick embryo liver appear to be inactive in mouse liver, evidence is presented that the results obtained with the chick embryo liver allow better prediction of results to be expected in the porphyric patients than the results in mouse liver.

3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine induces porphyrin biosynthesis in monolayer cultures of chick embryo liver cells, but not in the intact chick embryo. To investigate the reason for this finding, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine-^{14}C were prepared, injected into 17-day old chick embryos and the total amount of drug and metabolite(s) in the livers measured at various time periods. 3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine was found to undergo a more rapid metabolic degradation in liver than 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine and its inactivity in the chick embryo was attributed to its rapid metabolic degradation.

3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C was prepared, injected into 17-day old chick embryos and the total amount of drug and metabolite(s) in the liver measured at different time intervals. The amount of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C in the liver at any specific period in time after injection was considerably lower than the amount of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine found in liver after injection of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine-^{14}C. This observation was in accordance with the expectations that the inactive, sterically unhindered 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine was more readily metabolized and therefore could not achieve as high a concentration in the liver as the active, sterically hindered 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine.

ACKNOWLEDGMENTS

To my supervisor, Dr. G. S. Marks, I would like to express my sincere gratitude for his encouragement and guidance throughout the course of this investigation. I would also like to thank him for making the past three years an enjoyable and rewarding experience.

I would also like to express my thanks to Mrs. V. Bell for her skill and patience during the typing of this thesis, and to Mr. F. E. Loeffler and Mr. K. R. Burt for their draughting and photographic work.

This study was made possible by financial support from The National Research Council and the Medical Research Council of Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

TO DARILYN

CHAPTER I GENERAL INTRODUCTION

A. Porphyrin Chemistry

The porphyrins may be regarded as derived from the tetrapyrrole porphin (Fig. 1) by the substitution of the hydrogen atoms on positions 1 to 8 of the macromolecule. If all the hydrogen atoms in positions 1 to 8 of porphin are replaced by substituents of two different kinds (X and Y) as in etioporphyrin, uroporphyrin and coproporphyrin (Fig. 2), then four position isomers are possible (Falk, 1964). Mesoporphyrin and protoporphyrin (Fig. 3) present a more complicated picture as there are two substituents, X and Y, in rings A and B and two substituents, X and Z, in rings C and D. This type of substitution gives rise to fifteen isomers. The porphyrin obtained by removing the iron atom from protoheme has an arrangement of substituents leading to its assignment as the ninth isomer of the series and thus is designated protoporphyrin IX (Falk, 1964).

The porphin nucleus possesses a highly conjugated cyclic double bond system which is responsible for the characteristic absorption spectrum of this molecule and for its planarity. The most intense absorption band occurs in the region of 400 mμ and is known as the Soret band. In addition to the Soret band, the porphyrins in neutral solvents exhibit four absorption bands between 480 and 650 mμ (Fisher & Orth, 1937). These compounds exhibit characteristic fluorescence spectra when irradiated with energy in the region of 405 mμ. This serves as a sensitive tool for the detection and estimation of porphyrins.

There are two species of nitrogen atoms in porphin (Fig. 1) and other porphyrins. One of these species is the imino-type capable of accepting protons and therefore acting as a basic center. The second, a pyrrole-type, is capable of accepting or donating protons and thus acting

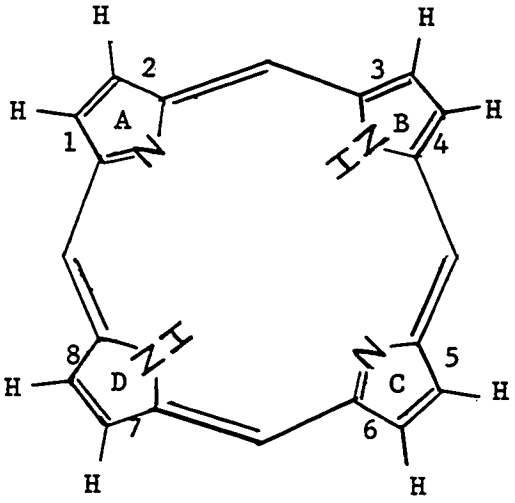
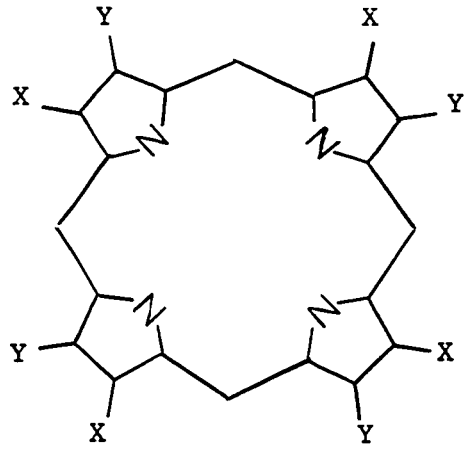
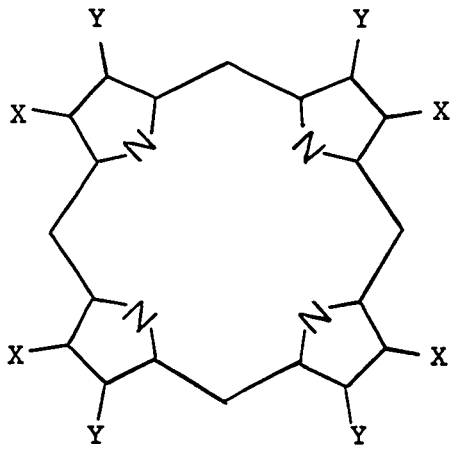


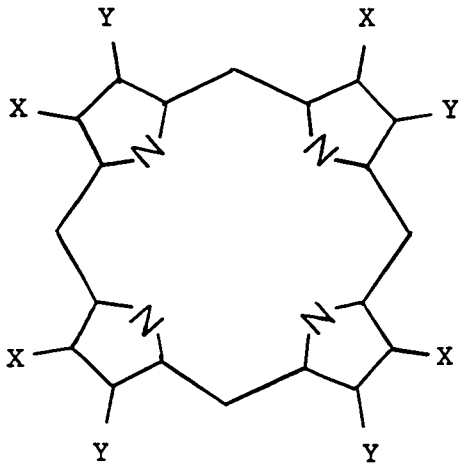
Fig. 1. Structure of Porphin



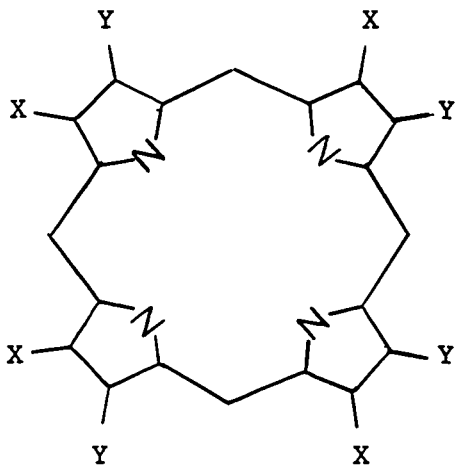
Type I



Type II



Type III



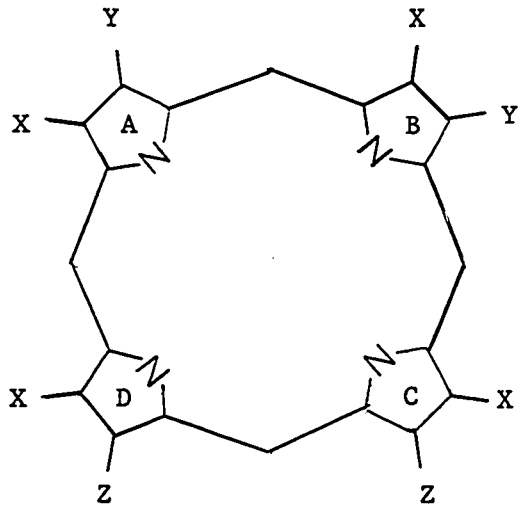
Type IV

For Etioporphyrin X = CH_3; Y = C_2H_5

For Coproporphyrin X = CH_3; Y = CH_2CH_2COOH

For Uroporphyrin X = CH_2COOH; Y = CH_2CH_2COOH

Fig. 2. Diagrammatic Representation of Isomers of Etio-, Copro- and Uroporphyrin



For Mesoporphyrin X = CH_3; Y = C_2H_5; Z = CH_2CH_2COOH

For Protoporphyrin X = CH_3; Y = $CH{=}CH_2$; Z = CH_2CH_2COOH

Fig. 3. Diagrammatic Representation of Structure of Meso- and Protoporphyrins

as either an acidic or basic center.

B. Protoheme Biosynthesis and Control of the Pathway

Since all mammalian cells contain cytochrome, it is not surprizing that all cells have the capacity to synthesize protoheme (Fig. 4C). The major sites of synthesis are erythropoietic tissues and the liver. Protoheme (Fig. 4C) serves as the prosthetic group of hemoglobin, cytochromes, peroxidases, catalases and tryptophan pyrrolase. Work carried out in the laboratories of Shemin and Granick in the United States and Rimington and Neuberger in Great Britain has resulted in almost complete elucidation of the protoheme biosynthetic pathway (DeMatteis, 1967). The enzymes of this pathway are located in the mitochondria and cytoplasm of the cell. It is believed that the initial and final stages occur in mitochondria and that intermediate steps occur in the cytoplasm (Sano & Granick, 1961; DeMatteis, 1967).

The first steps in the biosynthetic process involve the generation of succinyl Co-A and the formation of δ-aminolevulinic acid. Most of the succinyl Co-A is derived from the oxidation of α-ketoglutarate in the tricarboxylic acid cycle (Shemin & Kumin, 1952; Brown, 1958; Granick & Urata, 1963). Small amounts of succinyl Co-A can be generated directly from condensation of succinate and Co-A under the influence of succinyl Co-A synthetase (Shemin & Kumin, 1952).

The succinyl Co-A condenses with glycine in the presence of δ-aminolevulinic acid synthetase (δ-ALA synthetase), yielding an unstable intermediate, α-amino-β-ketoadipic acid, which rapidly decarboxylates (Fig. 4A) to yield δ-aminolevulinic acid (Neuberger, 1961). Pyridoxal phosphate is required as co-factor in this condensation (Lascelles, 1957;

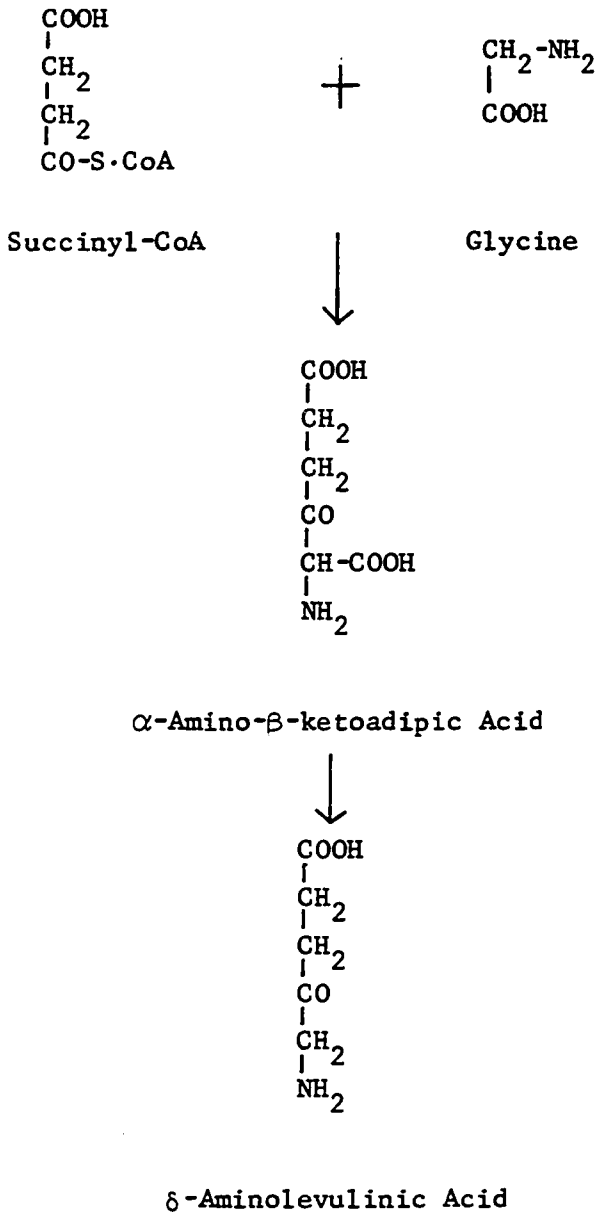


Fig. 4A. Biosynthesis of Protoheme.

Step I - Mitochondrial Formation of δ-Aminolevulinic Acid

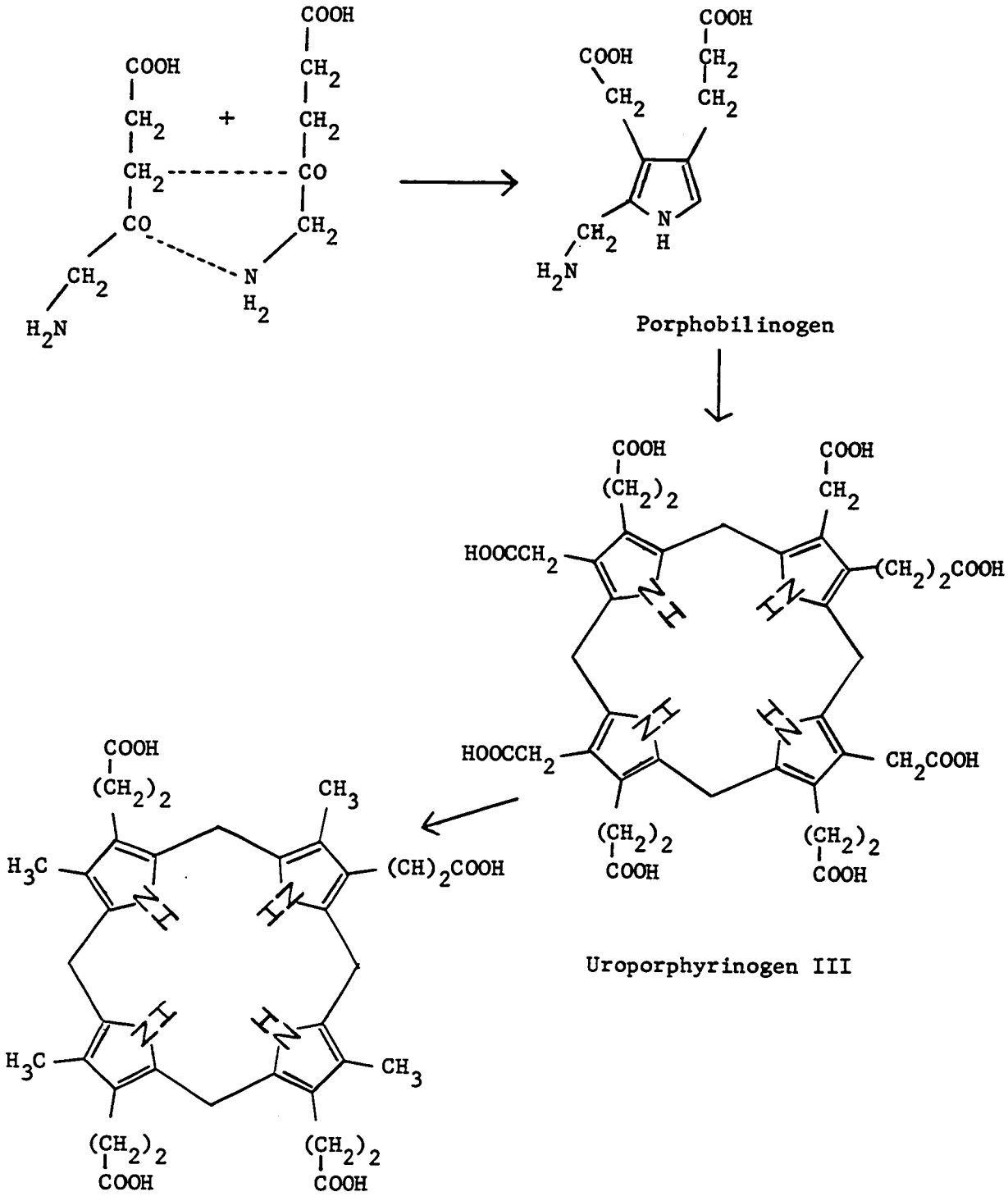


Fig. 4B. Biosynthesis of Protoheme (continued)

Step II - Cytoplasmic Formation of Coproporphyrinogen III

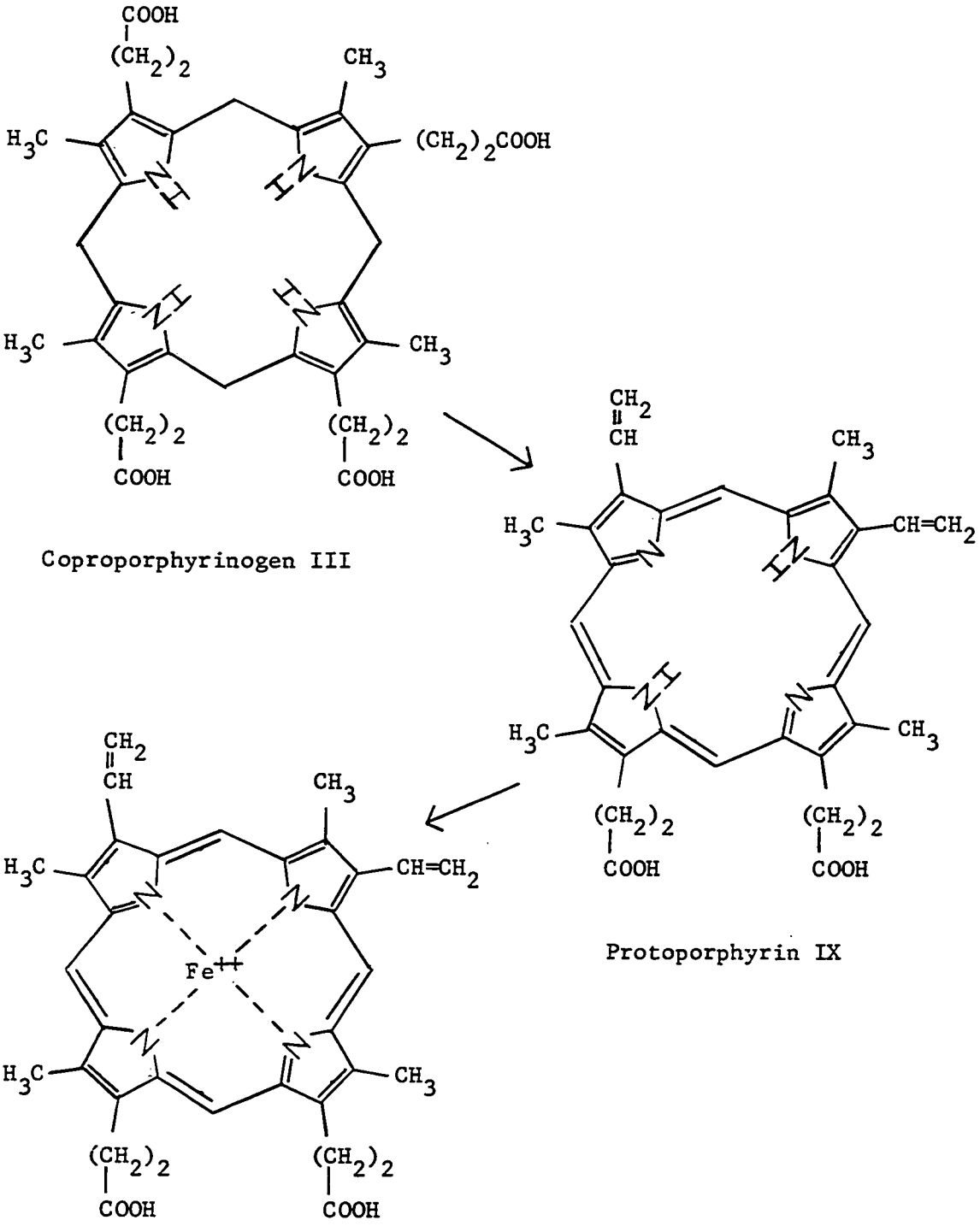


Fig. 4C. Biosynthesis of Protoheme (continued)

Step III - Mitochondrial Formation of Protoheme

Schulman & Richert, 1957).

In the next step, δ-aminolevulinic acid (δ-ALA) formed in the mitochondria diffuses into the cytoplasm where it is converted by the enzyme δ-ALA dehydrase (Gibson et al., 1955) to the monopyrrole porphobilinogen (PBG)(Fig. 4B). Four moles of PBG are then condensed to form uroporphyrinogen III (Fig. 4B) in the presence of two enzymes, uroporphyrinogen III synthetase and urophophyrinogen III cosynthetase (Bogorad, 1958a; Bogorad 1958b; Cornford, 1964). In the next step, the four acetic acid side chains of uroporphyrinogen III are decarboxylated by uroporphyrinogen III decarboxylase (Mauzerall & Granick, 1958), with the formation of coproporphyrinogen III (Fig. 4B). The coproporphyrinogen III synthesized in the cytoplasm diffuses into the mitochondria to be oxidatively decarboxylated to protoporphyrin IX (Fig. 4C) by the enzyme coproporphyrinogen III oxidase (Sano & Granick, 1961). The final step involves insertion of an atom of iron by the enzyme ferrochelatase (Labbe & Hubbard, 1960).

It is worth noting that protoporphyrin IX and the porphyrinogens which are the precursors of this biologically important porphyrin are related to etioporphyrin III. Moreover all metalloporphyrins known to have a metabolic function are derived from the uroporphyrin III isomer.

The localization of the biosynthetic enzymes has prompted Sano and Granick (1961) to suggest that permeability changes of the mitochondrial membrane to δ-ALA and coproporphyrinogen III may control the rate of tetrapyrrole synthesis.

Lascelles (1956) obtained evidence that end-product inhibition played a role in the control of protoheme biosynthesis by means of the following experiments. Growth of Rhodopseudomonas spheroides in an

iron-free medium resulted in the accumulation of porphyrins in the growth medium. The addition of a small amount of iron to the medium greatly decreased the porphyrin accumulation. Since the amount of iron added was very much less than that required to convert porphyrins to metalloporphyrins, it was possible that iron acted catalytically by incorporation into protoporphyrin IX to produce a small amount of protoheme which in turn inhibited the enzyme δ-ALA synthetase. This idea was strongly supported by the demonstration that protohemin was capable of inhibiting the action of δ-ALA synthetase (Gibson et al., 1961; Burnham & Lascelles, 1963). It is worth noting that protohemin was utilized in these experiments due to the difficulty of maintaining protoheme in the reduced state. Recently Karibian and London (1965) have demonstrated that protohemin inhibits the incorporation of glycine-2^{14}C into protoheme in immature rabbit erythrocytes, indicating that protoheme exerted feedback control on its synthesis by inhibiting the activity of δ-ALA synthetase.

Granick and Urata (1963) measured the levels of the enzymes involved in protoheme biosynthesis in guinea pig liver. They showed that while the activity of δ-ALA synthetase, the first enzyme in the pathway, was barely detectable, all the other enzymes were present in considerable quantities. These workers also showed that when guinea pigs are fed the potent porphyria-inducing drug, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)(I), a marked increase in δ-ALA synthetase activity occured; the level of activity of the other enzymes remained unaltered. On the basis of these observations it was suggested that control of tetrapyrrole synthesis is exerted via control of the activity of δ-ALA synthetase. However, it was necessary to determine

whether activation of an inactive enzyme or 'de novo' synthesis was responsible for the increase in the activity of δ-ALA synthetase. To answer this question, Granick (1965) established an elegant technique for growing chick embryo liver cells in culture. Porphyria-inducing drugs were added to the culture medium and the porphyrins which accumulated in the cells were observed under a fluorescence microscope. Granick assumed that the intensity of porphyrin fluorescence reflected the activity of the δ-ALA synthetase. In a subsequent report, Granick (1966) showed that inhibitors of protein synthesis almost completely abolished the increase in the activity of δ-ALA synthetase caused by DDC (I). This observation suggested that DDC acted by causing a 'de novo' synthesis of the enzyme, δ-ALA synthetase, which in turn caused the increased synthesis of the porphyrins. If DDC had acted by activating an inactive form of δ-ALA synthetase, the action of DDC would not have been prevented by an inhibitor of protein synthesis.

C. Defects of Protoheme Biosynthesis: The Porphyrias

Inborn or acquired defects in tetrapyrrole biosynthesis manifested by a large increase in the excretion of porphyrins, their precursors or both have been recognized and are known as porphyrias. In some cases of porphyria, the porphyrins accumulate in bone marrow and in other cases in the liver. For this reason, the porphyrias have been subdivided into two major groups: viz., erythropoietic and hepatic (Schmid et al., 1954).

i. Erythropoietic Pophyrias

Congenital erythropoietic porphyria is a rare disease of

porphyrin metabolism which is recognized at birth or during the first two years of life (Granick & Levere, 1964). This disease is marked by an increased concentration of uroporphyrin I and coproporphyrin I in circulating erythrocytes and in bone marrow, resulting in severe photosensitivity.

Congenital Erythropoietic Protoporphyria was recently described by Magnus and coworkers (1961). Clinically it is differentiated from congenital erythropoietic porphyria by the milder photosensitivity observed. This follows from the fact that it is the level of protoporphyrin IX rather than the level of the highly photosensitizing uroporphyrin I that is increased.

ii. Hepatic Porphyrias

Acute Intermittent Porphyria (AIP) is the most common of the inherited porphyrias. The disease is characterized clinically by gastrointestinal and neurological symptoms, but photosensitivity reactions do not occur (Goldberg & Rimington, 1962). During an acute attack, large quantities of ALA and PBG are excreted in the urine. However, in the latent phase of the disease, the excretion of these porphyrin precursors may be within normal limits or only slightly elevated. Acute attacks of the disease may be precipitated by a number of factors such as drugs, menstruation, pregnancy and infections. The hepatic level of δ-ALA synthetase in a patient with AIP has been shown to be seven times that of non-porphyric controls (Tschudy et al., 1965).

Cutaneous Hepatic Porphyria is most prevalent among the white South African population and usually manifests itself at adolescence. The disease resembles AIP clinically and acute attacks with abdominal pain and neurological symptoms are observed. However, in contrast to

AIP, photosensitivity invariably accompanies the disease. Biochemically, this disease can be distinguished from AIP since fecal concentrations of coproporphyrin and protoporphyrin are elevated even during remissions. In the acute phase of the disease, urinary excretion of uroporphyrin, coproporphyrin, ALA and PBG is increased.

While the porphyrias described above are all genetically predetermined, in recent years evidence has been provided for the occurrence in man of a purely acquired form of hepatic porphyria. The studies of Cam (1959) showed that a hepatic form of porphyria in a large number of Turks was due to the consumption of wheat treated with the fungicide hexachlorobenzene. Photosensitivity was the predominant clinical manifestation (Schmid, 1960). A similar porphyria which is apparently acquired by ingestion of a noxious agent has been described among the Bantus of South Africa (Barnes, 1955). Clinically the disease is characterized by photosensitivity. The etiologic agent or agents which precipitate the condition has not been specifically identified, but some suspicion has been cast upon the large quantities of an adulterated alcoholic beverage consumed by the Bantus (Barnes, 1959).

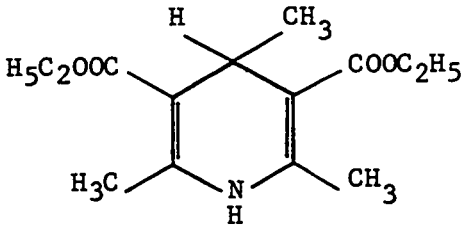
D. Drug Induced Porphyrin Biosynthesis: Experimental Porphyrias

The possibility that barbiturates were involved in the precipitation of attacks of human acute porphyria was suspected soon after the introduction of these drugs into clinical medicine. Convincing evidence for this view was later presented by Waldenstrom (1939). In the past decade a variety of therapeutic agents have been found to affect tetrapyrrole biosynthesis in a manner similar to the barbiturates. These drugs include sulfonamides, non-barbiturate sedatives and hypnotics,

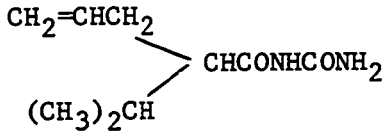
anticonvulsants and griseofulvin (DeMatteis, 1967). Very small quantities of these drugs can trigger an acute and often fatal attack in a patient in the latent phase, or aggravate both the clinical and biochemical features if administered to a porphyric patient in the overt phase of the disease (DeMatteis, 1967). On the other hand, large amounts of these drugs are required to cause increased porphyrin excretion in a person with no known genetic disposition to the disease. Even these large amounts in normal humans do not produce the clinical symptoms of the disease.

Two compounds related to the barbiturates, viz. Sedormid (II) and allylisopropylacetamide (AIA)(III), were found to cause a disordered porphyrin metabolism in livers of normal animals. Such disorders of porphyrin metabolism are known as experimental porphyrias (Goldberg & Rimington, 1955). Solomon and Figge (1959) found that 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC)(I), although chemically unrelated to AIA (III) or Sedormid (II), also produced a similar experimental porphyria. The experimental porphyrias are biochemically similar to the human hepatic porphyria, but the clinical symptoms do not appear to be manifested. Despite this, valuable information relating to the biochemical lesion in the human disease can be obtained from a study of experimental porphyria.

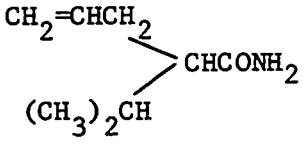
In his cell culture experiments, Granick (1966) found that at low concentrations of the inducing drugs, porphyrin production could be reduced by the addition of protoheme. The metalloporphyrin did not affect the conversion of δ-ALA to porphyrin and did not appear to affect the activity of δ-ALA synthetase in vitro. On the basis of these experiments, and on the ideas of Jacob and Monod (1961) with respect to end-product repression, Granick has postulated a mechanism whereby porphyria-



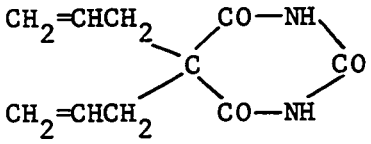
(I)



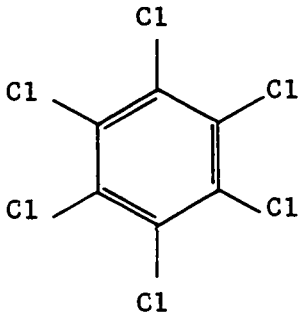
(II)



(III)



(IV)



(V)

Fig. 5. Structures of 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl-pyridine (DDC)(I); Sedormid (II) ; Allylisopropylacetamide (III); 5,5-Diallylbarbituric acid (IV); and Hexachlorobenzene (V).

inducing drugs act.

In the model of end-product repression as proposed by Jacob and Monod (1961) there are three distinct segments of the DNA molecule which are essential for the regulation of genetic expression. These are a regulator gene, an operator gene and a structural gene. The structural gene contains the genetic information needed to synthesize a given enzyme. Transcription of this information into messenger RNA (m-RNA) can be initiated at one site only, the operator site. This operator exists in open and closed positions. In the open position, the structural gene is available to code for the synthesis of m-RNA. On the other hand, when the operator gene is in the closed position, no m-RNA can be synthesized. The operator is closed when it is engaged by a specific repressor synthesized by the regulator gene. In certain cases, this repressor is synthesized in an inactive form or apo-repressor. The apo-repressor becomes active upon combination with a specific co-factor or co-repressor, usually the end product of the metabolic pathway.

The following is a mechanism of action (Fig. 6) suggested by Granick (1966) to account for the drug-induced porphyrin biosynthesis. Protoheme is considered to be the specific co-repressor and when combined with the apo-repressor engages the operator and curtails transcription. When there is insufficient protoheme available for combination with the apo-repressor, the operator will be open, allowing synthesis of new m-RNA. The porphyria-inducing drugs are thought to act by displacing protoheme from the apo-repressor forming an inactive repressor and thus leaving the operator open. This allows the structural gene to code for new m-RNA which in turn leads to the synthesis of more δ-ALA synthetase.

Since the clinical manifestations of AIP cannot be correlated

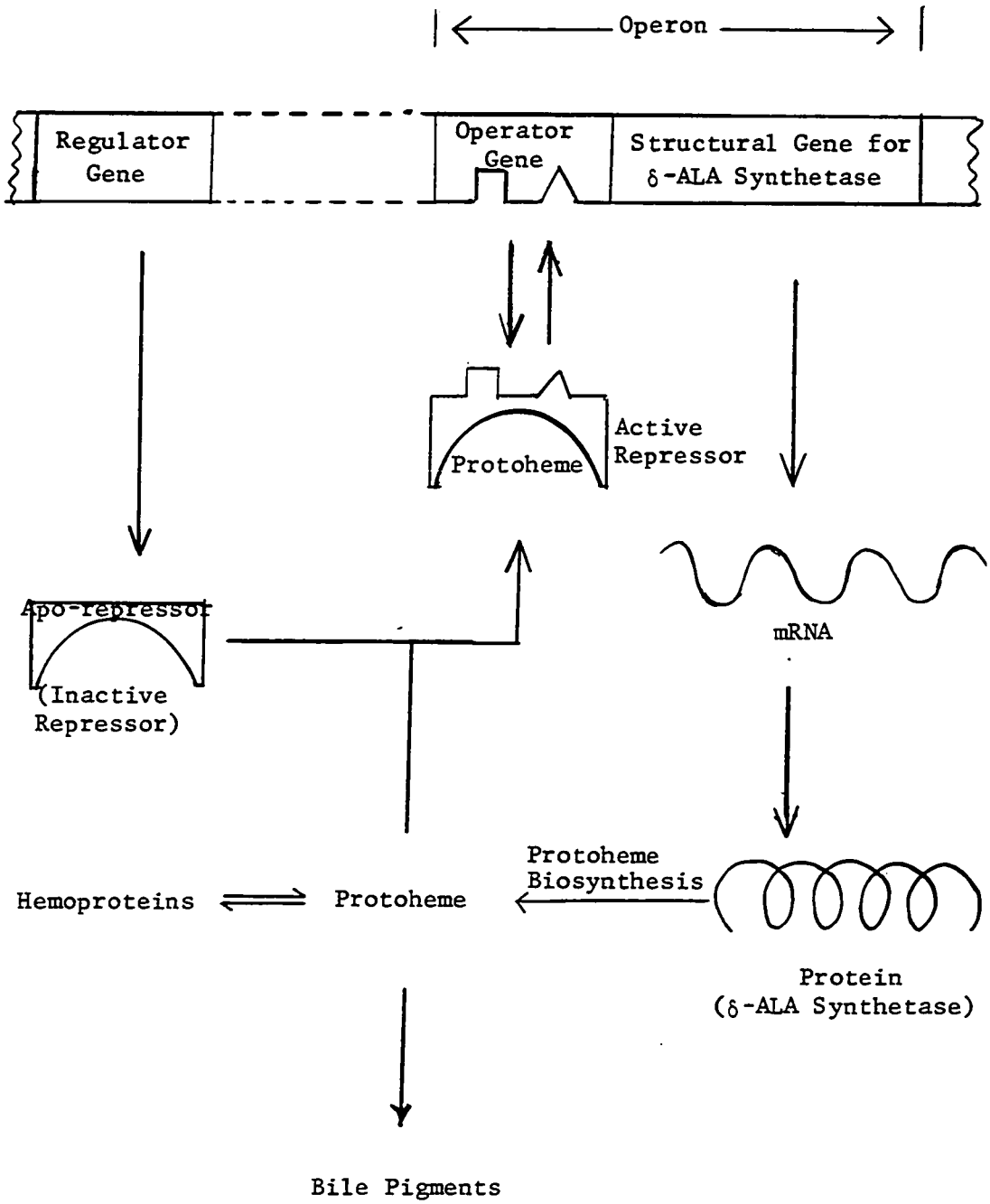


Fig. 6. The Operon Concept Applied to the Regulation of the Synthesis of δ-ALA Synthetase (from DeMatteis, 1967).

with the overproduction of δ-ALA and PBG, Tschudy (1965) has suggested that some other genetic defect is the primary defect which leads secondarily to the induction of δ-ALA synthetase. Many of the drugs which induce porphyria are also inhibitors of biological oxidation (Cowger & Labbe, 1965). These workers have shown that these drugs block the oxidase of reduced nicotinamide-adenine dinucleotide and on this basis have suggested that porphyria represents a specific block in the terminal oxidase. Tschudy (1965) has suggested that, since δ-ALA synthetase is the rate-controlling enzyme in the synthesis of the protoheme moiety required as the prosthetic group for some respiratory enzymes, certain types of respiratory defects may lead to induction of this enzyme.

E. Structure-Activity Relationships

The barbiturates and related compounds which are the drugs most often implicated in porphyria were the first to be studied from the standpoint of structure-activity relationships (Goldberg, 1954). Goldberg and Rimington (1962) suggested that the following structural features were required for activity in this series of compounds: an allyl group together with an amide as in allylisopropylacetamide (AIA) (III), or a ureide as in Sedormid (II), or a cyclic ureide as in the barbiturates, e.g. diallylbarbituric acid (IV). Similar results have been obtained by Stich and Decker (1955) who emphasized the need for a free allyl group, and by Talman et al. (1957).

Recently, several compounds structurally unrelated to the barbiturates have been found to be potent porphyria-inducing compounds, e.g. DDC (I)(Solomon & Figge, 1959); and hexachlorobenzene (V)(Ockner & Schmid, 1961). These findings indicated that the structural

features previously thought to be absolute requirements for activity were incorrect. The above studies, carried out in whole animals, are in some respects unsuitable for the interpretation of pharmacological action on a molecular level. Utilizing the in vitro system of chick embryo liver cells, Granick (1965) was able to examine the activity of some porphyria-inducing drugs at the cellular level. He suggested that steric rather than chemical factors are important and that for optimum activity, a compound should possess a planar portion with a side chain out of this plane. Marks et al. (1965) prepared a series of DDC analogues which were tested in guinea pigs and in the chick embryo liver cell system of Granick. On the basis of these studies, the importance of steric features for activity was emphasized. Thus the vital feature in the DDC molecule was shown to be an ethoxycarbonyl group which was sterically hindered from hydrolysis by two methyl groups. On the basis of these results, it was hard to understand why an allyl group had previously been considered essential for activity. For this reason, Hirsch et al. (1966) re-examined the importance of a free allyl group for activity using the in vitro system devised by Granick. They found that at the cellular level, the allyl group was not essential for activity. It was suggested that the essential feature for activity in the AIA molecule was the amide group which was sterically hindered from hydrolysis by the branching in the molecule.

It would be highly desirable if the molecular features responsible for porphyria-inducing activity could be defined. This might allow the design of drugs without these undesirable features. In order to obtain further information regarding the structural requirements for porphyria-inducing activity, studies have been carried out with a series

of DDC analogues and glutethimide stereoisomers using the chick embryo liver cell system of Granick. These studies are reported in Chapter II of this thesis.

F. Screening Drugs for Porphyria-Inducing Activity

In order to examine the barbiturates and other drugs for porphyria-inducing activity, these drugs were either injected intramuscularly or administered orally to a variety of animals followed by the measurement of urinary and fecal porphyrins and precursors (Solomon & Figge, 1959; Goldberg, 1954; DeMatteis & Rimington, 1963; Rimington & Zeigler, 1963; Marks et al., 1965). Talman et al. (1957) studied the relative porphyria-inducing potencies of the barbiturates and related compounds by injecting the drugs into the yolk sac of eight-day embryonated eggs and measuring the porphyrins which accumulated in the allantoic fluids. On the basis of the latter study, Granick (1964, 1965, 1966) developed an elegant chick embryo liver cell culture system and found useful therapeutic agents to possess marked porphyria-inducing ability. For this reason, Granick (1964) cautioned against the administration of these drugs to known porphyric patients and relatives of these patients. Watson (1966) suggested that many pharmaceuticals, especially the sedatives and hypnotics, should be screened in the cell culture system as latent cases of acute intermittent porphyria are more prevalent than has hitherto been thought.

A comparison of the ability of pyridine compounds to induce porphyria when fed to guinea pigs and when tested in the chick embryo liver cell system, revealed that some compounds which are highly active in cell culture are only weakly active in guinea pigs (Marks et al., 1965).

In view of these results, it appeared possible that many useful drugs might unnecessarily be excluded from use in porphyric patients. We have therefore re-examined the porphyria-inducing activity of therapeutic agents in the intact chick embryo and the results were compared to those previously obtained utilizing the isolated chick embryo liver cell system. By this means, we were able to compare the responsiveness of chick embryo liver cells in culture to those in the intact chick embryo. These results are reported in Chapter III of this thesis.

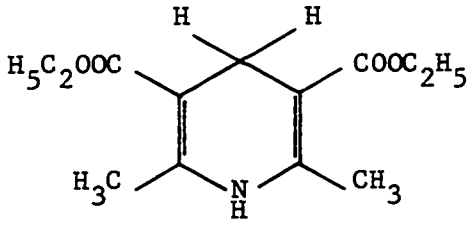
G. Drug Metabolism and Experimental Porphyria

Cytochrome P-450, a protoheme-containing enzyme of the endoplasmic reticulum, is thought to play a key role in the oxidative metabolism of drugs (Conney, 1967). Phenobarbital, in addition to inducing the synthesis of δ-ALA synthetase, also increases the activity of the liver drug metabolizing enzymes and the amount of cytochrome P-450. The increase in activity of drug metabolizing enzymes has been suggested to be caused by an increase in the synthesis of cytochrome P-450 (Orrenius & Ernster, 1964; Remmer & Merker, 1965).

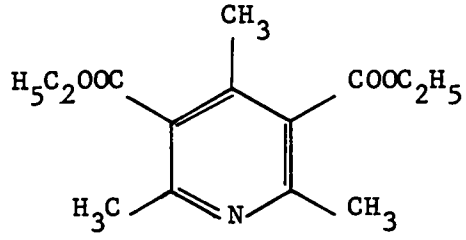
On the basis of these observations and his ideas of de-repression, Granick (1966) has suggested that the porphyrin formed following de-repression of δ-ALA synthetase in response to porphyria-inducing drugs is utilized for the formation of protoheme. This protoheme serves as the prosthetic group of cytochrome P-450 which functions in the detoxification of drugs. This sequence of events would facilitate removal of the drug from the liver. If this hypothesis is correct, all porphyria-inducing drugs should be oxidatively metabolized. Hirsch and coworkers (1967) have recently suggested that the underlying critical

feature for activity in the AIA (III) and DDC (I) series of compounds is an ester or amide group, sterically hindered from hydrolysis. These workers also suggested that drugs which could not be metabolized by a hydrolytic mechanism were oxidatively metabolized, a process requiring increased protoheme and porphyrin formation. Thus on the basis of these ideas, the active porphyria-inducing drug DDC (I) should not be metabolized by a hydrolytic mechanism, whereas the inactive analogue of DDC, viz. 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) should be readily hydrolyzed and thus easily removed from the liver. In order to examine the validity of these ideas, the metabolism of these compounds has been studied in chick embryo liver. These results are reported in Chapter V.

The metabolism of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) in the intact chick embryo has been studied in order to elucidate the cause of its relative inactivity in vivo despite its high activity in the liver cell culture system.



(VI)



(VII)

Fig.7. Structures of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-pyridine (VI), and 3,5-Diethoxycarbonyl-2,4,6-trimethyl-pyridine (VII).

CHAPTER II RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PORPHYRIA-INDUCING ACTIVITY IN MONOLAYER CULTURE OF CHICK EMBRYO LIVER CELLS

Introduction

Resolution of several racemic pharmacologically active compounds into their antipodes and study of their biological activity has revealed that in many cases activity predominates in one of the isomers. Thus, in the case of dl-epinephrine, activity has been shown to reside almost exclusively in the l-isomer. Small molecules whose biological action is specific and structure dependent are thought to have a molecular structure complimentary to the site at which they act. On the basis of this idea, Easson and Stedman (1933) formulated a structure for the α-adrenergic receptor which was complimentary to the structure of l-epinephrine. Beckett and Casy (1954) studied a series of optical enantiomorphs of narcotic analgesics and found that the most active isomer of each pair had a spatial configuration related to that of D-(-)alanine. On the basis of these results and on those obtained from structure-activity relationships, they suggested a possible structure for the analgesic receptor.

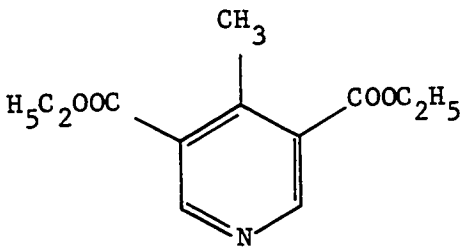
Glutethimide, a sedative and hypnotic drug, has been shown to possess marked porphyria-inducing activity and Granick (1964) has warned against the use of this drug in porphyric patients. Glutethimide has an asymmetric carbon atom and the (+) form of the drug has twice the hypnotic activity of the (-) form (Schmid et al., 1965). We have examined the relative porphyria-inducing potencies of the two isomers for two reasons: (1) if the porphyria-inducing activity resides almost exclusively in one of the optical isomers, a three dimensional picture of the site of action of these drugs could be formulated; (2) if one of the isomers was devoid of porphyria-inducing activity, it could be utilized with greater safety

than the racemic compound in the treatment of porphyric patients.

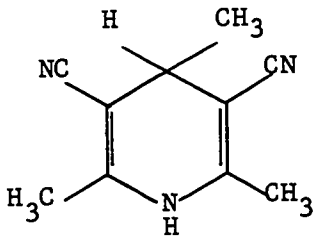
Marks et al. (1965) prepared a series of derivatives of DDC (I) and the corresponding pyridine (VII). They found that the derivatives containing a 4-alkyl substituent possessed marked porphyria-inducing activity. A study of the Fischer-Hirshfelder-Taylor models and the ultraviolet absorption spectra indicated that the 2-, 4- and 6-alkyl groups force the 3- and 5-ethoxycarbonyl substituents out of the plane of the ring. It was suggested that this non-planar configuration of the molecule was essential for activity.

In a subsequent paper, Hirsch et al. (1967) tested the validity of this concept as follows. The 2- and 6-methyl groups of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) were removed, yielding 3,5-diethoxycarbonyl-4-methylpyridine (VIII), a compound in which the ethoxycarbonyl groups are co-planar with the pyridine ring. This compound was shown to be inactive, a result which supported the idea that the two specifically oriented ethoxycarbonyl groups are essential for porphyria-inducing activity. Further studies of Hirsch et al. (1967) led to a revision of the idea that specifically oriented ethoxycarbonyl groups were essential for activity. Instead it was suggested that the critical feature required for activity was an ethoxycarbonyl group protected from hydrolysis by two ortho-methyl substituents. In the present study, we have examined the activity of a series of DDC (I) analogues in which the 4-methyl substituent was replaced by a series of different substituents. If the hypothesis of Hirsch et al. (1967) is correct, it would be anticipated that provided the 4-substituent shields the ethoxycarbonyl groups from hydrolysis, these compounds should be active porphyria-inducing substances. In a previous study, Marks et al. (1965) showed that

replacement of the ethoxycarbonyl substituents with acetyl groups led to a complete loss of activity. To obtain further information on the importance of the ethoxycarbonyl groups for activity, we have examined the activities of analogues (IX and X) containing cyano groups in place of ethoxycarbonyl groups.



(VIII)



(IX)

Fig. 8. Structures of 3,5-Diethoxycarbonyl-4-methylpyridine (VIII), and 3,5-Dicyano-1,4-dihydro-2,4,6-trimethylpyridine (IX).

Experimental

i. Source of Compounds

Racemic glutethimide and its optical isomers were obtained from Dr. H. Keberle, Forschungslaboratorien, der Ciba Aktiengesellschaft, Pharmazeutische Abteilung, Basel. 3,5-Dicyano-1,4-dihydro-2,4,6-trimethylpyridine (IX) and 3,5-dicyano-1,4-dihydro-2,6-dimethyl-4-t-butylpyridine (X) were synthesized by Mr. G.L. Bubbar in our laboratory (Schneck et al., 1968). The other dihydropyridines were supplied by Dr. B. Loev; Smith, Kline and French, Philadelphia, Pa.

ii. Testing of Compounds for Porphyria-Inducing Activity in Chick Embryo Liver Cell Culture

The procedures described below are essentially those of Granick (1966).

Preparation of Reagents

(a) Calcium and magnesium-free Earle's medium

The following substances were dissolved in one liter of water and the pH adjusted with dilute HCl to 6.8:

NaCl	6.8 g
KCl	0.4 g
$NaH_2PO_4 \cdot H_2O$	0.125 g
Dextrose	1.0 g
$NaHCO_3$	2.2 g

The solution was sterilized by filtration through a millipore filter (porosity 0.45 μ) and stored at room temperature.

(b) Medium for culturing liver cells

The cell culture medium had the following composition:

1. 100 Ml Eagle's Basal medium without phenol red (Microbiological Associates).
2. 10,000 I.U. Penicillin G dissolved in 0.1 ml sterile water (Ayerst, McKenna and Harrison).
3. 10 Mg streptomycin sulfate dissolved in 0.25 ml sterile water (Glaxo-Allenburys).
4. 2000 I.U. Mycostatin (Squibb) suspended in 0.2 ml sterile water.
5. 10 Ml Fetal Bovine Serum (Microbiological Associates).
6. 1 Ml of 200 mM Glutamine (Microbiological Associates).

(c) Pangestin solution

One gram of Pangestin (1:75 Difco certified) was suspended in 100 ml of calcium and magnesium-free Earle's medium and allowed to stand for 12 hours at 4°. The pH of the solution was adjusted to 6.8 and the suspension filtered by gravity. The filtrate was sterilized by filtration through a Millipore filter (porosity 0.45 μ) and the sterile Pangestin solution stored in 5 ml aliquots at -15°. The Pangestin used in these experiments is an active preparation of pancreatic enzymes containing primarily amylopsin, trypsin and steapsin.

(c) Preparation of cover slips and vials

The cover slips (size 5/8 inch, thickness 0) were immersed in a mixture of concentrated hydrochloric and nitric acids (1:1) for 24 hours. They were then washed several times by decantation with double distilled water. A single cover slip was then placed into each vial with the aid of forceps. The vials were capped and autoclaved.

(e) Preparation of enzyme solution for digestion of cells

Immediately before use, 6 ml of calcium and magnesium-free Earle's medium were added to a vial containing 100 mg sterile, crystallized and lyophillized trypsin (Worthington Biochemical). Three milliliters of the Pangestin solution were added to the vial and the contents agitated to form a clear solution.

(f) Preparation of liver cells

The livers of two 17-day old chick embryos were removed under aseptic conditions and washed three times with calcium and magnesium-free Earle's solution to remove blood and other debris. These livers were placed in a small petri dish containing the enzyme solution and chopped with a sterile razor blade into tiny fragments. The mixture was then incubated at 38° until most of the cells were separated (approximately 20 minutes). During this period, the suspension was gently drawn up and down in a large bore Pasteur pipette to aid in separating the cells. The separated cell suspension was transferred to a centrifuge tube and allowed to stand undisturbed for 5 minutes to allow the larger particles to settle. The suspension minus the large particles was then transferred to a second centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and discarded and the cells resuspended in 9 ml of calcium and magnesium-free Earle's medium. Aliquots of this cell suspension (0.05 ml) were added to the vials containing a cover slip and 1 ml of culture medium. The cells were incubated at 37° in an atmosphere of moist 95% air and 5% carbon dioxide and after a 24 hour period the culture medium was removed and replaced with fresh medium. The drugs dissolved in 1 or 2 μliters of 95% ethanol were then added to the culture medium and the cells incubated

for another 24 hour period.

(g) Counting the liver cells

A small aliquot of the liver cell suspension was diluted 1:100 with 0.9% saline in a serological pipette, and counted on a hematocytometer. The average number of cells found was 3.4×10^5 per 0.05 ml of cell suspension.

(h) Measurement of fluorescence intensity

The cover slips supporting a monolayer of liver cells were carefully removed from the vials, inverted onto glass microscope slides and gently blotted. The cover slips were then sealed to the slide with a ring of molten paraffin to prevent dehydration of the cells. The cover slip surfaces were again washed with water and blotted dry. Each slide was then examined under a fluorescence microscope to detect porphyrins present in the cells.

(g) Testing drugs for porphyria-inducing activity

In each experiment, the drug was tested in triplicate at each concentration and all drugs were tested in at least two separate experiments. Several vials to which 1 or 2 microliters of ethanol had been added were included as controls in each experiment. To reduce bias in scoring results, the vials were identified only by numbers and so arranged that the scorer was unaware of its drug content. DDC (I) was tested in each experiment as a standard porphyria-inducing agent in order to be able to compare the responsiveness of cells from one experiment to another. The fluorescence intensity was scored according to the system devised by Granick (1966) as follows:

4 All colonies fluoresce intensely

3 Most colonies fluoresce intensely

2 Most colonies fluoresce partially

1 Some colonies fluoresce partially

Fig. 9A is a phase contrast photomicrograph of a colony of chick embryo liver cells grown as a monolayer on a coverslip. According to Granick (1966), the cytoplasmic streaming observed at the periphery of the colony is indicative of healthy cellular growth. Fig. 9B is a photomicrograph of ultraviolet irradiated cells which had been incubated in the presence of a porphyria-inducing drug for twenty-four hours. The results obtained in the cell culture experiments are recorded in Table I.

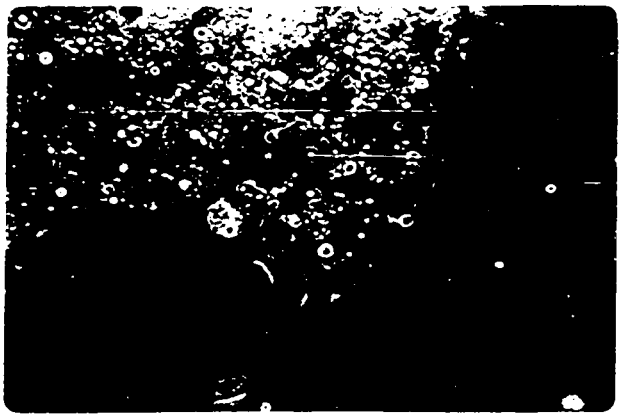
Results and Discussion

The results in Table I reveal that the two optical antipodes of glutethimide, previously demonstrated to differ in hypnotic potency, are apparently equipotent as porphyria-inducing drugs. The reasons for this difference might be explained by one or all of the following: (1) The asymmetric center of glutethimide may be involved in eliciting hypnotic response but not in eliciting porphyria-induction. (2) The antipodes of glutethimide are metabolized differently in vivo (Keberle et al., 1962). Therefore it is possible that the difference in hypnotic potency observed is simply a reflection of this factor. (3) It is possible that a small difference in porphyria-inducing activity exists between the two antipodes which cannot be demonstrated by the cell culture technique. We thus concluded from this study that: (1) A three dimensional picture of the site of action of porphyria-inducing

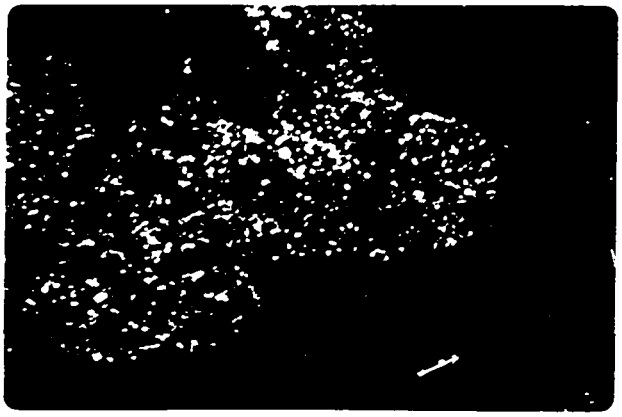
A

B

Fig. 9. Photomicrograph of Chick Embryo Liver Cells Under Phase Contrast Microscope (A);
Photomicrograph of Fluorescing Cells When Irradiated With Ultraviolet Light (B).



A



B

Fig. 9. Photomicrograph of Chick Embryo Liver Cells Under Phase Contrast Microscope (A);
Photomicrograph of Fluorescing Cells When Irradiated With Ultraviolet Light (B).

TABLE I. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY GLUTETHIMIDE AND DIHYDROPYRIDINES AND MEASURED BY FLUORESCENCE MICROSCOPY

Compound	Concentration		Intensity of Fluorescence			Mean
	(μM)	(μg/ml)	Expt.1	Expt.2	Expt.3	
Glutethimide (± isomer)	46	10	2.5	1	1.5	
			2.5	2.5	2.5	
			1.5	2	2	2
	9	2	0.5	0.5	1	
			0.5	0.5	1.5	
			0.5	0.5	T	0.5
Glutethimide (- isomer)	46	10	3	2.5	1.5	
			2.5	2	1.5	
			3.5	1.5	2	2
	9	2	0.5	T	1	
			0.5	0.5	1	
			0.5	0.5	0.5	0.5
Glutethimide (+ isomer)	46	10	2.5	1.5	1.5	
			2.5	1.5	1.5	
			2.5	1.5	2	2
	9	2	0.5	0.5	T	
			0.5	0.5	T	
			0.5	T	0.5	0.5

TABLE I Continued

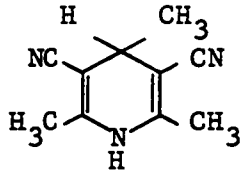
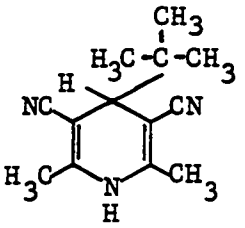
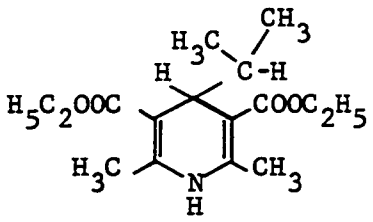
Compound	Concentration (μM)	Concentration (μg/ml)	Intensity of Fluorescence Expt.1	Expt.2	Expt.3	Mean
 (IX)	292	50	0	0		
			0	0		
			0.5	0		0
	29	5	0	0		
			T	0		
			0	0		0
 (X)	233	50	1.5	0	0.5	
			1	T	T	
			1	0.5	0.5	0.5
	23	5	T	T	T	
			T	0	0	
			T	T	0	T
 (XI)	170	50	2.5	2.5	2.5	
			3	3	2	
			3	2.5	2.5	2.5
	17	5	2	2	3	
			2	1.5	2.5	
			2	2	2	2
	1.7	0.5		0.5		
				0.5		
				0.5		0.5

TABLE I Continued

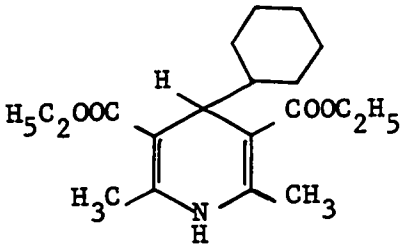
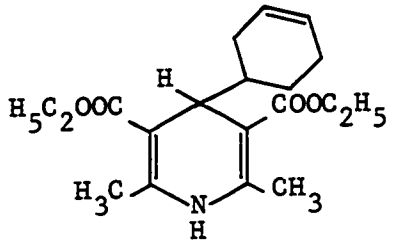
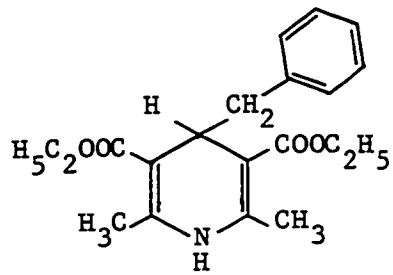
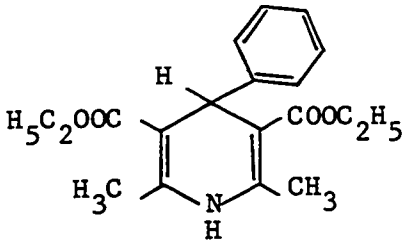
Compound	Concentration (μM)	Concentration (μg/ml)	Intensity of Fluorescence Expt.1	Expt.2	Expt.3	Mean
 (XII)	143	50	0	0.5	0	
			0	T	0	
			0	0.5	T	T
	14	5	0.5	0	0.5	
			T	0	0.5	
			0.5	T	T	T
	1.4	0.5	0	0		
			0	0		
			T	0		0
 (XIII)	144	50	1.5	1.5		
			0.5	1		
			T	1.5		1
	14	5	0	1		
			T	1		
			T	1		0.5
 (XIV)	146	50	T	1		
			0.5	0.5		
			0.5	0.5		0.5
	15	5	0.5	T		
			0.5	T		
			T	0		T
	1.5	0.5		0		
				0		
				0		0

TABLE I Continued

Compound	Concentration (μM)	Concentration (μg/ml)	Intensity of Fluorescence Expt.1	Expt.2	Expt.3	Mean
 (XV)	15	5	3	1.5	1.5	
			3.5	1	1.5	
			3.5	T	2	2
	1.5	0.5	0.5	T	T	
			0.5	T	T	
			0.5	T	0.5	0.5

T = Trace.

drugs could not be formulated on the basis of the structure of the glutethimide isomers. (2) Neither of the antipodes of glutethimide could be used with safety in the treatment of porphyric patients.

The inactivity of the two compounds in which the ethoxycarbonyl groups are replaced by cyano groups (Table I; IX and X) is consistent with the studies of Marks et al. (1965) indicating that an ethoxycarbonyl group with two ortho-methyl groups is essential for activity in this series of compounds.

The high degree of activity observed with 3,5 diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-isopropylpyridine (XI) is in accord with the hypothesis of Hirsch et al. (1967) that the 2-,4- and 6-alkyl substituents shield the ethoxycarbonyl groups from hydrolysis. The weak activity of the dihydropyridine analogues (XII, XIII and XIV) was not anticipated since these compounds all have large substituents in the four-position and in addition, have methyl groups in the 2- and 6-positions. Thus the degree of activity of the 3,5-diethoxycarbonyldihydropyridines (XII, XIII and XIV) with substituents in the 2-, 4- and 6-positions was not predictable on the basis of the hypothesis of Hirsch et al. (1967). A possible factor complicating the interpretation of the results is the following: Loev and Snader (1965) have demonstrated that oxidation of the dihydropyridines containing a 4-isopropyl (XI), 4-cyclohexyl (XII), 4-cyclohex-3-enyl (XIII), or 4-benzyl (XIV) substituent results in loss of the 4-substituent in addition to aromatization. This transformation occurs when there is crowding at the 4-position due to the large size of the 4-substituent and where a stable carbonium ion can be formed from the 4-substituent. It is possible that oxidative liver enzymes remove the substituent in the 4-position leaving the ethoxycarbonyl substituents

open to hydrolysis and subsequent inactivation of the drug.

The moderate porphyria-inducing activity of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine (Table I; XV) was surprizing because this compound had been reported to be inactive when administered to guinea pigs via gastric intubation (Marks et al., 1965). This phenomenon of a compound exhibiting activity in the cell culture system and yet possessing no activity in vivo has been observed previously with other compounds (Marks et al., 1965). The reason for this has been investigated and is reported in a subsequent chapter of this thesis.

CHAPTER III DRUG-INDUCED PORPHYRIN BIOSYNTHESIS IN THE INTACT CHICK EMBRYO

Introduction

The ability of drugs to induce porphyria has been studied by a variety of methods. A common method has been oral administration to mice (DeMatteis et al., 1961; DeMatteis & Rimington, 1963; Nakao et al., 1967), rats (Ockner & Schmid, 1961), or rabbits (Schmid & Schwartz, 1952; Stich & Decker, 1955; DeMatteis et al., 1961). A second method involved drug administration by gastric intubation to guinea pigs, rabbits and rats (Goldberg, 1954; Stich & Decker, 1955; Rimington & Ziegler, 1963; Granick, 1965; Marks et al., 1965). Administration of drugs to animals by intramuscular or intraperitoneal injection has been another way of studying porphyria-inducing activity (Case, Aldrich & Nevé, 1953; Goldberg, 1954). In these studies, the degree of porphyria was assessed by measuring the amount of porphyrins and their precursors in the urine and feces. Rimington and Ziegler (1963) examined the ability of chlorinated benzenes to induce porphyria by feeding these compounds to rats. They assessed the degree of porphyria by measuring the amount of porphyrins in the liver. Talman et al. (1957) developed the following procedure for studying porphyria-inducing activity: drugs were injected into the yolk sacs of 8-day embryonated eggs and the degree of porphyria was assessed by measuring the porphyrins which accumulated in the allantoic fluids. These screening procedures were time consuming and required at least several days to perform. Since the relationship between dose and response was not determined in any of these studies, it is possible that some compounds reported to be inactive may have been active at higher doses. In order to screen a large number of drugs for porphyria-inducing activity in vivo, it is important to have a screening procedure which can

be performed easily and yield reliable results. This chapter describes a simplified procedure for determining porphyria-inducing activity in the intact chick embryo by measuring porphyrin accumulation in the liver.

Utilizing the chick embryo liver cell system, Granick (1964, 1965, 1966) examined a large number of drugs for porphyria-inducing activity. On the basis of these results, he cautioned against the use of glutethimide, methsuximide, phensuximide, the barbiturates, mephenytoin, griseofulvin and other drugs in patients or relatives of patients with porphyria. It has been suggested that many pharmaceuticals, expecially sedatives and hypnotics, should be screened in the cell culture system for porphyria-inducing activity (Watson, 1966).

The results presented in Chapter II of this thesis indicated that liver cells, cultured as a monolayer, may respond differently to porphyria-inducing agents than do liver cells in vivo. This phenomenon of a drug inducing porphyria in vitro but possessing no activity in vivo has been observed previously: (1) Marks et al. (1965) found that 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) was active in vitro but inactive when administered by gastric intubation to guinea pigs. (2) Goldberg and Rimington (1962) found α-propylvaleramide to be devoid of activity in vivo, yet when subsequently tested in the chick embryo liver cell culture system, this compound was shown to possess moderate porphyria-inducing activity (Hirsch et al., 1966).

It appeared possible that on the basis of the in vitro test, several valuable drugs might be unnecessarily excluded from use in porphyric patients. For this reason, we have utilized the procedure developed with the intact chick embryo to determine the porphyria-inducing activity of drugs whose activity had previously been determined

in chick embryo liver cell culture. Differences were expected in view of the following considerations. (1) In cell culture, the effect of drugs is studied on cells in an artificial environment without interference by nervous and humoral factors present in the intact organism. (2) In cell culture, the cells are released from the normal 3-dimensional framework and the influence of intercellular contact is diminished. (3) Drug activities observed in cell culture are independent of the effects of absorption, distribution and excretion which complicates the activities observed with a drug in the intact organism (Schindler, 1969).

Experimental

i. Source of Compounds

The pyridines (VII), dihydropyridines (I, VI and XVI), 1,4-diethoxycarbonyl-2,3,5,6-tetramethylbenzene (XVII) and α-propylvaleramide were prepared previously in our laboratory (Marks et al., 1965; Hirsch et al., 1967). Hexachlorobenzene was purchased from Fisher Scientific, Fair Lawn, N.J., and coproporphyrin I tetramethyl ester (grade B) from Calbiochem, Los Angeles. Allylisopropylacetamide and methyprylon were obtained from Hoffman La Roche, Montreal, glutethimide from Dr. H. Keberle, Forschungslaboratorien, der Ciba Aktiengesellschaft, Pharmazeutische Abteilung, Basel, and α-isopropylvaleramide from Prof. C. Rimington, University College Hospital, London. Mephenytoin was supplied by Sandoz Pharmaceuticals, Dorval, Quebec. Sodium phenobarbital, sodium diphenylhydantoin and meprobamate were supplied by Dr. L. G. Chatten, Faculty of Pharmacy, University of Alberta; Bemegride by Abbott Laboratories, Montreal, Quebec; methsuximide and phensuximide by Parke, Davis & Co., Ann Arbor, Michigan. Sodium secobarbital was supplied by Eli Lilly & Co., Indianapolis,

Ind., diallylbarbituric acid by Ciba Pharmaceutical Products, Summit, N.J., and amobarbital and aprobarbital by Drs. R. F. Labbe and M. L. Cowger, University of Washington, Seattle, Wash. All reagents and chemicals used were of reagent grade and available commercially.

ii. Source of Embryos

Fertilized eggs used were of a white Leghorn strain obtained from the University of Alberta Farm. They were incubated at 38° and a relative humidity of 68% in a Humidaire Model 50 incubator allowing automatic rotation of the eggs every hour. The age of the embryo was taken as the number of days from the onset of incubation.

iii. Procedure for Administration of Drug to 17-Day Chick Embryos

The compound under study was dissolved in dimethylsulfoxide (DMSO; 0.1 ml). DMSO was the solvent selected because it readily dissolved most of the drugs to be tested and it produced no apparent toxicity. For accurate injection of this small volume, a sterile 1 inch 21 gauge disposable needle was attached to the tip of a graduated 0.2 ml pipette. The egg was cleansed with 70 percent ethanol and a small hole was made in the egg shell above the air sac. The drug was injected through the chorioallantois into the fluids surrounding the embryo. The opening in the shell was covered with cellophane tape and the chick embryo incubated at 38°. At the completion of the incubation period, the embryo was sacrificed and the entire liver, weighing approximately 0.4 g was removed for extraction of porphyrins.

iv. Calibration of the Fluorescence Curve for Coproporphyrin I

A stock solution of coproporphyrin I in 1 N HCl was prepared by the procedure of Talman (1958) as modified by Schwartz and described

below. An accurately weighed quantity of coproporphyrin I tetramethyl-ester (approx. 0.5 mg) was hydrolyzed overnight with 1 ml of 7.5 N HCl and then made up to 100 ml with 1 N HCl. This stock solution was then diluted with 1 N HCl so that calibration curves covering a concentration range of 0.1 to 10 μg/100 ml were obtained. When stored in an amber-colored bottle at 4° this stock solution is stable for several months. Granick (1966) has shown that coproporphyrin III constitutes over 80 percent of the total porphyrins present in induced liver cells. Since the fluorescence properties of coproporphyrin I and coproporphyrin III are identical and since coproporphyrin I is more readily available, it was selected as the standard.

All fluorometric determinations were performed utilizing a Turner Model 110 Fluorometer with a 405 mμ band pass primary filter and a Wratten No.25 (595 mμ) sharp cut secondary filter. The latter filter passes all light above 595 mμ. The resulting calibration curves are shown in Fig.10.

v. Extraction and Estimation of Porphyrins in Chick Embryo Liver

The procedure was a modification of that described by Schwartz et al. (1960). The liver (approx. 0.4 g) and 5 ml of ethyl acetate-acetic acid (4:1) were placed in a Potter-Elvehjem apparatus and the mixture homogenized. After centrifugation of the homogenate at 2500 rpm, the supernatant was decanted into a separatory funnel. The residue was resuspended in 5 ml of ethyl acetate-acetic acid (4:1) and homogenized. After centrifugation the supernatant was added to the first extract in the separatory funnel. This extraction was repeated for a third time. Ten ml of sodium acetate solution (3%) and 1 drop of 0.1 percent iodine in ethanol were added to the combined ethyl acetate-

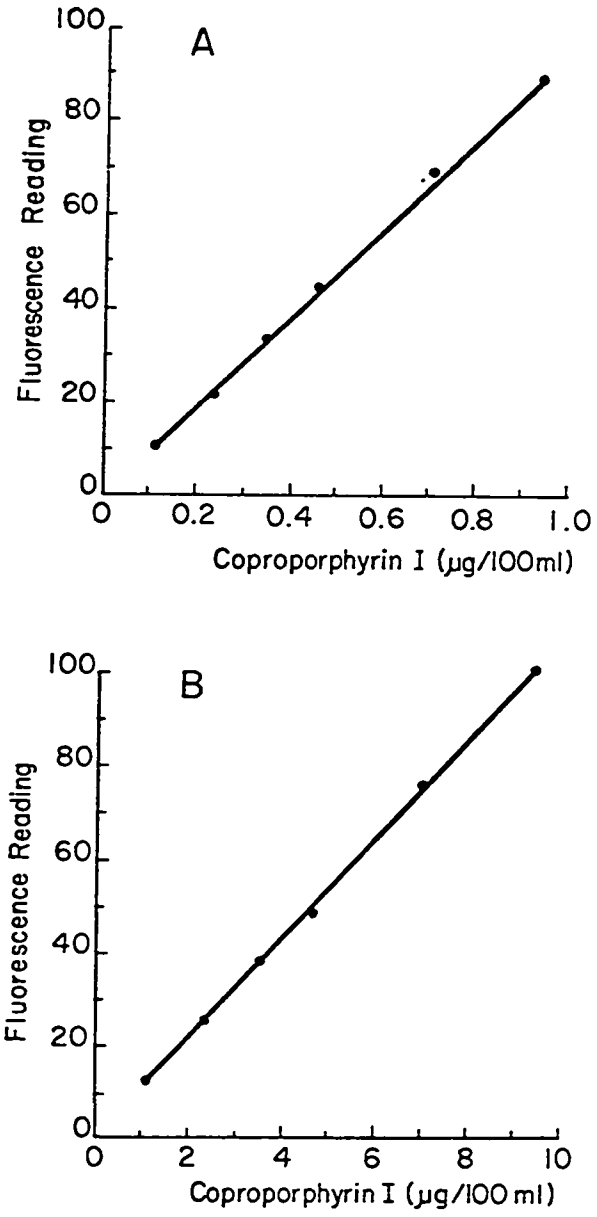


Fig. 10. Porphyrin Standard Calibration Curves; Instrument Sensitivity 30X (A) and Instrument Sensitivity 3X (B).

acetic acid extract. After thorough agitation, the lower layer containing the aqueous phase was removed and discarded. The organic layer was washed with 10 ml of 3 percent sodium acetate solution.

Porphyrins (coproporphyrin and/or protoporphyrin) were extracted from the organic phase with three portions (10 ml) of 3N HCl. The first of these acid extracts was examined for the presence of porphyrin fluorescence under a long wavelength ultraviolet lamp. The limit of detection by this qualitative method was 1 μg of porphyrin per gram liver. The volume of the combined acid extracts was made up to 100 ml with distilled water and the porphyrin content determined fluorometrically using 1N HCl as the reagent blank. Results are expressed as μg coproporphyrin per gram wet weight of liver.

vi. Recovery of Added Porphyrin to Liver

Coproporphyrin I in HCl solution was added to livers obtained from untreated embryos. The acid was neutralized with an equivalent amount of NaOH and the porphyrin extracted as described above. Recovery was found to be 97.3 ± 1.5 % (± SE of mean, n = 5).

vii. Relationship Between Dose of DDC Administered and Porphyrin Accumulation in Chick Embryo Liver

3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (0.1 mg) in DMSO (0.1 ml) was injected into chick embryos in the manner described above. After incubation at 38° for 24 hours, the liver porphyrin content was determined. This experiment was repeated using 0.5, 1, 2, 4, 8, 16 and 32 mg of DDC. The results are shown in Table II.

viii. The Rate of Porphyrin Accumulation in Liver Following Injection of 4 mg DDC into Chick Embryos

DDC (4 mg) dissolved in 0.1 ml DMSO was injected into the fluids surrounding the embryo as described above. The embryos were sacrificed at 0.5, 3, 6, 10, 12, 16, 20, 24, 30, 36 and 48 hours following drug administration and porphyrin accumulation determined. The results are shown in Table III.

ix. Induction of Porphyria in Mice

The compounds dissolved in saline or sesame oil (approx. 0.2 ml) were injected intraperitoneally once daily into mice. Insoluble compounds were suspended in sesame oil prior to injection. In one series of experiments only one injection was given. In a second series, the drug was injected once daily for 4 days and in a third series, once daily for 14 days. DMSO was found to be unsuitable as a solvent for drugs as it was toxic to mice when administered once daily for several days. After the desired time period, animals were sacrificed and a portion (weighing approx. 0.2 g) of each lobe of the liver was removed for extraction of porphyrins. The above studies in mice were carried out with the assistance of T. Theman and D. Eliason, summer students in this department. The conclusions from this work are summarized in Table V for comparison with the results obtained in the chick embryo. If no significant increase in porphyrin content of the liver was detected, the compound was designated as inactive.

x. Induction of Porphyria in Chicks

Hexachlorobenzene was administered as a 1.5% mixture by weight in the diet while griseofulvin was administered as a 2.5% mixture.

Feeding, ad libitum, was begun on the first day after hatching and continued for 6 days. At this time the animals were sacrificed and the livers removed for analysis of porphyrins.

Results and Discussion

The first objective of these studies was to determine the relationship between the porphyrin accumulation in chick embryo liver and the administered dose of a known porphyria-inducing drug, viz. DDC (I). The latter was to be used throughout as the standard of comparison. This information (Table II) was required for rational selection of dose ranges for the drugs subsequently to be tested.

The next objective was to determine the time required for a porphyria-inducing agent to induce maximal porphyrin accumulation. The accumulation of porphyrins in the chick embryo liver at different time periods following administration of DDC is recorded in Table III. Porphyrin accumulation was detected as early as three hours after injection of the drug so that the time required for the induction of the enzyme, δ-ALA synthetase, is relatively short. From the results in Table III it was decided to select 24 hours as an appropriate and convenient time period for studying drug-induced porphyrin accumulation since little or no significant increase occurred after this time.

The first series of compounds tested were those possessing dihydropyridine, pyridine and benzene rings. Having been previously screened for porphyria-inducing activity both in vivo and in monolayer cultures of chick embryo liver cells, these compounds served as a means whereby the validity of the intact chick embryo procedure as a screening

TABLE II. PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVERS 24 HOURS AFTER INJECTION OF DIFFERENT DOSES OF DDC

Dose (mg/egg)	Porphyria-Inducing Activity (μg porphyrin/g liver)*		Number of Experiments
Control	0.46	(0.23 - 0.99)	20
0.1	6.2	(3.9 - 9.0)	5
0.5	13.5	(3.2 - 16.4)	5
1.0	19.7	(4.9 - 40)	10
2.0	103	(15.0 - 195)	8
4.0	136	(72 - 208)	10
8.0	156	(72 - 327)	10
16.0	184	(64 - 225)	8
32.0	200	(65 - 558)	8

* Expressed as coproporphyrin I/wet weight liver, the extreme values are given in parentheses.

TABLE III. PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER WITH TIME FOLLOWING INJECTION OF 4 MG DDC

Time (Hr)	Porphyria-Inducing Activity (μg porphyrin/g liver)*		Number of Experiments
0.5	0.37	(0.35 - 0.39)	5
3	1.10	(0.91 - 1.28)	5
6	9.1	(5.9 - 12.9)	7
10	55	(32 - 65)	5
12	43	(30 - 73)	10
16	87	(68 - 103)	5
20	112	(84 - 159)	5
24	136	(72 - 208)	10
30	173	(92 - 272)	5
36	155	(43 - 212)	5
48	179	(94 - 296)	7
Control (no drug added)	0.46	(0.23 - 0.99)	20

*Expressed as coproporphyrin I/wet weight liver, the extreme values are given in parentheses.

test could be studied. The results obtained with the dihydropyridines (I, VI and XVI) in the intact chick embryo (Table IV) corresponded with results obtained previously using monolayer cultures of chick embryo liver cells (Marks et al., 1965; Granick, 1966), and oral administration to guinea pigs (Solomon & Figge, 1959; Granick, 1965; Marks et al., 1965). In all three test procedures, dihydropyridines I and XVI, exhibited marked porphyria-inducing activity while VI was inactive. On the other hand, the related pyridine compound (VII) and the benzenoid compound (XVII) which possessed marked activity in monolayer cultures of chick embryo liver cells (Hirsch et al., 1967) were practically devoid of activity in the intact chick embryo (Table IV). The pyridine compound (VII) had been shown previously to possess no porphyria-inducing activity when administered via gastric intubation to guinea pigs (Marks et al., 1965). Administration of compounds I, VII and XVII to mice by intraperitoneal injection (Table V) yielded results similar to those obtained in the intact chick embryo. As a system for detecting porphyria-inducing activity, the intact chick embryo appears from these preliminary studies to yield results similar to those from other in vivo screening procedures. Attempts to test 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine (XV) and 3,5-diethoxycarbonyl-1,4-dihydro-4-methylpyridine in the intact chick embryo were unsuccessful because of their toxicity.

The results obtained with allylisopropylacetamide (AIA), α-propylvaleramide, sodium secobarbital, diallylbarbituric acid, and phenobarbital (Table VI) using the intact chick embryo corresponded to the results previously obtained with monolayer cultures of chick embryo liver cells (Hirsch et al., 1966, 1967; Schneck et al., 1968). Thus,

TABLE IV. PORPHYRIN ACCUMULATION IN LIVERS OF CHICK EMBRYOS INDUCED BY COMPOUNDS CONTAINING PYRIDINE, DIHYDROPYRIDINE AND BENZENE RINGS

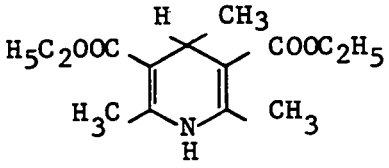
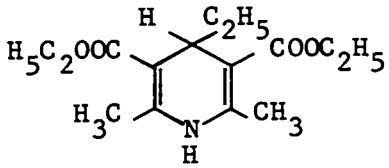
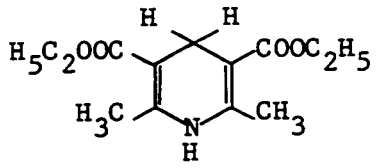
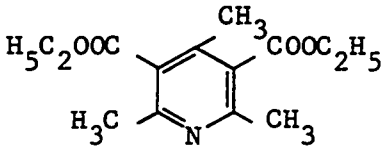
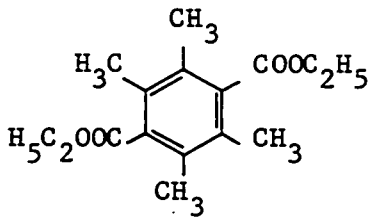
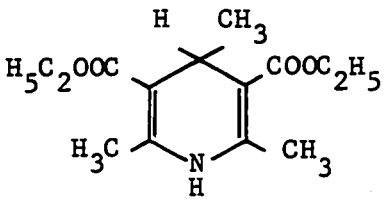
Compound	Dose (mg/egg)	Porphyria-Inducing Activity* (μg porphyrin/g liver)		No. of Expts.
Control (only solvent given)		0.46	(0.23 - 0.99)	20
 (I)	0.1	6.2	(3.9 - 9.0)	5
	1.0	19.7	(4.9 - 40)	10
	4.0	136	(72 - 208)	10
 (XVI)	0.1	6.5	(2.4 - 10.4)	5
	0.5	84	(44 - 204)	5
	1.0	186	(102 - 249)	5
	4.0	217	(201 - 247)	5
 (VI)	3.8	0.35	(0.32 - 0.38)	5
	7.6	0.46	(0.37 - 0.71)	5

TABLE IV. Continued

Compound	Dose (mg/egg)	Porphyria-Inducing Activity* (μg porphyrin/g liver)	No. of Expts.
 (VII)	4.0	0.70 (0.37 - 0.94)	8
	8.0	1.1 (0.78 - 1.7)	5
	16.0	2.1 (0.36 - 9.7)	7
 (XVII)	4.2	0.51 (0.32 - 0.72)	5
	8.4	0.42 (0.37 - 0.50)	5

* Expressed as coproporphyrin I/wet weight liver, the extreme values are given in parentheses.

TABLE V. PORPHYRIN ACCUMULATION IN LIVERS OF MICE INDUCED BY DRUGS

Compound	Dose (mg/kg)	Porphyria-Inducing Activity (μg porphyrins/g liver)*	
Control		0.26	(0.21 - 0.35)
 (I)	25	6.9	(0.46 - 25.1)
	50	54	(1.6 - 175)
	100	62	(3.5 - 135)
	150	86	(15.4 - 158)
Allylisopropylacetamide (III)	75	1.2	(0.76 - 3.2)
	150	4.6	(1.6 - 7.8)

The following compounds did not induce porphyria in mice, compounds VII and XVII, α-propylvaleramide, sodium secobarbital, glutethimide, mephenytoin, methyprylon, sodium diphenylhydantoin, meprobamate, bemegride, methsuximide and phensuximide.

* Expressed as coproporphyrin I/wet weight liver, the extreme values are given in parentheses.

Each drug was tested in at least 5 animals at each dose.

TABLE VI. PORPHYRIN ACCUMULATION IN LIVERS OF CHICK EMBRYOS INDUCED BY BARBITURATES AND RELATED COMPOUNDS

Compound	Dose (mg/egg)	Porphyria-Inducing Activity (μg porphyrin/g liver)*		No. of Expts.
Control (only solvent given)		0.46	(0.23 - 0.99)	20
Allylisopropyl-acetamide	1.0	9.7	(0.77 - 28)	5
	4.0	33	(10.7 - 106)	6
	8.0	25	(7.4 - 46)	5
α-Propylvaler-amide	2.2	10.6	(0.68 - 30)	5
	4.3	33	(0.74 - 152)	6
	8.6	5.8	(0.80 - 25)	5
α-Isopropylvaler-amide	4.0	4.3	(1.6 - 15.5)	6
	8.0	0.80	(0.75 - 1.3)	3
Sodium secobarbital	3.9	10.1	(0.65 - 45)	6
	7.8	20.3	(11.3 - 37)	5
Sodium amobarbital	3.7	0.67	(0.55 - 0.79)	5
	7.4	0.66	(0.49 - 1.0)	6
	14.8	3.1	(1.1 - 4.8)	3
Aprobarbital	3.5	4.1	(0.73 - 14.1)	5
	7.0	31	(1.1 - 81)	6
Diallylbarbituric acid	3.1	0.63	(0.55 - 0.69)	5
	6.2	0.80	(0.39 - 1.3)	5
	12.4	6.4†		1
Sodium phenobarbital	3.8	0.69	(0.57 - 1.0)	5
	7.6	1.1	(0.57 - 2.5)	6
	15.2	1.2	(0.86 - 1.6)	3

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

† Only one embryo of 5 survived this dose.

in both test procedures, AIA, α-propylvaleramide and sodium secobarbital exhibited marked porphyria-inducing activity while phenobarbital (Granick, 1966) and diallylbarbituric acid had only weak activity (Hirsch et al., 1966). In spite of the fact that it had shown comparable activity to AIA in monolayer cultures of chick embryo liver cells, the related compound α-propylvaleramide was considerably less active than AIA in the intact chick embryo. Aprobarbital, which possessed marked activity and amobarbital, which was weakly active have not been tested in monolayer cultures of chick embryo liver cells. It is of interest to compare our results with those reported by Talman et al. (1957) who injected drugs into the yolk sac of 8-day embryonated eggs and measured the degree of porphyria by estimating porphyrin accumulation in the allantoic fluids. Thus, secobarbital, aprobarbital and diallylbarbituric acid, found to be active in our procedure, were also found to be active by the above authors. To facilitate further discussion and comparison with the work of previous authors, a table summarizing our results and those of previous workers has been prepared (Table VII).

While AIA was found to possess activity when injected intraperitoneally into mice, α-propylvaleramide and sodium secobarbital were inactive (Table V). It is of interest to compare these results with those reported by other laboratories (Table VII). Secobarbital, diallylbarbituric acid, aprobarbital and phenobarbital have been found to be active porphyria-inducing agents in rabbits while amobarbital was found to exhibit no activity (Goldberg, 1954). Stich and Decker (1955) demonstrated porphyria-inducing activity with aprobarbital and diallylbarbituric acid but not with phenobarbital when these compounds were administered orally to rabbits. Thus, in this series of compounds, the present

TABLE VII. SUMMARY OF PORPHYRIA-INDUCING ACTIVITY OF DRUGS IN ANIMALS AND MONOLAYER CULTURES OF CHICK EMBRYO LIVER CELLS

Compound	Cell Culture	Chick Embryo	Mice	Guinea Pigs	Rabbits	Rats
DDC	+	+	+	+	+	+
Dihydropyridine (XVI)	+	+		+		
Dihydropyridine (VI)	-	-		-		
Pyridine (VII)	+	-	-	-		
Benzene Compound (XVII)	+	-	-	-		
Allylisopropyl-acetamide	+	+	+	+	+	+
α-Propylvaler-amide	+	+	-			
α-Isopropylvaler-amide	+	+			-	
Sodium secobarbital	+	+	-		+	
Sodium amobarbital	+	+			-	
Aprobarbital	+	+			+	

TABLE VII Continued

Compound	Cell Culture	Chick Embryo	Mice	Guinea Pigs	Rabbits	Rats
Diallylbarbituric acid	+	+			+	
Sodium phenobarbital	+	+	-		+,-*	
Glutethimide	+	+	-			
Mephenytoin	+	+	-			
Methyprylon	+	+	-			
Sodium diphenylhydantoin	+	+	-			
Meprobamate	+	+	-			
Bemegride	+	-	-			
Methsuximide	+	+	-			
Phensuximide	+	+	-			
Griseofulvin	+	-	+			+
Hexachlorobenzene	+	-	+	+	+	+

* Goldberg (1954) reported this compound to be active, however, Stich and Decker (1955) could not demonstrate activity.

procedure using the intact chick embryo yielded results similar to those obtained in monolayer cultures of chick embryo liver cells, and with the exception of α-isopropylvaleramide which has been reported to be inactive when administered to rabbits (Goldberg & Rimington, 1962), the results correspond to other in vivo test procedures. The results obtained in vivo, led previous workers (Stich & Decker, 1955; Talman et al., 1957; Goldberg & Rimington, 1962) to emphasize the importance of an allyl group for activity since compounds possessing an allyl group were active while those lacking such a group were not. Studies of Hirsch et al. (1966) showed that an allyl group was not required for activity when these compounds were tested in monolayer cultures of chick embryo liver cells. Our present studies where the activity of α-propylvaleramide and α-isopropylvaleramide was compared with that of AIA in the intact chick embryo showed that an allyl group while not essential does enhance the activity of these compounds (Table VI).

Using the intact chick embryo, porphyria-inducing activity was revealed in the following compounds in agreement with results in monolayer cultures of chick embryo liver cells (Granick, 1966): glutethimide, mephenytoin, methyprylon, meprobamate, methsuximide, phensuximide and diphenylhydantoin (Table VIII). However, bemegride, griseofulvin and hexachlorobenzene which were shown to be active in monolayer cultures of chick embryo liver cells (Granick, 1966) could not be shown to be active in the intact chick embryo (Table VIII). In the case of bemegride, this discrepancy might be explained by the fact that its toxicity in the chick embryo prevented the administration of a dose sufficiently large to demonstrate activity. Hexachlorobenzene was only slightly soluble in dimethylsulfoxide (DMSO) and this might have accounted for the failure

TABLE VIII. PORPHYRIN ACCUMULATION IN LIVERS OF CHICK EMBRYOS INDUCED BY MISCELLANEOUS DRUGS

Compound	Dose (mg/egg)	Porphyria-Inducing Activity* (μg porphyrin/g liver)		No. of Expts.
Control (only solvent given)		0.46	(0.23 - 0.99)	20
Glutethimide	1.0	0.47	(0.27 - 0.64)	5
	4.0	4.9	(0.84 - 13.1)	5
	6.6	37	(3.5 - 91)	5
Mephenytoin	4.0	7.4	(0.79 - 27)	5
	8.0	1.9	(1.3 - 3.4)	5
Methyprylon	4.0	4.5	(1.1 - 12.8)	5
	8.4	26	(0.94 - 121)	5
Sodium diphenylhydantoin	4.1	0.42	(0.34 - 0.60)	5
	8.2	1.2	(1.1 - 1.3)	2
Meprobamate	3.3	1.6	(0.72 - 5.8)	6
	6.6	3.3	(1.2 - 6.7)	6
Bemegride	4.6	0.63	(0.50 - 0.76)	3
Methsuximide	4.0	18.2	(2.2 - 47)	5
	8.0	37	(0.75 - 72)	5
Phensuximide	4.0	0.82	(0.45 - 1.2)	6
	11.4	4.5	(0.99 - 7.6)	5
Griseofulvin	4.0	0.53	(0.41 - 0.64)	5
	8.0	0.68	(0.47 - 0.92)	5
	16.0	0.52	(0.09 - 1.2)	6
Hexachlorobenzene	5	0.43	(0.39 - 0.46)	5

* Expressed as μg coproporphyrin/wet weight liver, the extreme values are given in parentheses.

to demonstrate activity with this compound. The compounds in Table VIII which exhibited porphyria-inducing activity in the intact chick embryo were all devoid of activity when administered to mice (Table V).

Hexachlorobenzene and griseofulvin have previously been shown to induce porphyria in man (DeMatteis, 1967) and in several animal species (Ockner & Schmid, 1961; DeMatteis & Rimington, 1963). It was thus of interest that these compounds were active in monolayer cultures of chick embryo liver cells but inactive in the intact chick embryo. There appears to be three possible explanations for this finding. (1) The monolayer cultures of chick embryo liver cells possess altered properties when compared to the intact liver. (2) The drugs were unable to achieve an adequate concentration in the liver after injection into the fluids surrounding the chick embryo. (3) These drugs require longer than a 24 hour period to produce a significant accumulation of porphyrins in the chick embryo liver. Ockner and Schmid (1961) observed that some rats treated with hexachlorobenzene for a period of several weeks died without exhibiting any abnormality in porphyrin metabolism. They did, however, show the toxic manifestations of the drug. It was thus desirable to determine whether porphyrin accumulation in the liver could be induced when these drugs were administered orally to young chicks for a period considerably longer than 24 hours. After feeding the drug for 6 days, porphyria-inducing activity was detected with both drugs (Table IX). Therefore, these two drugs appeared to be unusual in the long period of time required to induce porphyrin accumulation.

The failure of all compounds tested except DDC (I) and AIA (III) to induce porphyria when injected into mice (Table V), indicates that a major difference might exist between the responsiveness of avian

TABLE IX. PORPHYRIN ACCUMULATION IN THE LIVERS OF YOUNG CHICKS FOLLOWING ORAL ADMINISTRATION OF DRUGS

Compound	% Drug Administered in Diet	Porphyria-Inducing Activity* (μg porphyrins/g liver)	No. of Expts.
Hexachlorobenzene	1.5	0.83 ±0.05†	6
Griseofulvin	2.5	0.34 ±0.01†	6
Control	0	0.16 ±0.01	6

* Expressed as coproporphyrin I/wet weight liver, mean ± SE of mean.

† Significantly greater than control, $P < 0.05$.

and mammalian (mouse) liver cells to drugs. We have assumed that porphyrin accumulation in the liver can be equated with increased synthesis of δ-aminolevulinic acid synthetase in view of the fact that Granick (1966) has marshalled considerable evidence to support this idea in monolayer cultures. While it is possible that this assumption does not hold in the intact animal where there is the possibility of excretion of porphyrins and precursors, there have not been any reports of porphyria-inducing drugs which failed to cause the accumulation of coproporphyrin and/or protoporphyrin in the liver (DeMatteis & Prior, 1962; Onisawa & Labbe, 1963). In view of the failure of demonstrate porphyria-inducing activity in mice, the question arises as to the relevance of results obtained in an avian species to the results anticipated in the porphyric patient. To resolve this question, it is valuable to consider what has occurred when some of these drugs have been administered to patients with porphyria. Thus, secobarbital, meprobamate, methsuximide and diphenylhydantoin which have been shown to be active in the monolayer culture of chick embryo liver cells and the intact chick embryo have also been shown to produce a clinical relapse in porphyric patients (Cowger & Labbe, 1965). Since these drugs are inactive in mice, it seems that the results obtained using avian liver cells allow better prediction of the results to be expected in the porphyric patient than do the results obtained with mice. The reaons for this are not clear, but a possible explanation emerges after consideration of studies in chapter IV of this thesis. Kappas et al. (1968) using 5-β-H steroids have also observed marked species variation in porphyria-inducing activity. These workers were able to enhance δ-ALA synthetase formation in chick embryo liver but not in guinea pig or rat liver by administration of these substances. It was

suggested that the lack of responsiveness of guinea pig and rat liver to these steroids probably denotes the existence of additional control mechanisms for protoheme biosynthesis in mammalian liver as compared to chick embryo liver.

In summarizing our results, it is apparent that comparable results were obtained in the chick embryo and in monolayer cultures of chick embryo liver cells with 18 out of 23 drugs tested. These findings are in accord with those of Kappas et al. (1968) where they showed that the monolayer cultures of chick embryo liver cells and the intact chick embryo liver responded in the same manner to steroid metabolites. It appears that several compounds which exhibit porphyria-inducing activity in monolayer cultures of chick embryo liver cells, are inactive in the intact chick embryo. The reasons for this will be discussed in chapter IV. In the procedure designed by Granick (1966) utilizing monolayer cultures of chick embryo liver cells, drug permeability problems are reduced and the effect of the drug on the liver is examined independently of other body tissues. For these reasons, this technique is invaluable for studying the mechanism of action of porphyria-inducing drugs.

Previous studies in whole animals suffer from the following disadvantages: (1) Treatment must generally be carried out for relatively long periods of time. (2) It is inconvenient to carry out a large number of tests simultaneously due to the demands for equipment and laboratory space. By contrast, the intact chick embryo tests appear to reflect the activity of the drug in the chick embryo liver cell culture system and in the porphyric patient. This test can be carried out very quickly with a minimum of effort and equipment necessary. However, it must be born in mind that this procedure will not always reveal porphyria-

inducing activity in all compounds exhibiting such activity in the isolated liver cell culture system.

While DDC (I) has been shown to induce porphyria in adult rats, rabbits and guinea pigs, it has recently been claimed that the drug has no effect on porphyrin metabolism in the newborn of these animals (Woods & Dixon, 1969). These workers suggested that the regulation of protoheme synthesis in perinatal animals may be less functional than in adults, so that the control mechanism in perinatal animals is not as readily disturbed as in adult animals. Since increased activity of δ-ALA synthetase is readily induced by drugs in intact chick embryos, it appears that there is a major species difference in control mechanisms which merits further investigation. It is conceivable that in the porphyric patient, the normal mammalian control mechanism for protoheme biosynthesis is lost and that the patient becomes as responsive to porphyria-inducing drugs as the avian species.

CHAPTER IV INVESTIGATION OF THE DIFFERENCES IN RESPONSE OF ISOLATED LIVER CELLS AND THE LIVER OF THE INTACT CHICK EMBRYO TO PORPHYRIA-INDUCING DRUGS

Introduction

The dihydropyridine, DDC (I), exhibits porphyria-inducing activity in monolayer cultures of chick embryo liver cells and in the intact chick embryo, while the corresponding pyridine, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) is active in monolayer culture of chick embryo liver cells but practically devoid of activity in the intact chick embryo. The latter compound was inactive when administered orally to guinea pigs, and this has been attributed to dynamic phenomena which control drug concentration in the liver (Marks et al., 1965). In view of their structural similarity, it was difficult to reconcile the fact that both DDC (I) and the pyridine (VII) were active in monolayer cultures of chick embryo liver cells but only DDC (I) was active in vivo. There were two possible reasons for this result: (1) after administration to animals, the pyridine (VII) did not reach the liver in sufficient quantities to induce porphyrin production; (2) the pyridine (VII) was rapidly metabolized and inactivated in the liver when administered to animals. To examine these possibilities, ^{14}C-labelled 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) and DDC (I) were prepared, injected into 17-day old chick embryos and the total amount of drugs and metabolite(s) in the livers was measured at various time periods.

Experimental

i. Source of Compounds

Ethyl acetoacetate-3-^{14}C (4.17 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Massachusetts. Unlabelled ethyl

acetoacetate was purified by distillation at reduced pressure and the fraction with b.p. 78.5 - 79.5°/18 mm was collected. 2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) were purchased from Nuclear Chicago Corp., Des Plaines, Illinois. Phenethylamine was purchased from Eastman Organic Chemicals, Rochester, New York, and distilled at reduced pressure before use. Silica gel G was purchased from Brinkmann Instruments Inc., Westbury, New York. Cab-O-Sil, a product of Godfrey L. Cabot, Inc., Boston, Massachusetts, was obtained from Van Waters and Rogers Ltd., Edmonton.

ii. Instrumentation

Gas-liquid chromatography was carried out with a Microtek Model 2000 MF Gas Chromatograph fitted with a 5-foot stainless steel column packed with Celite. The hydrogen flame ionization detector and helium carrier gas were used for all separations. A Bausch and Lomb Spectronic 505 was used for recording ultraviolet spectra. All radioactive samples were counted in a Nuclear Chicago Model 6850 Liquid Scintillation System. Counts were corrected for quenching by the channels ratio method and the background count subtracted. The thin-layer chromatograms containing radioactive samples were scanned using a Nuclear Chicago Actigraph III radiochromatography system. All radiochromatograms were scanned at a detector voltage of 960 volts and gas pressure of 7 lbs. Melting points are uncorrected.

iii. Preparation of Acetaldehyde (Fieser, 1957)

Paraldehyde (20 ml) was placed in a 100 ml round bottom flask fitted with a fractionating column. Concentrated sulphuric acid (0.5 ml) and 0.5 ml of water were added and the flask heated gently

with a small flame. The acetaldehyde which formed was distilled at a temperature not higher than 35° and collected in an ice-cold receiver.

iv. General Method Used for Determining the Specific Activity of Compounds

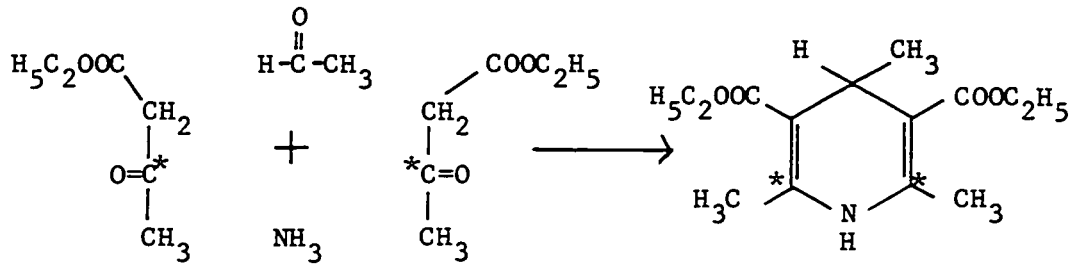
Toluene scintillation solution was prepared by dissolving 6 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) in 1 liter of toluene (Wang & Willis, 1965). Three individual dried samples of the labelled compounds were weighed on a Cahn electrobalance and dissolved in 10 ml of toluene. Triplicate 1 ml aliquots of each solution were then added to 14 ml of the toluene scintillation solution, counted, and the specific activity determined.

v. Synthesis and Radiochemical Purity of 3,5-Diethoxycarbonyl-1,4-Dihydro-2,4,6-Trimethylpyridine-^{14}C (DDC-^{14}C)

Two procedures (a and b) described below were explored for the synthesis of DDC-^{14}C.

(a) Synthetic Procedure of DeMatteis and Prior (1962) for the Preparation of the Unlabelled Compound

The procedure involves the condensation of two moles of ethyl acetoacetate with one mole of ammonia and one mole of acetaldehyde as illustrated below.



Ethyl acetoacetate-3-^{14}C (4.17 mCi/mmole) was diluted in a vial with redistilled ethyl acetoacetate (0.26 g; 0.002 moles) to give a product of 0.048 mCi/mmole. The contents of the vial were transferred with the aid of a syringe to a 10 ml centrifuge tube. The vial was washed twice with 1 ml portions of ether and these washings transferred to the centrifuge tube. The ether was removed by warming the centrifuge tube in a water bath and passing a current of air over the surface of the solution. Acetaldehyde (0.050 g; 0.001 moles) and 5 ml of 10% ammonium carbonate were added to the ethyl acetoacetate and the mixture thoroughly agitated. The reaction mixture was kept for 72 hours at 4° with occasional mixing. The DDC (I) which crystallized was collected by filtration and resuspended in 2 ml of 3N HCl. After a period of 5 hours, the crystals were collected and dried at 100° and 18 mm Hg. The product (yield, 41%) was purified by successive crystallizations from ethanol-water until the specific activity of the compound remained constant. This was achieved after three crystallizations. The product, m.p. 128.5 - 129°, was found to have a specific activity of 0.088 mCi/mmole, λ max. (ethanol) 232 and 351 mμ (ϵ, 18,100 and 8,100). Marks et al. (1965) reported m.p. 128.5 - 129, λ max. (ethanol) 232 and 351 mμ (ϵ, 18,200 and 8,050). No evidence for the presence of an impurity was obtained by gas-liquid chromatography (column temperature 160°).

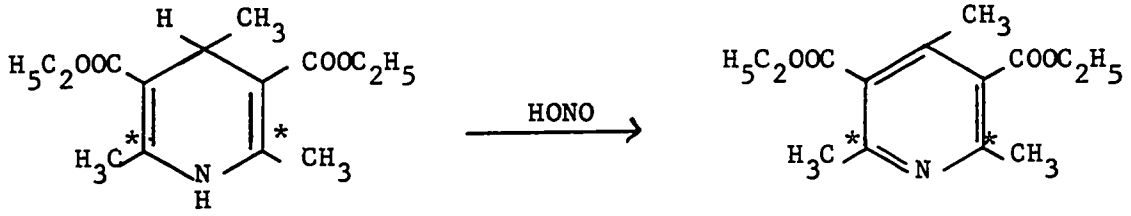
(b) Procedure of Loev and Snader (1965)

Redistilled ethyl acetoacetate (2.1 g; 0.016 moles) was added to a vial containing 0.1 mCi of ethyl acetoacetate-3-^{14}C to give a product of 0.006 mCi/mmole. The contents of the vial were transferred to a 25 ml flask with 4 ml of ethanol. Acetaldehyde (0.44 g;

0.01 moles) and 2 ml of concentrated ammonium hydroxide (0.015 moles) solution were added and the mixture refluxed for 3 hours. After cooling, the solution was poured into 20 ml of ice cold water whereupon the product crystallized (yield, 65%). The crystals were collected and purified by successive crystallizations from a mixture of ethanol and water. The dihydropyridine (I), m.p. 128 - 129°, has a specific activity of 0.010 mCi/mmole.

vi. Synthesis and Radiochemical Purity of 3,5-Diethoxycarbonyl-2,4,6-Trimethylpyridine-^{14}C

This compound was prepared by the oxidation of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine-^{14}C (DDC-^{14}C) according to the following reaction:



The procedure described below is essentially that of Loev and Snader (1965). One gram of DDC-^{14}C (0.004 moles; 0.010 mCi/mmole) was dissolved in 10 ml of glacial acetic acid in a 50 ml round bottom flask and sodium nitrite (1.0 g) added in small portions over a period of an hour with constant stirring. After stirring for an additional 90 minutes, the mixture was poured into 100 ml of ice-cold water and the product which separated as an oil extracted with three 50 ml portions of ether. The pyridine (VII) was extracted from the

ethereal solution with three successive portions (20 ml) of 10% HCl. Following neutralization of the acidic solution with sodium carbonate, the aqueous phase was extracted with three 50 ml portions of ether. The ethereal solution was dried (sodium sulfate) and the ether removed. Distillation (bulb-tube) afforded 3,5-diethoxycarbonyl-2,4,6-trimethyl-pyridine-^{14}C (VII) as a colorless oil, b.p. 105 - 115 °/0.07 mm, λ max. (ethanol) 269.5 mμ (ε,3,040). The yield was 70%. Marks et al. (1965) reported λ max. (ethanol) 269 mμ (ε,3,250). The specific activity of this compound was found to be 0.010 mCi/mmole. No evidence for the presence of an impurity was obtained by gas-liquid chromatography (column temperature 160°).

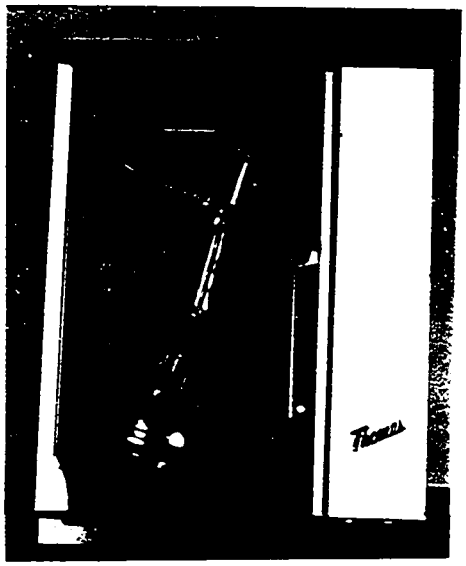
vii. Amount of ^{14}C in the Livers at Different Time Intervals After Injection of ^{14}C-labelled drugs into the Fluids Surrounding the Chick Embryo

An accurately weighed amount of labelled drug was mixed with unlabelled drug and the mixture dissolved in a quantity of dimethyl-sulfoxide calculated to give a dilution of drug such that the amount of drug to be injected was contained in 0.1 ml of solution. In order to have accurate information on the amount of drug injected, an aliquot of this solution (0.1 ml) was mixed with toluene scintillation solution (14.9 ml) and the mixture counted. The drug dissolved in 0.1 ml of dimethylsulfoxide was then injected into 17-day old chick embryos as described in Chapter III, section iii. At the end of various incubation periods, the livers of the chick embryos were removed, washed with saline, weighed and placed on a piece of cellophane (2 inches by 3 inches) where they were dried under an infrared heat lamp for six hours. The remainder of the chick embryos and

their surrounding fluids were frozen for subsequent extraction of labelled compounds (Chapter IV, section ix). The oxygen flask combustion technique of Davidson and Oliverio (1967) was employed to prepare the dried liver samples for liquid scintillation counting. The combustion apparatus (Fig.11) consisted of a 2 liter heavy walled Erlenmeyer filter flask fitted with a Neoprene No.9 stopper containing one hole. A sample holder constructed from 16 mesh nichrome wire was attached to the end of a glass rod mounted in the stopper so that the sample holder remained 2 to 3 inches above the bottom of the flask. The side arm of the flask was fitted with Tygon tubing which was closed with a pair of rubber tipped forceps.

The dried liver sample wrapped in cellophane and a small blackened piece of filter paper were placed in the nichrome basket. The flask was thoroughly flushed with oxygen and the Neoprene stopper fitted with the sample holder secured in position. The flask was placed in a Thomas-Ogg igniter, in a fume hood fitted with a shatter proof door, and the infrared lamp focused on the darkened filter paper to ignite the sample. The sample usually ignited within 5 seconds and combustion was complete within 1 minute. The flasks were removed from the igniter and cooled in an ice-water bath for 5 minutes. Scintillation solvent consisting of phenethylamine (270 ml), methanol (270 ml), PPO (5 g), POPOP (100 mg) and toluene (460 ml) was prepared (Davidson & Oliverio, 1967). A pipette containing the scintillation solvent (15 ml) was inserted into the Tygon side arm, the forceps released thus allowing the liquid to run slowly down the side of the flask. The flask was then swirled gently to distribute the solvent over the entire bottom of the flask and set aside for 20 minutes to allow for

(A)



(B)

Fig. 11. Photograph Illustrating the Wrapped Sample Being Inserted Into the Nichrome Sample Holder of the Flask Head, (A), and Oxygen Combustion Flask in the Thomas-Ogg Igniter, (B).

(A)

(B)

Fig. 11. Photograph Illustrating the Wrapped Sample Being Inserted Into the Nichrome Sample Holder of the Flask Head, (A), and Oxygen Combustion Flask in the Thomas-Ogg Igniter, (B).

the absorption of the liberated carbon dioxide. An additional 3 ml of scintillation solvent was added and the flask swirled. After removing the stopper, a 15 ml aliquot of the scintillation solvent was transferred to a vial for counting. Correction for quenching in the samples was carried out by the channels ratio method outlined below.

viii. Determination of Balance Point and Counting Efficiency of Liquid Scintillation Counter

(a) General Considerations

The Unilux Model 6850 Liquid Scintillation system consists of a pulse height analyzer with two separate variable attenuators which allows analysis of two distinct portions of the energy spectrum of a given isotope. These measured portions of the spectrum are referred to as channels. By means of channels ratio counting, an estimate of the amount of quenching in a sample can be obtained and a suitable correction made. In the channels ratio method of quench correction, the two attenuator channels are positioned relative to an isotope's energy spectrum so that the ratio of net count rates in the two channels can be used as a measure of the degree of spectral shifting or quenching which is occuring and hence affecting the counting efficiency. A series of samples of known activity and varying degrees of quenching is counted in two channels and from the results obtained, a curve is constructed relating the counting efficiency to the ratio of the counts in the two channels (channels ratio) for each quenched sample. The channels ratio of each sample whose radioactivity is to be measured is then determined and the counting efficiency obtained by reference to the curve.

(b) Procedure

The balance point was determined in the upper channel, by adjusting the amplifier gain until the unquenched toluene-^{14}C standard was counted at a maximal rate. A region of gain values exist where the pulses observed do not change appreciably with a small change in gain. This region is the "balance point".

The amplifier gain on the lower channel was then adjusted so that the number of pulses counted was one-third the value obtained in the upper channel. A series of toluene-^{14}C standards of known activity but varying degrees of quenching was then counted. The channels ratios of these standards were obtained by dividing the observed counts in the lower channel by the observed counts in the upper channel. The efficiency of the standards was calculated from the observed count in the upper channel and the known amount of activity contained in each standard. The channels ratio curve was plotted against the efficiency (Fig. 12).

All samples, unless otherwise indicated, were counted for a period of time, sufficient in duration so that the percentage standard error for the corrected count did not exceed $\pm$ 2%.

ix. Extraction of Labelled Compounds From 17-Day Old Chick Embryos and Surrounding Fluids After Injection of DDC-^{14}C or 3,5-Diethoxycarbonyl-2,4,6-Trimethylpyridine-^{14}C

After removal of the liver for determination of radioactivity (Chapter IV, section vii), the chick embryo and surrounding fluids were homogenized in 150 ml of chloroform-methanol (2:1) in a Sorvall Omni-mixer. The resulting homogenate was filtered by gravity, the filtrate set aside and the residue homogenized with another 150 ml portion of

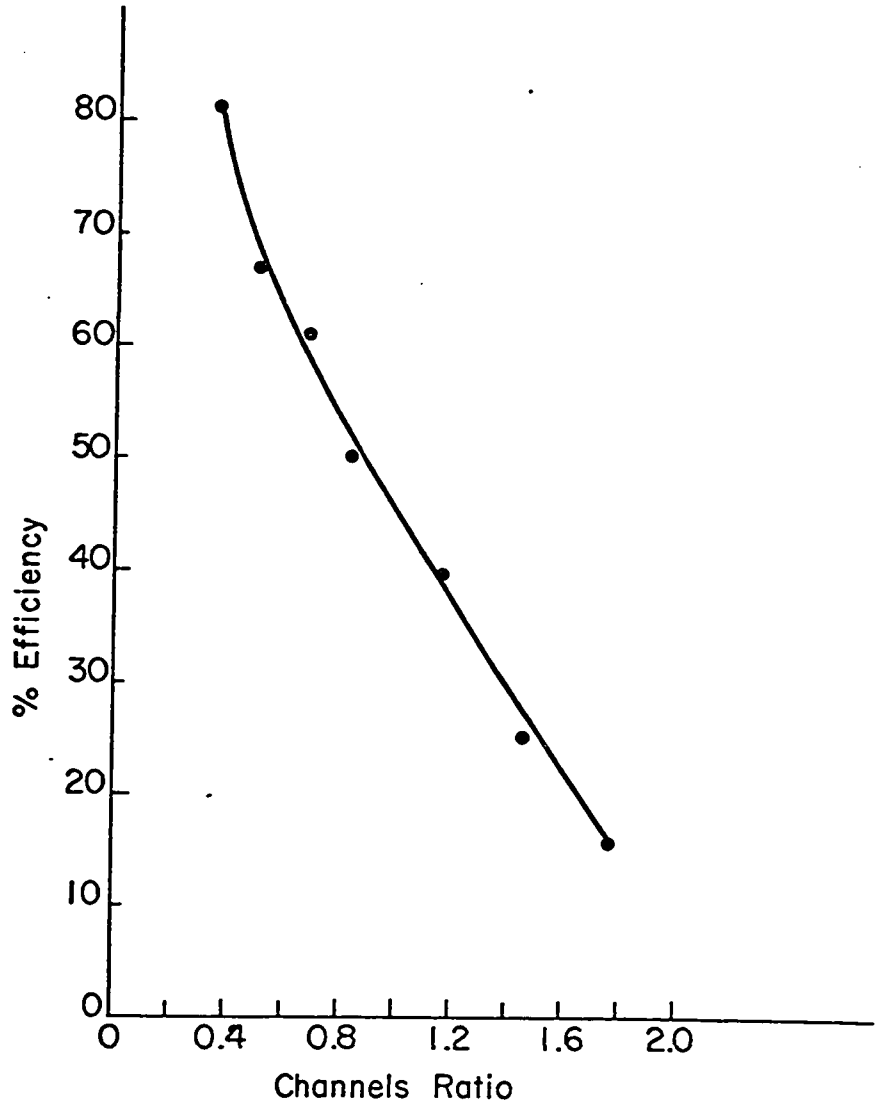


Fig. 12. Carbon-14 Quench Correction Curve.

chloroform-methanol (2:1). After filtration, the extraction procedure was repeated for a third time. The combined filtrates were placed in a rotary evaporator and the solvent removed. The extracts of all chick embryos and fluids remaining from studies with DDC-^{14}C were combined to facilitate further work. In a similar manner, the extracts obtained from studies with the pyridine (VII) were combined.

x. Investigation of Labelled Compounds in Chick Embryo Extracts by Isotope Dilution Analysis

(a) Studies with DDC-^{14}C (I)

The extracts were dissolved in petroleum ether-methanol (1:2) and three 1 ml aliquots added to three vials containing 14 ml of toluene scintillation solution. From the count obtained with these aliquots, it was possible to calculate how large a volume of the petroleum ether-methanol solution contained 1 mg of DDC (I) or metabolite(s). This calculated volume of solution was placed in a separatory funnel, 99 mg unlabelled DDC added and the solution thoroughly agitated to ensure complete mixing. DDC was extracted from the petroleum ether-methanol solution with three successive 30 ml portions of methanol-water (2:1). The methanol-water was removed on a rotary evaporator, the residue dissolved in hot methanol and the solution filtered. Water was then added to the filtrate and the DDC crystals which separated were collected by filtration and recrystallized to constant specific activity (0.051 μCi/mmole). Assuming all the radioactivity in the original extract from the chick embryo and fluids was represented by DDC-^{14}C, it could be calculated that the specific activity of the recrystallized product would have been 0.088 μCi/mmole.

(b) Studies with 3,5-Diethoxycarbonyl-2,4,6-Trimethylpyridine-^{14}C

The extract was dissolved in 30 ml of petroleum ether-methanol (1:2) and three 1 ml aliquots added to 3 vials containing 14 ml of toluene scintillation solution. From the count obtained with these aliquots, it was possible to calculate how large a volume of the petroleum ether-methanol solution contained 3 mg of the drug or metabolite. This volume was placed in a separatory funnel and 297 mg of unlabelled drug added and thoroughly mixed to ensure uniform distribution of the labelled and unlabelled substances. The pyridine (VII) was then extracted from the petroleum ether-methanol solution with three 25 ml portions of 1N HCl. The acid extracts were neutralized with sodium carbonate and the pyridine (VII) extracted from the aqueous solution with three 50 ml portions of ether. The ether solution was extracted with three 25 ml portions of 1N HCl and the acid solution neutralized with sodium carbonate. The aqueous solution was extracted with three 25 ml portions of ether. The ether solution was dried (sodium sulfate), the ether removed and the residue distilled (bulb-tube) affording 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) as a colorless oil, b.p. 95 - 105 °/0.07 mm, λ max. (ethanol) 269 mμ (ϵ,3,470), specific activity 0.054 μCi/mmole. Marks et al. (1965) reported λ max. (ethanol) 269 mμ (ϵ, 3,250). Assuming all the radioactivity in the original extract from the chick embryo and fluids was represented by 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (VII) it could be calculated that the specific activity of the recrystallized product would have been 0.104 μCi/mmole.

xi. Preparation of Thin-Layer Plates

Silica gel G (25 g) was placed in a wide mouth bottle and

50 ml of distilled water added. The mixture was thoroughly agitated to form a smooth slurry and then poured into a Desaga spreader for spreading over 2 inch glass plates to give a layer 250μ thick. The plates were allowed to dry at room temperature and were activated by heating at 110° for 30 min just prior to use (Stahl, 1965a).

xii. Amount of Unchanged DDC-^{14}C in the Liver at Different Time Periods After Injection of DDC-^{14}C into the Fluids Surrounding the Embryo

DDC-^{14}C (4 mg) was administered by the procedure outlined in Chapter IV, section vii of this thesis. The livers were removed, washed several times with saline and then stored frozen until the extraction of the drug and its metabolites could be carried out.

The liver was weighed, homogenized in 3 ml of methanol in a Potter-Elvehjem apparatus and the homogenate transferred to a centrifuge tube. After centrifugation, the supernatant was decanted into a small flask and the residue resuspended in an additional 3 ml of methanol for homogenization and centrifugation. The extraction procedure was repeated and the three supernatants combined. The tissue residue was allowed to dry in air, combusted and counted. No significant radioactivity was found to be present in this residue. The methanol was removed from the liver extracts with a rotary evaporator and the residue dissolved in 0.5 ml of methanol. A 0.1 ml aliquot of this solution was added to a vial containing 14 ml of toluene scintillation solution for counting and a 0.3 ml aliquot was spotted as a band on a 2 inch thin-layer plate, coated with 250μ of silica gel G. The plate was developed with benzene-methanol (14:1). This solvent system was modified from Stahl (1965b). The Rf value for DDC was found to be 0.45 in this system. The thin-layer plates were scanned for

radioactivity with a Nuclear Chicago Actigraph III radiochromatogram scanner and the radioactive portions removed and counted by the following procedure. The radioactive silica gel portions were placed in liquid scintillation vials containing 0.45 g of Cab-O-Sil and 15 ml of toluene scintillation solution (Noujaim, 1969). The vial was agitated to suspend the silica gel and correction for quenching was carried out by reference to a curve constructed as described below (section xiii).

To measure the effectiveness of our procedure, a known amount of DDC-^{14}C was added to livers from untreated embryos. The drug was extracted as described above and the extract spotted on thin-layer plates and chromatographed with benzene-methanol (14:1). The radioactive areas were removed and counted.

xiii. Preparation of Quenched Standards and Channels Ratio Efficiency Curve for Samples Containing Cab-O-Sil

In order to prepare a series of standards with varying degrees of quenching, six vials were prepared containing the following constituents:

Vial no.	Cab-O-Sil (g)	Silica Gel (mg)	Methanolic Liver Extract (ml)
1	0.45	0	0
2	0.45	25	0
3	0.45	50	0.1
4	0.45	100	0.2
5	0.45	150	0.3
6	0.45	200	0.4

An accurately weighed sample of DDC-^{14}C was dissolved in 100 ml of toluene scintillation solution and 15 ml of this solution was added to each of the 6 vials. The contents of the vials were thoroughly agitated, counted and their channels ratios and counting efficiencies calculated. The channels ratio efficiency curve is plotted in Fig.13.

xiv. Amount of Unchanged 3,5-Diethoxycarbonyl-2,4,6-Trimethyl-pyridine-^{14}C in the Liver at Different Time Periods After Injection of Labelled Drug into Fluids Surrounding the Embryo

The procedure for the extraction of this drug and its metabolites after injection of 4 mg into the fluids surrounding the chick embryo was identical to that followed for DDC-^{14}C. The Rf value for this compound (VII) in benzene-methanol (14:1) was 0.72. The liver residue after extraction was found to contain no radioactivity.

Results and Discussion

Whenever tracer techniques are utilized to study the action of a drug, it is necessary that the labelled drug be radiochemically pure, as a small quantity of impurity of relatively high specific activity could produce misleading results. The first objective of this study was to synthesize DDC-^{14}C (I) and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (VII) in radiochemically pure form. DDC-^{14}C was synthesized by two procedures both of which gave radiochemically pure products. The synthetic procedure of Loev and Snader (1965) was found to be preferable to that of DeMatteis and Prior (1962) since a yield of 65% was obtained with the former procedure as compared to a yield of 41% with the latter. There was no difficulty in obtaining a

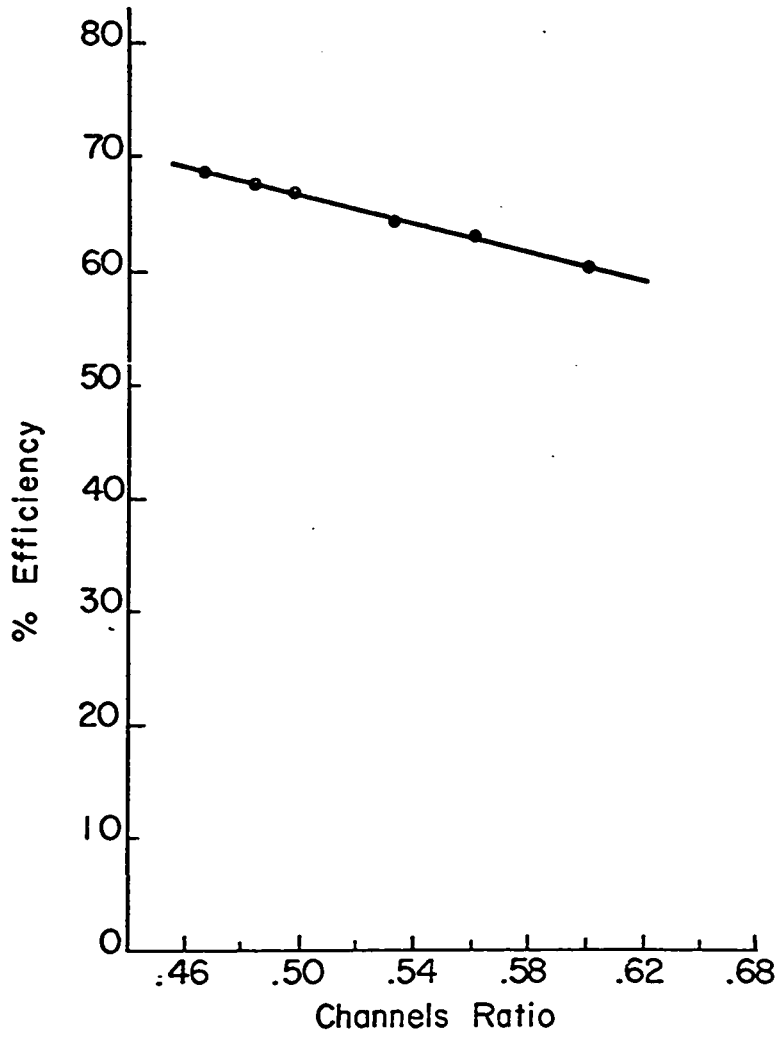


Fig. 13. Carbon-14 Quench Correction Curve in Cab-O-Sil.

radiochemically pure sample of DDC-^{14}C (I) since the compound was a solid and could be crystallized to constant specific activity. In the case of the pyridine (VII), synthesized by oxidation of DDC-^{14}C with nitrous acid, it was more difficult to establish radiochemical purity because it was an oil and too small a quantity was available for repeated distillation. To overcome this problem, the radiopurity of the pyridine (VII; 0.010 mCi/mmole) was assessed by comparing its specific activity to that of DDC-^{14}C, 0.010 mCi/mmole.

The second objective of our study was to establish a procedure for converting the radioisotope in liver into a form suitable for counting. The oxygen flask combustion method (Davidson & Oliverio, 1967) is useful for preparing samples containing colored materials for counting. In our laboratory we have found the technique to possess several advantages over the conventional procedures in which organs are solubilized with hyamine or similar materials. With the conventional technique, difficulty is often experienced in solubilizing organs and moreover, highly colored solutions are obtained. The counting efficiency of our procedure was found to be in the region of 35% and investigation showed phenethylamine to be the major quenching agent. Purification of phenethylamine by fractional distillation did not appreciably improve counting efficiency. The recovery of known amounts of DDC-^{14}C added to livers from control chick embryos was 99.9 $\pm$ 0.9% ($\pm$ SE mean, n = 10).

The third and primary objective of this study was to measure the total amount of radioactivity and unchanged drugs in the livers of 17-day old chick embryos at various time intervals after injection of DDC-^{14}C and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (VII). The total amount of radioactivity in the livers of chick embryos at

different times after injection of DDC-^{14}C and the pyridine (VII) is shown in Tables X and XI. From the data in these tables, the curves shown in Fig. 14 were constructed. The amount of radioactivity after injection of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (VII) reached a maximum in the liver after approximately 0.5 hours and then dropped rapidly. The amount of radioactivity after injection of DDC-^{14}C reached a maximum in the liver after approximately 6 hours and remained elevated for a considerable period of time. Since DDC and the pyridine (VII) are approximately equipotent in monolayer cultures of chick embryo liver cells and the radioactivity from the pyridine (VII) attained a level in the liver approximately five-fold higher than that reached by the radioactivity from DDC-^{14}C, it is clear that the inactivity of the pyridine (VII) in the intact chick embryo cannot be ascribed to an inability to reach the liver in sufficient quantity to induce porphyrin formation.

It was therefore necessary to examine the possibility that the inactivity of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) in chick embryo liver was due to its conversion to a metabolite. For this purpose, the technique of reverse isotope dilution analysis was used initially, followed later by radiochromatography. Folch et al. (1957), utilized chloroform-methanol (2:1) for the extraction of lipids, and since DDC and the pyridine (VII) are both lipid soluble, this solvent system was employed for the extraction of the radioactive drug and metabolites from the embryos and surrounding fluids for reverse isotope dilution analysis. The recovery of drug and metabolite(s)

TABLE X. AMOUNT OF RADIOACTIVE DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 4 MG (15,000 mμMOLES) DDC-^{14}C

Time After Injection (hours)	Amount of Radioactive Drug* mμmoles/100 mg liver†	
0.25	22.3 ± 4.3	(8)
0.5	14.1 ± 2.6	(9)
3	20.4 ± 1.9	(8)
6	36.2 ± 1.2	(10)
12	24.2 ± 2.9	(9)
18	32.5 ± 2.4	(9)
24	24.5 ± 2.3	(8)
48	17.0 ± 1.5	(6)

The number of experiments is given in parentheses.

* The results in this table are calculated on the basis that all the drug in the liver is DDC. Later results showed that some of the radioactivity is contributed by a metabolite of DDC.

† Mean ± SE of mean.

TABLE XI. AMOUNT OF RADIOACTIVE DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 4 MG (15,000 mμMOLES) 3,5-DIETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE-^{14}C

Time After Injection (hours)	Amount of Radioactive Drug* mμmoles/100 mg liver†
0.25	118.7 ± 18.0 (9)
0.5	181.6 ± 10.6 (8)
1.5	135.9 ± 10.1 (7)
3	85.4 ± 17.6 (7)
6	34.9 ± 6.3 (7)
12	22.6 ± 3.8 (9)
24	11.2 ± 1.1 (6)
48	3.7 ± 0.6 (7)

The number of experiments is given in parentheses.

* The results in this table are calculated on the basis that all the drug in the liver is 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII). Later results showed that some of the radioactivity is contributed by a metabolite of the pyridine (VII).

† Mean ± SE of mean.

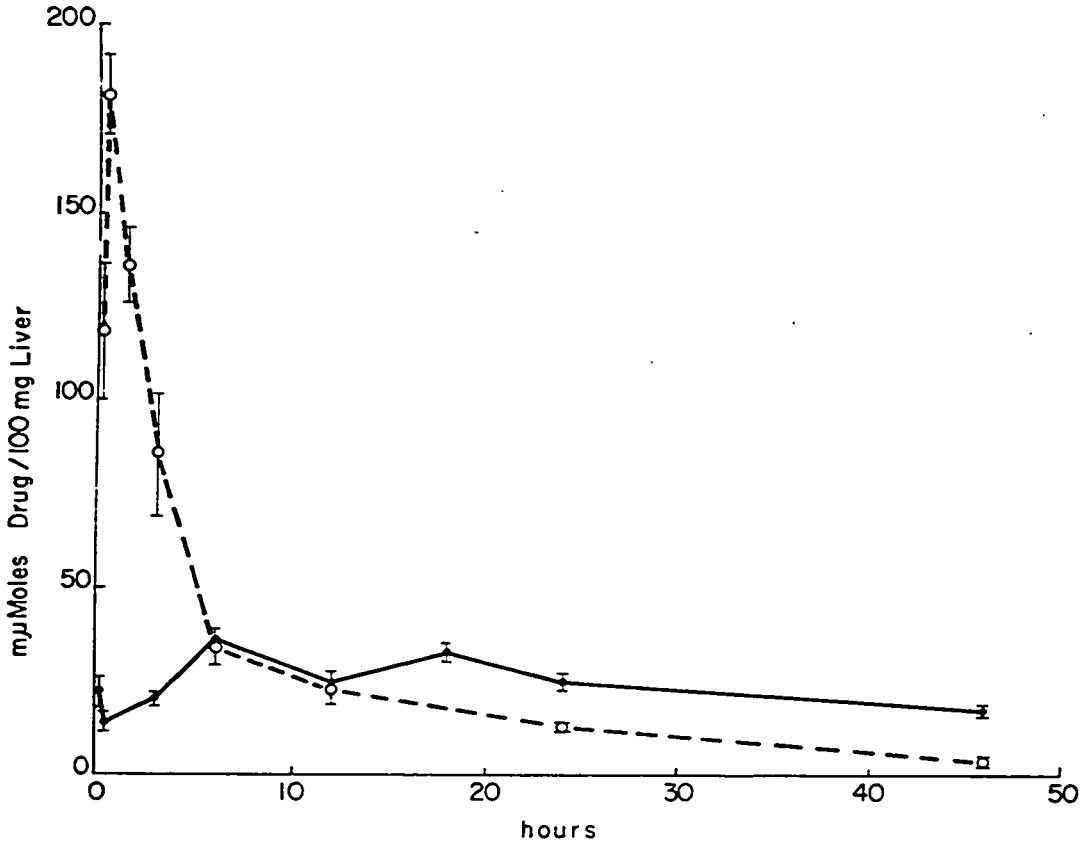
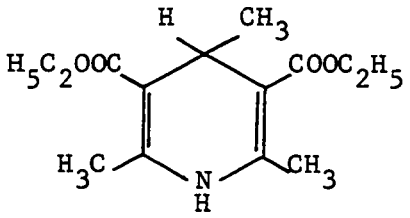
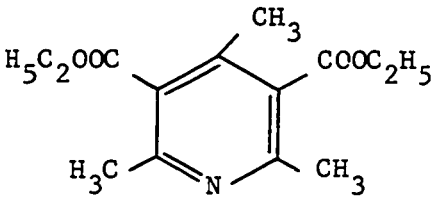


Fig. 14. Amount of Radioactive Drug in Livers of 17-Day Old Chick Embryos at Different Time Intervals After Injection of DDC-^{14}C (o———o) and 3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine (O— — — O). Each Point Represents Mean ± SE of Mean.

was 80% for DDC and 50% for the pyridine (VII). For reverse isotope dilution analysis, a mixture of drug and metabolite (approx. 1 mg) containing a known amount of radioactivity was diluted with a known amount (99 mg) of unlabelled drug and the specific activity of the mixture calculated, assuming all the radioactivity was contributed by unchanged drug. The drug was purified to constant specific activity. From the difference between the calculated and the observed specific activity, the percent of unchanged drug in the original radioactive sample was calculated (Table XII). From the data it was evident that both compounds were metabolized in the chick embryo. However, it was necessary to extend these studies to determine how much of the radioactive material in the liver at various time intervals was in the form of unchanged drug.

For this reason, the total radioactive material in the livers of 17-day chick embryos was extracted at various time intervals and separated by means of thin-layer chromatography using benzene-methanol (14:1) as the developing solvent. In the case of DDC-^{14}C, two radioactive areas were detected, one at the origin and the other with an Rf corresponding to that of DDC (Fig. 15). In order to rule out the possibility that the radioactive area at the origin was unchanged drug, prevented from migrating on the chromatogram by binding to some tissue constituent at the origin, the following experiment was performed. The radioactive area at the origin was scraped off the plate, treated with methanol and the methanol solution spotted on a fresh plate which was developed with methanol. The radioactive area which had an Rf of 0.4 on this plate and had moved away from the tissue constituents at the origin was eluted and rechromatographed with benzene-methanol (14:1).

TABLE XII. REVERSE ISOTOPE DILUTION ANALYSIS OF RADIOACTIVE EXTRACTS FROM EMBRYOS INJECTED WITH DDC-^{14}C AND 3,5-DIETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE-^{14}C

Compound	Unchanged Drug % of Total Radioactive Drug Extracted*
 (I)	59 58
 (VII)	57 58

*Duplicate analyses were performed.

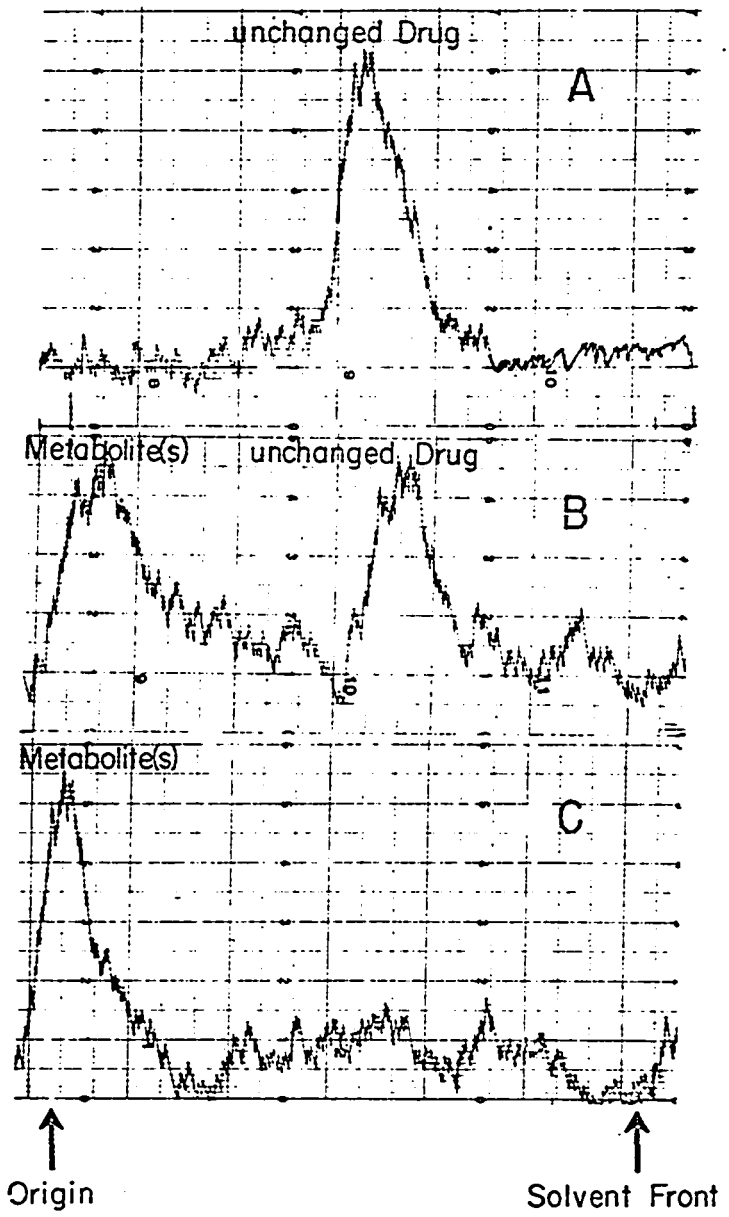


Fig. 15. Metabolism of DDC-^{14}C; Radiochromatogram Scanner Records Showing DDC-^{14}C Added to Control Liver (A), Liver Extract 6 Hours After Injection of DDC-^{14}C (B), and Liver Extract 24 Hours After Injection of DDC-^{14}C (C).

In this solvent system, the radioactive material was confined to the origin, thus demonstrating that it was a derivative of DDC rather than DDC itself. In the case of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (VII), only one radioactive area could be detected which had an Rf different to that of the pyridine (VII) (Fig. 16). The areas of silica gel containing drugs and metabolites were counted as a suspension in Cab-O-Sil. The addition of Cab-O-Sil has the advantage that a labelled compound adhering to the silica gel is counted in addition to labelled material dissolved in the toluene scintillation solution. After addition of labelled compounds to control livers, extraction and thin-layer chromatography, the recovery of DDC-^{14}C was 90.4 $\pm$ 3.6% ($\pm$ SE of mean, n = 5) and of the pyridine (VII) was 89.4 $\pm$ 3.8% ($\pm$ SE of mean, n = 5).

The results of the experiments are shown in Tables XIII and XIV, and plotted in Fig. 17. Six hours after the administration of DDC (4 mg; 15,000 mμmoles) and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (4 mg; 15,000 mμmoles) there was 9.9 $\pm$ 1.69 mμmoles of DDC in 100 mg of liver as compared to 0.47 mμmoles of the pyridine (VII). At 24 hours there was 2.1 $\pm$ 0.5 mμmoles of DDC in 100 mg of liver as compared to 0.05 mμmoles of the pyridine (VII). These results clearly demonstrate that 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) undergoes more rapid metabolic degradation in liver than does DDC. Thus it is clear that the inactivity of the pyridine (VII) in the chick embryo is due to its rapid metabolic degradation. It is likely that the chick embryo liver cells when grown in monolayer culture are unable to metabolize and thus inactivate 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (VII) at a sufficient rate to prevent it exerting

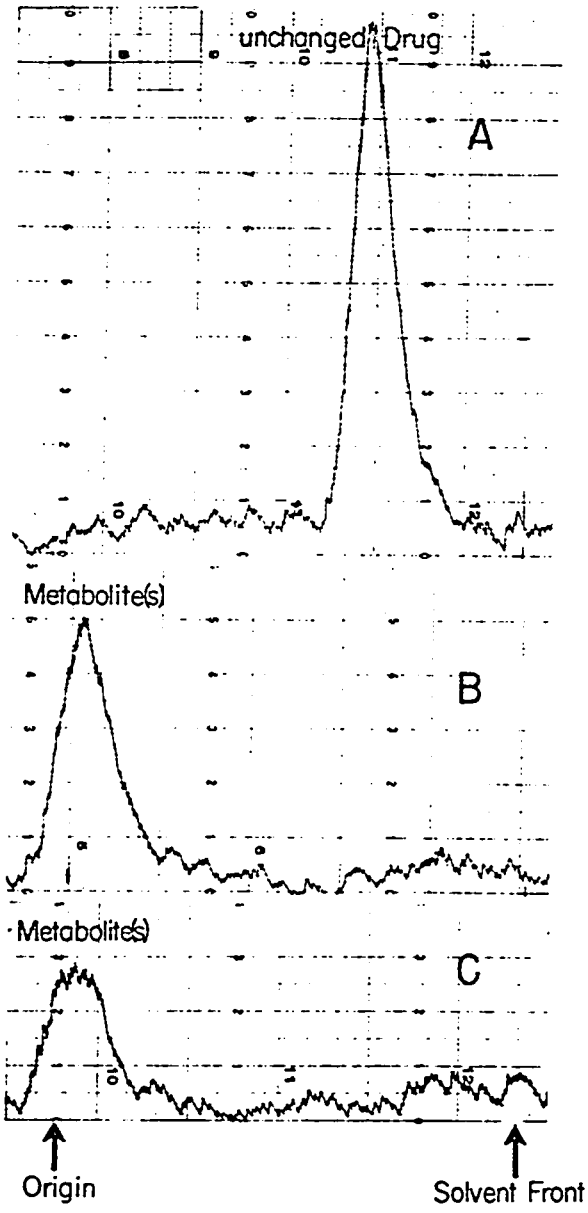


Fig. 16. Metabolism of 3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C; Radiochromatogram Records Showing Drug Added to Control Liver (A), Liver Extract 6 Hours After Injection of Drug (B), and Liver Extract 24 Hours After Injection of Drug (C).

TABLE XIII. AMOUNT OF UNCHANGED DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 4 MG (15,000 mμMOLES) DDC-^{14}C

Time After Injection (hours)	Amount of Unchanged Drug in Liver	
	% of Total Activity*	mμmoles/100 mg*
3	41.1 ± 2.2 (4)	12.2 ± 1.9 (4)
6	37.2 ± 3.0 (6)	9.9 ± 1.7 (6)
12	29.9 ± 1.2 (5)	7.1 ± 1.0 (5)
24	12.4 ± 1.6 (5)	2.1 ± 0.5 (5)
36	15.3 ± 4.2 (3)	2.3 ± 0.5 (3)

The number of experiments is given in parentheses.

* Mean ± SE of mean.

TABLE XIV. AMOUNT OF UNCHANGED DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 4 MG (15,000 mμMOLES) 3,5-DIETHOXYCARBONYL-2,4,6-TRIMETHYLPYRIDINE-^{14}C

Time After Injection (hours)	Amount of Unchanged Drug in Liver	
	% of Total Activity*	mμmoles/100 mg*
6	3.2 ± 0.9 (7)	0.47 ± 0.10 (7)
24	1.1† (5)	0.05† (5)

The number of experiments is given in parentheses.

* Mean ± SE of mean.

† Count rate not sufficient to be statistically valid.

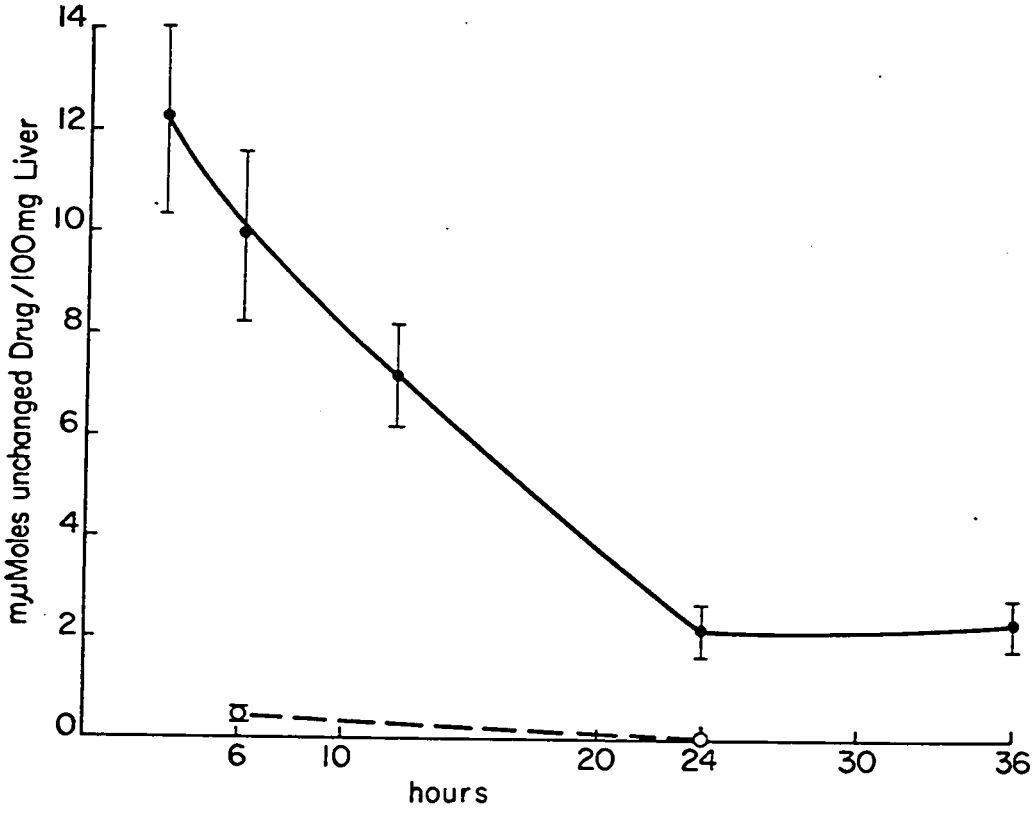


Fig. 17. Amount of Unchanged Drug in Livers of 17-Day Old Chick Embryos at Different Time Intervals After Injection of DDC-^{14}C (o———o) and 3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (O— — — —O). Each Point Represents Mean ± S E of Mean.

its porphyria-inducing activity.

It is likely that the test of porphyria-inducing activity using monolayer cultures of chick embryo liver cells gives false positive results for porphyria-inducing activity due to the fact that metabolism of drugs is not as effective as in vivo. A further question of interest raised by these studies is the following. It has recently been suggested that liver cell cultures might be used as a system for screening drugs for hepatotoxicity (Dujovne et al., 1969). Since it is possible that drugs are metabolized at a slow rate in cell culture, many false positive results may be obtained. It thus becomes of considerable interest to compare the rates of metabolism of drugs in the intact animal and in monolayer cultures of liver cells.

CHAPTER V COMPARISON OF THE METABOLISM OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,4,6-TRIMETHYLPYRIDINE AND 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYLPYRIDINE IN THE CHICK EMBRYO

Introduction

In a series of investigations, the porphyria-inducing activity of several analogues of DDC (I), the related pyridine (VII), and the benzene compound (XVII) were investigated in monolayer cultures of chick embryo liver cells. It was shown that the critical feature required for activity was an ethoxycarbonyl group with two ortho methyl substituents as shown in Fig. 18 (Marks et al., 1965; Hirsch et al., 1967).

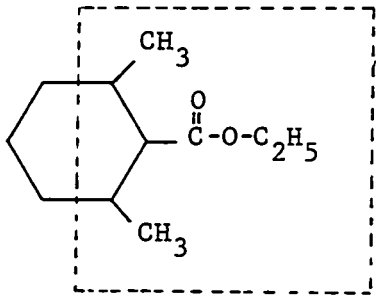
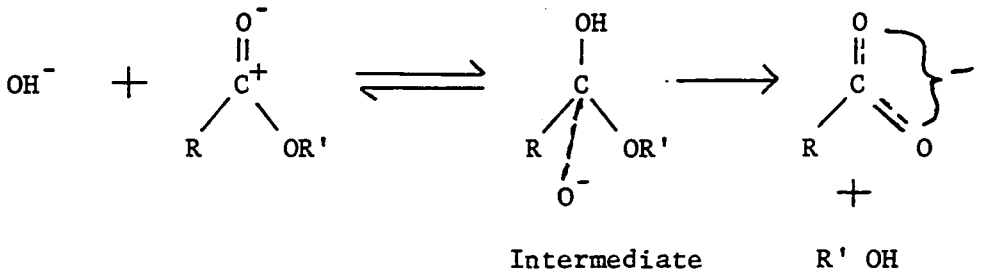


Fig. 18. Critical Feature for Porphyria-Inducing Activity in Dihydropyridine, Pyridine and Benzene Compounds

The possible significance of this arrangement of substituents becomes apparent after consideration of the mechanism of acid, base and enzymic hydrolysis of esters.

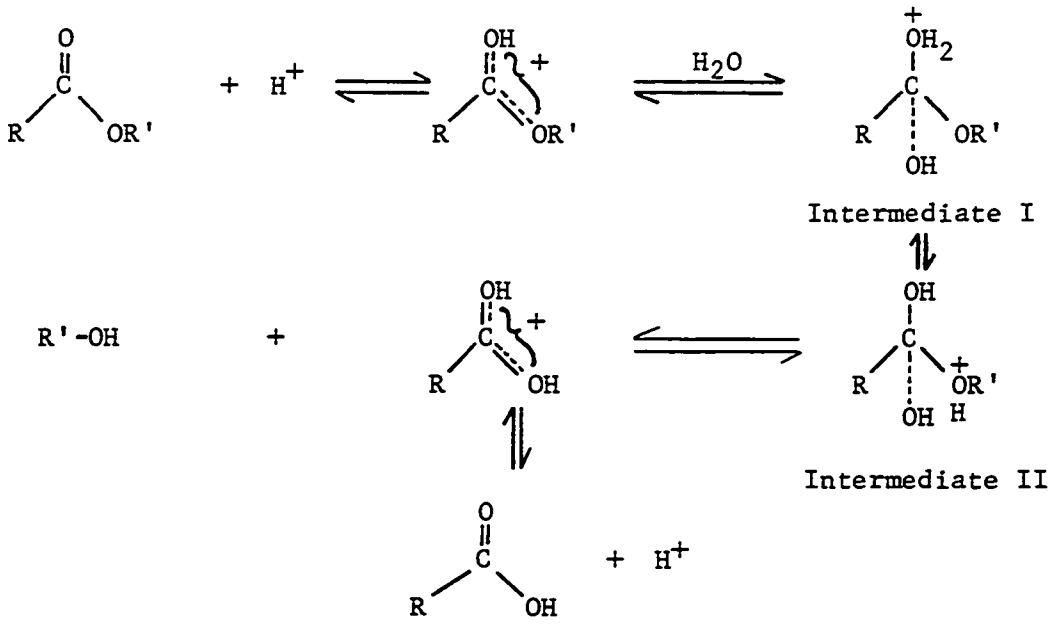
Alkaline hydrolysis of esters proceeds by the following reaction mechanism (Morrison & Boyd, 1966):



Intermediate R' OH

The first step in the hydrolysis is thought to be a nucleophilic attack of the hydroxyl ion on the carbonyl carbon, bearing a partial positive charge, with the formation of a tetrahedral intermediate. In an ester, the groups R and OR' lie in the same plane as the unsaturated function and the attack by hydroxyl ion comes from above or below this plane. This is followed by the loss of the alkoxide ion and the formation of the carboxylate anion. The reaction is essentially irreversible since the carboxylate anion shows little tendency to react with an alcohol. The slow step in the reaction is the addition of the hydroxyl ion to the carboxyl carbon to form the tetrahedral intermediate.

Acid hydrolysis of an ester is considered to proceed according to the mechanism illustrated below (Morrison & Boyd, 1966).

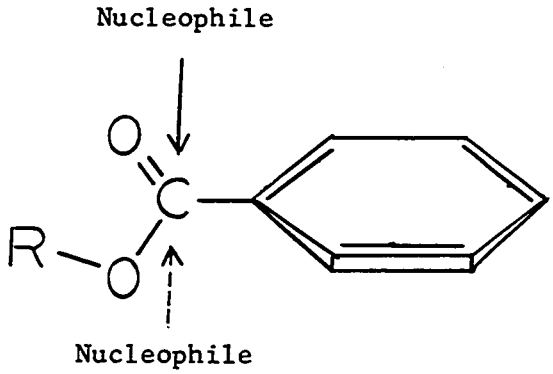


Intermediate I

Intermediate II

The electrophilic hydrogen ion attacks the oxygen atom of the carbonyl function bearing a partial negative charge. This is followed by nucleophilic attack of water from above or below the plane of the molecule on the carbonyl carbon with the formation of the tetrahedral intermediate I which is in equilibrium with intermediate II. The reaction is completed by the liberation of the alcohol moiety and the formation of the planar protonated acid which releases a proton. As in the case of alkaline hydrolysis, the slow step is the formation of the tetrahedral intermediate.

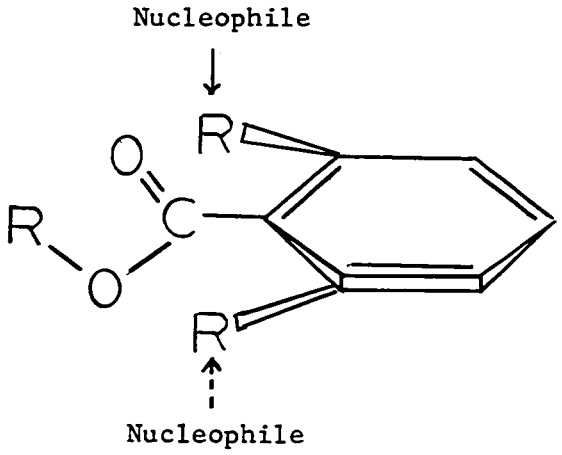
While the esters of benzoic acid are readily hydrolyzed in acidic or basic media, the esters of di-ortho substituted benzoic acids are not. This ability of the two ortho substituents (larger than hydrogen) to prevent hydrolysis of the ester group is referred to as steric hindrance. The reason for this phenomenon is the following (Kadesch, 1944; Newman, 1956). For hydrolysis of aliphatic or aromatic esters, the attacking reagent must approach the carbonyl carbon from a direction perpendicular to the plane of the carbonyl double bond. If this plane is essentially co-planar with the ring, there is little steric hindrance from the ortho substituents since the attacking reagent has a clear path of approach perpendicular to the plane of the ring (Fig. 19A). This is the case in aromatic esters such as ethyl benzoate where there are hydrogen substituents ortho to the ethoxycarbonyl group. The ethoxycarbonyl group then lies in the same plane as the benzene ring due to conjugation of the carbonyl double bond with the aromatic ring. On the other hand, if the ortho substituents are large, the ethoxycarbonyl group cannot be accommodated in the same plane as the ring (Fig. 19B), and lies in a plane perpendicular to the



(A)

No Large Ortho Substituents:

The ester function lies in the same plane as the benzene ring. Nucleophilic attack can readily occur from a direction perpendicular to the plane of the ring.



(B)

Two Large Ortho Substituents:

The ester function lies in a plane perpendicular to the benzene ring. Nucleophilic attack must come from a plane perpendicular to the plane of the ester function, access is blocked by the large ortho substituents (R).

Fig. 19. Steric Hindrance in Aromatic Esters.

ring. In this orientation, the approach of the attacking reagent is blocked by the two ortho substituents, preventing hydrolysis (Fig 19B).

Recent investigation of enzymic hydrolysis of esters reveals that the mechanism is similar to that of alkaline hydrolysis (Westheimer, 1962). For this reason, the action of such enzymes should be sensitive to steric factors in substrates. On the basis of the above considerations, it has been suggested (Hirsch et al., 1967) that the ortho-methyl substituents in compounds I, VII and XVII, are required to protect the ethoxycarbonyl groups from hydrolysis by liver and other esterases. Since hydrolysis of the ethoxycarbonyl groups would make the molecule less lipid soluble and therefore easier to eliminate (Brodie & Hogben, 1957), it is likely that molecules which are readily hydrolyzed will not achieve a concentration in the liver adequate to induce increased porphyrin biosynthesis. It was suggested that DDC (I) and other active porphyria-inducing agents of this series were metabolized by an oxidative mechanism and that this type of metabolism increased the demand for protoheme and porphyrin formation for reasons outlined below.

The oxidative metabolism of drugs is mediated by enzymes located in the microsomal fraction of the mammalian liver. Cytochrome P-450, a protoheme containing enzyme of the endoplasmic reticulum, is thought to play a key role in this type of metabolism. A suggested scheme (Omura et al., 1965) for oxidative metabolism is shown in Fig. 20. According to this scheme, NADPH reduces a flavoprotein which in turn reduces a non-heme containing iron (ferric) protein. The ferrous protein reduces cytochrome P-450 to reduced cytochrome P-450 which combines with oxygen to form an "active oxygen" intermediate (cytochrome P-450•O). This "active oxygen" intermediate reacts with the

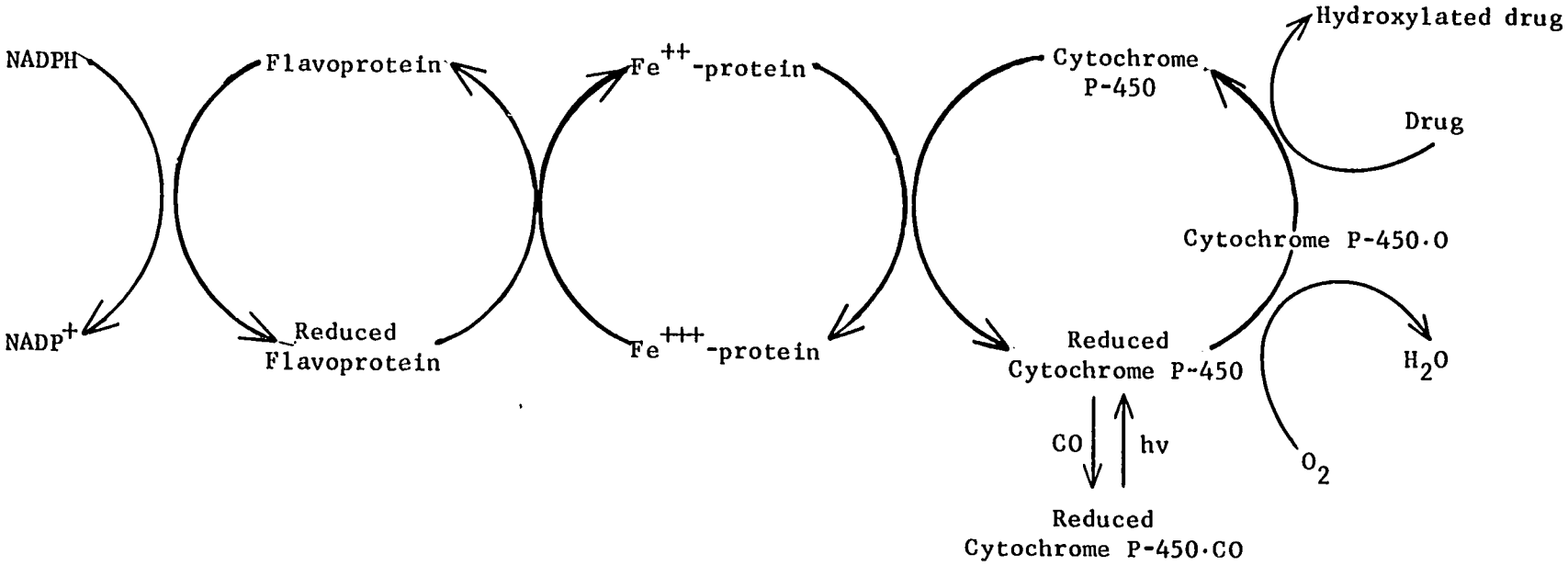


Fig. 20. Schematic Representation of the Pathway of Electron Transport for Cytochrome P-450 and the Activation of Oxygen for Hydroxylation Reactions. (From Omura et al., 1965.)

drug forming the oxidized drug and regenerating cytochrome P-450 (Gillette, 1966; Conney, 1967). Phenobarbital increases the amount of cytochrome P-450 and the activity of drug metabolizing enzymes and δ-ALA synthetase in liver (Orrenius & Ernester, 1964; Remmer & Merker, 1965). These observations prompted Granick (1966) to suggest that the porphyrin formed following de-repression of δ-ALA synthetase in response to phenobarbital and other porphyria-inducing drugs is utilized for the formation of protoheme. This protoheme in turn serves as the prosthetic group of cytochrome P-450 which is required for the oxidative metabolism of drugs. If this interpretation of Granick's is correct, then all porphyria-inducing drugs should be oxidatively metabolized.

To investigate the validity of the above ideas, ^{14}C-labelled 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) has been prepared, injected into 17-day old chick embryos and the total amount of drug and its metabolites in the liver was measured at different time intervals. These results were compared with those obtained with DDC-^{14}C. It was anticipated that 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-pyridine (VI) would be more readily metabolized and therefore not achieve as high a concentration in the liver as DDC (I). Moreover, it was anticipated that metabolites of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-pyridine (VI) would be obtained which resulted from hydrolysis. On the other hand, it was anticipated that metabolites of DDC would be obtained resulting from oxidative metabolism.

Experimental

i. Source of Compounds

Chloranil was obtained from Eastman Organic Chemicals, Rochester, New York. Neutral alumina (activity grade I) was purchased from Alupharm Chemicals, New Orleans, La.

ii. Instrumentation

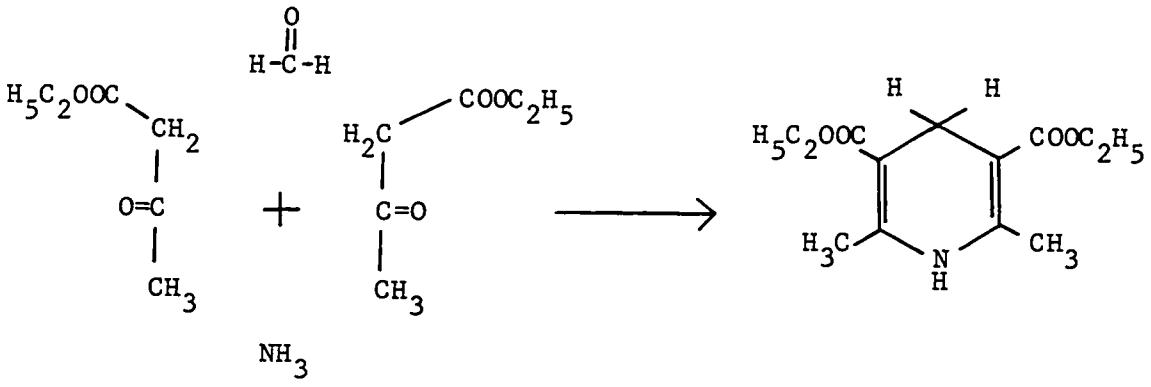
Nuclear magnetic resonance spectra were determined by the Department of Chemistry or the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta. All nuclear magnetic resonance spectra were determined in $CDCl_3$; internal reference, tetramethylsilane; oscillator frequency 60 Mc/s. Infrared spectra were obtained with a Perkin-Elmer 137 sodium chloride spectrophotometer.

iii. Preparation of Thin-Layer Plates

Silica gel G (50 g) was washed with three 100 ml portions of methanol, dried at 50° and suspended in 100 ml of distilled water. The suspension was poured into a Desaga adjustable spreader and spread onto 20 x 20 cm glass plates. The plates were allowed to dry at room temperature and activated at 110° for 30 minutes just prior to use.

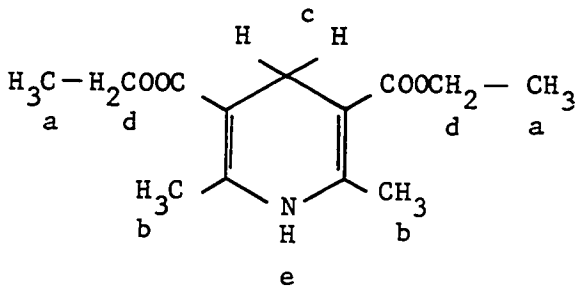
iv. Synthesis of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine

This compound was prepared by the procedure of Loev and Snader (1965) and involves the condensation of two moles of ethyl acetoacetate with one mole of formaldehyde and one mole of ammonia as illustrated on the next page.



Ethyl acetoacetate (26 g; 0.2 moles), formaldehyde (8.1 g; 0.1 moles) and 10 ml of concentrated ammonium hydroxide (0.15 moles) in 20 ml of ethanol were refluxed for 3 hours. The cooled reaction mixture was poured into 250 ml of ice cold water and the product which crystallized (yield 65%) was collected. The product was crystallized from ethanol-water, m.p. 158 - 163°, λ max. (ethanol) 231 and 365 mμ (ε, 16,200 and 6,830). Braude et al. (1960) reported m.p. 189 - 190°, λ max. 230 and 372 mμ (ε, 16,000 and 7,250). Examination of this product by gas-liquid chromatography revealed the presence of an impurity constituting approximately 5% of the sample.

The essential data derived from the nuclear magnetic resonance spectrum (Fig. 21) is given below:



τ Value	Proton Assignment	J (cps)	Relative Peak Area	
			Found	Theoretical
8.75	a	7.0	6.5	6
7.83	b		6.0	6
7.12	Not assigned		1.3	
6.75	c		1.8	2
5.85	d	7.0	4.1	4
4.7	e		0.6	1

Schroll et al. (1968) reported the following spectral characteristics:

8.70	a	7.0
7.78	b	
6.70	c	
5.78	d	7.0
4.42	e	

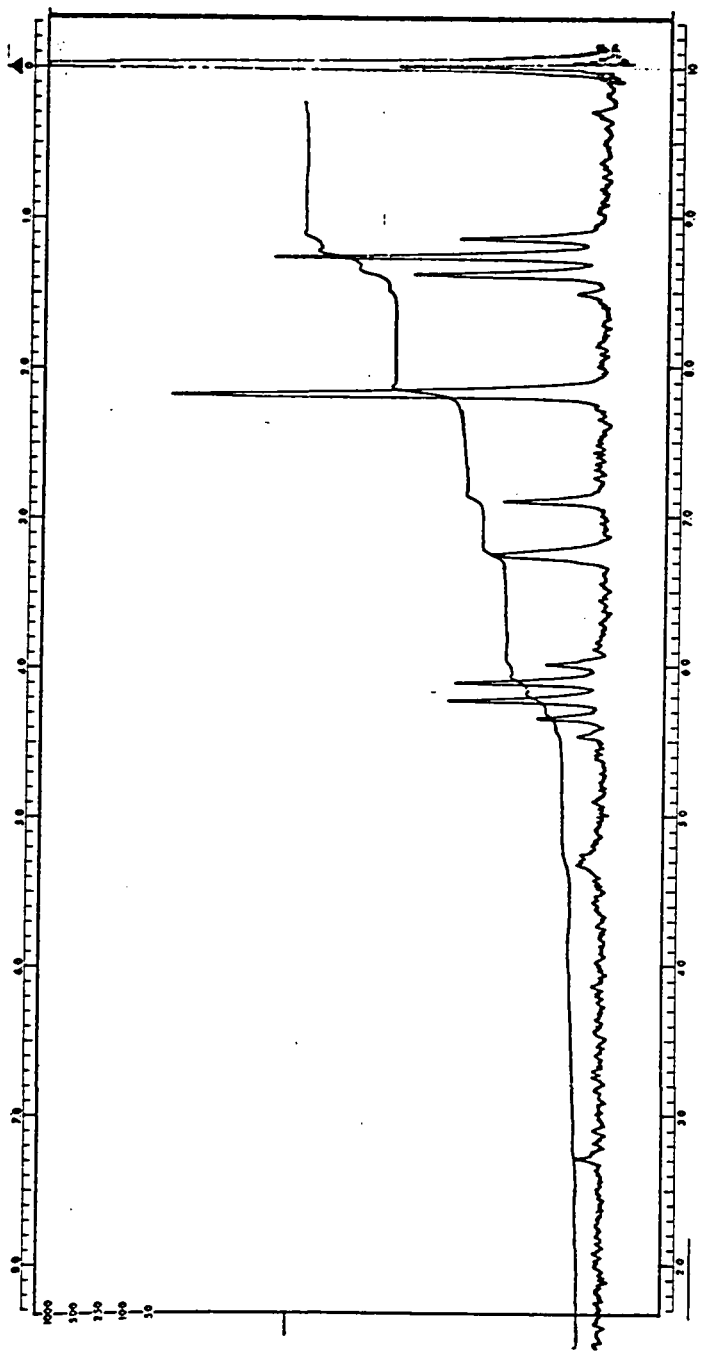


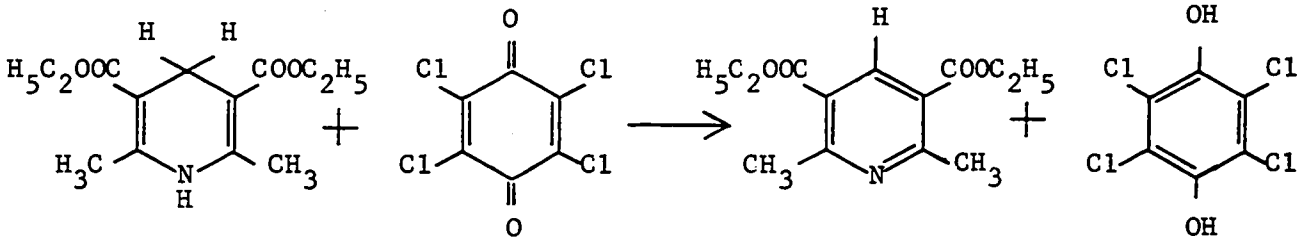
Fig. 21. Nuclear Magnetic Resonance Spectrum of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) in $CDCl_3$.

v. Attempted Purification of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine by Column Chromatography

A column (2 cm internal diameter) was packed in the usual manner to a height of 25 cm with either alumina (neutral activity grade I) or silica gel. The dihydropyridine was dissolved in a minimum amount of chloroform and placed on the column. Elution was carried out with petroleum ether, petroleum ether-benzene mixtures, benzene, benzene-chloroform mixtures and chloroform.

vi. Synthesis of 3,5-Diethoxycarbonyl-2,6-dimethylpyridine from 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine

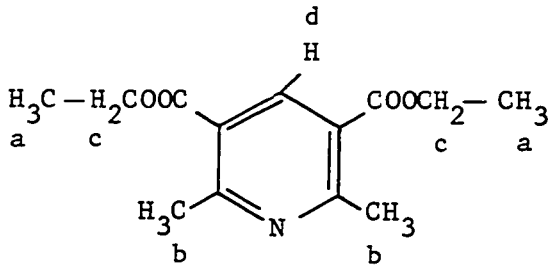
The conversion involves the oxidation of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) to the pyridine (XVIII) (Braude et al., 1960) as shown below:



(VI) (XIX) (XVIII)

A solution of 10 g of chloranil (XIX) (0.04 moles) in 250 ml of tetrahydrofuran was added to a solution of 10 g (0.04 moles) of the di-ester (VI) in 200 ml of tetrahydrofuran at 25°. After 1 hour, the solvent was removed with a rotary evaporator and the residue dissolved

in 100 ml of ether. The ethereal solution was extracted with three 50 ml portions of 1N HCl. The acidic solution was neutralized with sodium carbonate and the product, which crystallized, collected (yield 66%). The product was purified by repeated crystallizations from ethanol-water, yielding the pure di-ester (XVIII), m.p. 70 - 71°, λ max. (ethanol) 235, 273, and 281 mμ (ε, 11,500, 3,930 and 3,260). Braude et al. (1960) reported m.p. 73.5 - 74.5°, λ max. 236, 273 and 282 mμ (ε, 13,200, 4,120 and 3,380). The essential data derived from the nuclear magnetic resonance spectra is given below:



τ Value	Proton Assignment	J (cps)	Relative Peak Area	
			Found	Theoretical
8.58	a	7.0	5.7	6
7.14	b		6.0	6
5.58	c	7.0	3.7	4
1.29	d		0.9	1

vii. Reduction of 3,5-Diethoxycarbonyl-2,6-dimethylpyridine With Sodium Borohydride

Sodium borohydride (665 mg) was added in small portions over a 30 minute period to a solution of 2 g (0.008 moles) of the pyridine (XVIII) in 30 ml of ethanol (Brignell et al., 1966). The solution was kept at 25° for 72 hours, 15 ml of water added and the solution heated on a water bath. Upon cooling, the crystals which separated were collected (yield 30%) and purified by crystallization from ethanol-water, m.p. 146 - 150°, λ max. (ethanol) 231 and 375 mμ (ϵ, 16,000 and 6,920). Braude et al. (1960) reported m.p. 189 - 190 °, λ max. 230 and 372 mμ (ϵ, 16,000 and 7,250). The nuclear magnetic resonance spectrum was similar to that of the product obtained by the previous procedure (section iv).

viii. Synthesis and Radiochemical Purity of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C

To a vial containing 0.1 mCi (4.17 mCi/mmole) ethyl acetoacetate-3-^{14}C, was added 0.78 g (0.006 moles) of unlabelled ethyl acetoacetate to yield a product of 0.017 mCi/mmole. The contents were transferred to a 10 ml flask with 2 ml of ethanol. Formaldehyde (0.090 g; 0.003 moles) and 0.75 ml (0.005 moles) of concentrated ammonia solution was added to the flask and the mixture refluxed for 3 hours. After cooling, water was added to the reaction mixture and the product which crystallized was collected (yield 65%). The product was purified by successive crystallizations from ethanol-water until the specific activity of the compound remained constant. This was achieved after 3 crystallizations. The product, m.p. 150 - 153°, was found to have a specific activity of 0.024 mCi/mmole, λ max. (ethanol) 231 and 375 mμ (ϵ, 14,800

and 6,990). Braude et al. (1960) reported m.p. 189 - 190°, λ max. 231 and 372 mμ (ε, 16,000 and 7,250).

ix. Synthesis and Radiochemical Purity of 3,5-Diethoxycarbonyl-2,6-dimethylpyridine-^{14}C

A solution of 80 mg chloranil (XIX) (0.3 mmoles) in 10 ml of tetrahydrofuran was added to a solution of 75 mg (0.2 mmoles) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (0.024 mCi/mmole) in 10 ml of tetrahydrofuran. The product (yield 50%), isolated in the usual manner, was purified by successive crystallizations from ethanol-water until the specific activity of the compound remained constant. This was achieved after two crystallizations. The product, m.p. 70 - 71°, was found to have a specific activity of 0.024 mCi/mmole, λ max. (ethanol) 235, 273 and 281 mμ (ε, 11,500, 3,930 and 3,260). Braude et al. (1960) reported m.p. 73.5-74.5°, λ max. 236, 273 and 282 mμ (ε, 13,200, 4,120 and 3,380).

x. Amount of ^{14}C in the Livers at Different Time Intervals After Injection of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C Into Fluids Surrounding the Chick Embryo

The procedure employed was that used for DDC-^{14}C and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C, described in chapter IV.

xi. Extraction of Labelled Drug and Metabolites From 17-Day Old Chick Embryos and Surrounding Fluids After Injection of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C

The procedure employed for the extraction of the drug and its metabolites was the same as that described for DDC-^{14}C and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C in chapter IV. The recovery of radioactive material was 50% of the amount injected.

xii. Determination of the Amount of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C and 3,5-Diethoxycarbonyl-2,6-dimethylpyridine-^{14}C in Chick Embryo Extracts by Isotope Dilution Analysis

(a) 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C

The procedure for estimating the concentration of this compound by means of isotope dilution analysis was essentially the same as that described for DDC-^{14}C (chapter IV), except that extraction of the drug from the petroleum ether-methanol (1:2) solution was carried out with methanol-water (4:1) rather than methanol-water (2:1). The product was crystallized to constant specific activity (0.001 μCi/mmole), λ max. (ethanol) 231 and 375 mμ (ε, 14,900 and 7,000). Assuming all the radioactivity in the original extract from the chick embryo and fluids was represented by 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI), it could be calculated that the specific activity of the recrystallized product would have been 0.034 μCi/mmole.

(b) 3,5-Diethoxycarbonyl-2,6-dimethylpyridine-^{14}C

The procedure for estimating the concentration of this compound by isotope dilution analysis was the same as that described for 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C (chapter IV) except that 3,5-diethoxycarbonyl-2,6-dimethylpyridine, which is a solid, was allowed to crystallize from the final neutralized acid extracts. The compound (m.p. 70 - 71°) was then crystallized to constant specific activity (0.029 μCi/mmole) from ethanol-water, λ max. (ethanol) 235, 273 and 281 mμ (ε, 11,000, 3,860 and 3,150). Braude et al. (1960) reported λ max. 236, 273 and 282 mμ (ε, 13,200, 4,120 and 3,380). Assuming all the radioactivity in the original extract from the chick embryo and fluids was contributed by 3,5-diethoxycarbonyl-2,6-dimethyl-

pyridine-^{14}C (XVIII), it could be calculated that the specific activity of the recrystallized product would be 0.034 μCi/mmole.

xiii. Amount of Unchanged 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-pyridine-^{14}C in the Liver at Different Time Intervals After Injection of Labelled Drug into Fluids Surrounding the Embryo

The procedure for the extraction of this drug and its metabolite(s) from liver after injection of 3.8 mg (15,000 mμmoles) into the fluids surrounding the chick embryo was identical to that described for DDC-^{14}C (chapter IV). Because of the small amount of radioactive drug in the liver 24 hours after injection, the extracts from two livers were combined prior to chromatographic separation of unchanged drug and its metabolite(s). The Rf value on silica gel plates for the dihydropyridine (VI) using benzene-methanol (14:1) as developing solvent was 0.38 and for the pyridine (XVIII) was 0.71. The liver residue after extraction was found to contain no radioactivity.

xiv. Attempts to Determine the Nature of the Metabolite(s) of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C in Extracts of Chick Embryo

The drug and its metabolites were extracted from the embryo and surrounding fluids with chloroform-methanol (2:1) as outlined for DDC (chapter IV). The residue (R) obtained by removal of the solvent (Fig. 22) was treated successively with four 250 ml portions of methanol and the methanol extracts (ME) decanted from the insoluble sludge, combined and counted (Fig. 22). After removal of the methanol with a rotary evaporator, the residue was dissolved in 400 ml of petroleum ether-methanol (1:2). The bulk of the radioactive material was extracted from

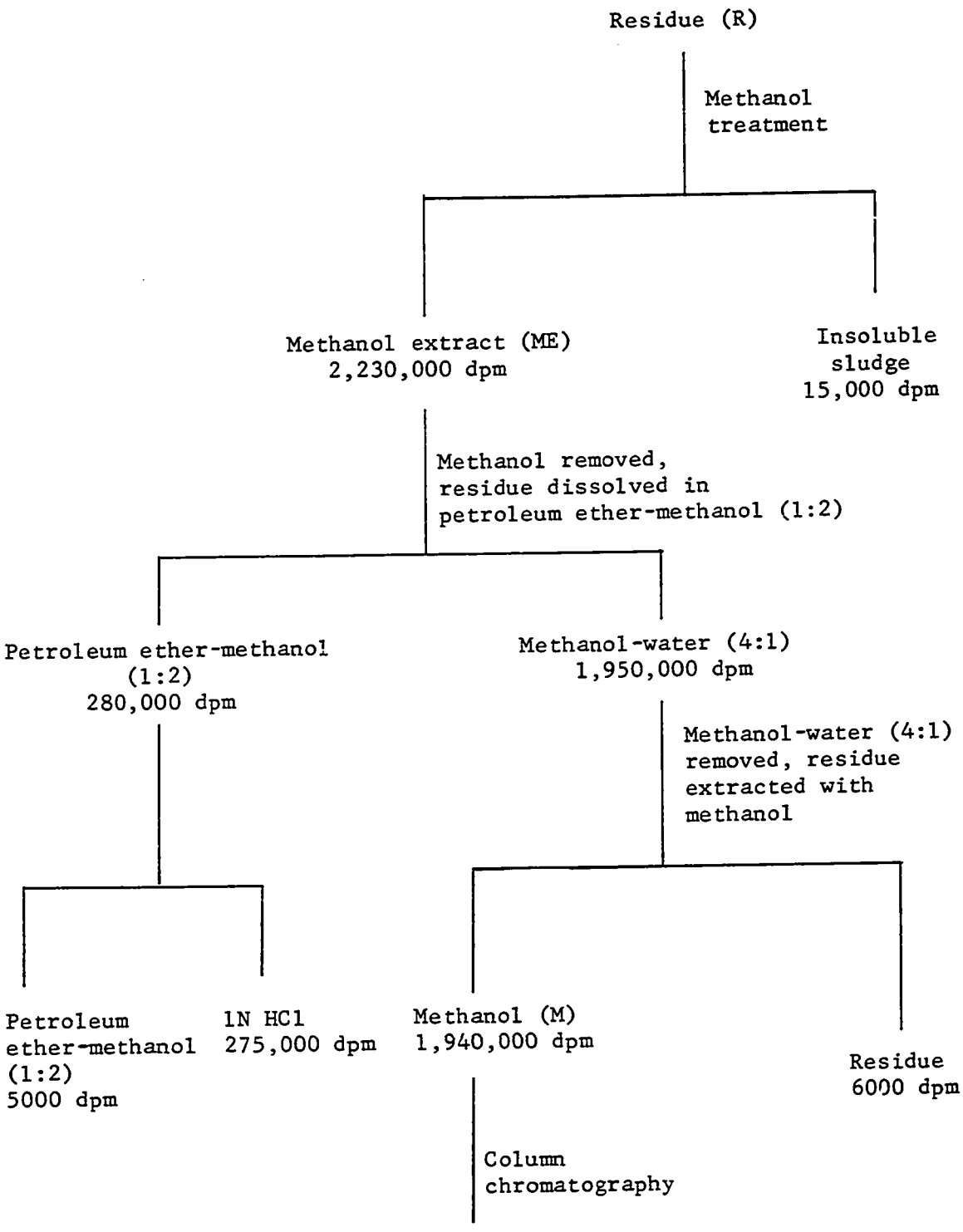


Fig. 22. Separation of 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-pyridine (VI) and its Metabolite(s)

the petroleum ether-methanol (1:2) solution with three 200 ml portions of methanol-water (4:1). The remaining radioactivity in the petroleum ether-methanol (1:2) solution was extracted with three 100 ml portions of 1N HCl (Fig. 22). For convenience, further investigation of the 1N HCl solution and the methanol-water (4:1) solution will be discussed separately below. For the sake of brevity, the abbreviation, dpm, is used throughout this chapter for counts per minute corrected for quenching (Noujaim, 1969).

(a) Investigation of the 1N HCl Solution

The 1N HCl solution was neutralized with sodium carbonate and the neutralized solution extracted with three 100 ml portions of ether. The ethereal solution was extracted with four 25 ml portions of 1N HCl, the acid solution neutralized with sodium carbonate and the neutralized solution extracted with three 50 ml portions of ether. The ether was removed, methanol (10 ml) added to the residue and 1 ml aliquots removed and added to vials containing 14 ml of toluene scintillation solution. A portion of the methanol solution (approximately 0.3 ml) was placed as a band on a thin-layer plate coated with 250μ of silica gel G and developed with benzene-methanol (14:1). The radioactive area was detected with the radiochromatogram scanner and the Rf of the compound was found to be 0.72. For purposes of estimating a suitable concentration for recording the ultraviolet absorption spectra, it was assumed that all the radioactivity in the solution was contributed by 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI). Examination of the ultraviolet absorption spectra in ethanol revealed absorption bands at 236, 273 and 281.5 mμ. 3,5-Diethoxycarbonyl-2,6-dimethylpyridine (XVIII) has an Rf of 0.71 in benzene-methanol (14:1). Braude et al. (1960) reported

λ max. 235, 273 and 281 mμ.

(b) Methanol-Water (4:1) Extract of Petroleum Ether-Methanol (1:2) Solution

The methanol-water (4:1) was removed with a rotary evaporator and the residue extracted with three 100 ml portions of methanol. The methanolic solution was concentrated to 10 ml and placed on top of an alumina (neutral activity grade I) column (2 cm x 25 cm). The drug and its metabolite(s) were eluted from the column by a series of solvents of increasing polarity and the fractions counted in either the toluene scintillation solution or in a dioxane scintillation solution prepared as follows. To 100 g naphthalene, 4 g PPO, and 0.1 g POPOP was added dioxane to make a final volume of 1 liter (Wang & Willis, 1965). The dioxane scintillation solution was utilized for counting water containing eluants. The eluting solvent used and the amount of radioactivity in each fraction is given below:

Fraction No.	Eluting Solvent	Volume (ml)	Total Radioactivity (dpm)
1	petroleum ether	200	830,000
2	chloroform	315	6,600
3	chloroform-methanol(1:1)	250	594,000
4	methanol	75	23,000
5	methanol-water (7:3)	200	289,000
6	methanol-water (1:1)	250	61,000
7	water	100	1,000

To facilitate discussion, fraction no.1 will be referred to as alumina fraction A-I, fraction 3 as alumina fraction A-II, and fractions 5 and 6, which were pooled, as alumina fraction A-III.

1. Alumina Fraction A-I

The solvent was removed, the residue dissolved in 5 ml of methanol and placed as bands on several silica gel plates (750 μ). After development with benzene-methanol (14:1) was carried out, one radioactive spot (Rf 0.72) was detected with the radiochromatogram scanner. The radioactive areas of silica gel were removed and eluted with three 25 ml portions of methanol. One ml aliquots of this methanolic solution were added to vials containing 14 ml of toluene scintillation solution and counted. Inspection of the ultraviolet spectral properties of this solution revealed absorption bands in ethanol at 236, 273 and 281.5 mμ. For purposes of estimating a suitable concentration for recording the ultraviolet absorption spectra, it was assumed that all the radioactivity in the solution was contributed by 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI).

2. Alumina Fraction A-II

The solvent was removed, the residue dissolved in 5 ml of methanol and a portion placed as a band on a 2 inch silica gel (250 μ) plate. The material did not migrate from the origin after development with benzene-methanol (14:1). The remainder of the solution was placed as bands on 20 x 20 cm silica gel (750 μ) plates. After development with butanol-methanol-water (9:1:1), one radioactive spot was detected with the radiochromatogram scanner. The radioactive areas of silica gel were removed and eluted with three 25 ml portions of methanol. After concentrating the methanolic solution, the residue was purified by

repetition of the chromatographic procedure two more times. The Rf of the radioactive material was 0.52. An aliquot (1 ml) of the purified material in methanol (75 ml) was counted and on the basis of the radioactivity, the solution was diluted with ethanol in order to record the ultraviolet absorption spectrum. The solution was found to have an absorption band at 241 mμ. Potassium chloride (200 mg) was added to an amount of methanolic solution containing approximately 1 mg of radioactive material. The solvent was removed and the residue dried at 100° under reduced pressure. The residue was compressed into a pellet and the infrared spectrum recorded. No information could be obtained from this spectrum due apparently to contaminating material.

3. Alumina Fraction A-III

This fraction was treated in the same manner as alumina fraction A-II. A wide radioactive band (Rf 0.5) was obtained on the thin-layer chromatogram, λ max. (ethanol), 251 mμ.

xv. Attempts to Determine the Nature of Metabolites of DDC-^{14}C in Extracts of Chick Embryo

The drug and its metabolite(s) were extracted from the embryo and surrounding fluids with chloroform-methanol (2:1) as outlined in chapter IV. This extract was treated in a manner similar to the extract from chick embryos which had been injected with 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI). However, methanol-water (2:1) was employed (Fig. 23) to extract the petroleum ether-methanol (1:2) solution rather than methanol-water (4:1) used previously. This was necessary because of the different solubilities of compounds encountered in this experiment. Following this treatment, the methanolic solution (designated M in Fig. 23) containing the radioactivity was concentrated

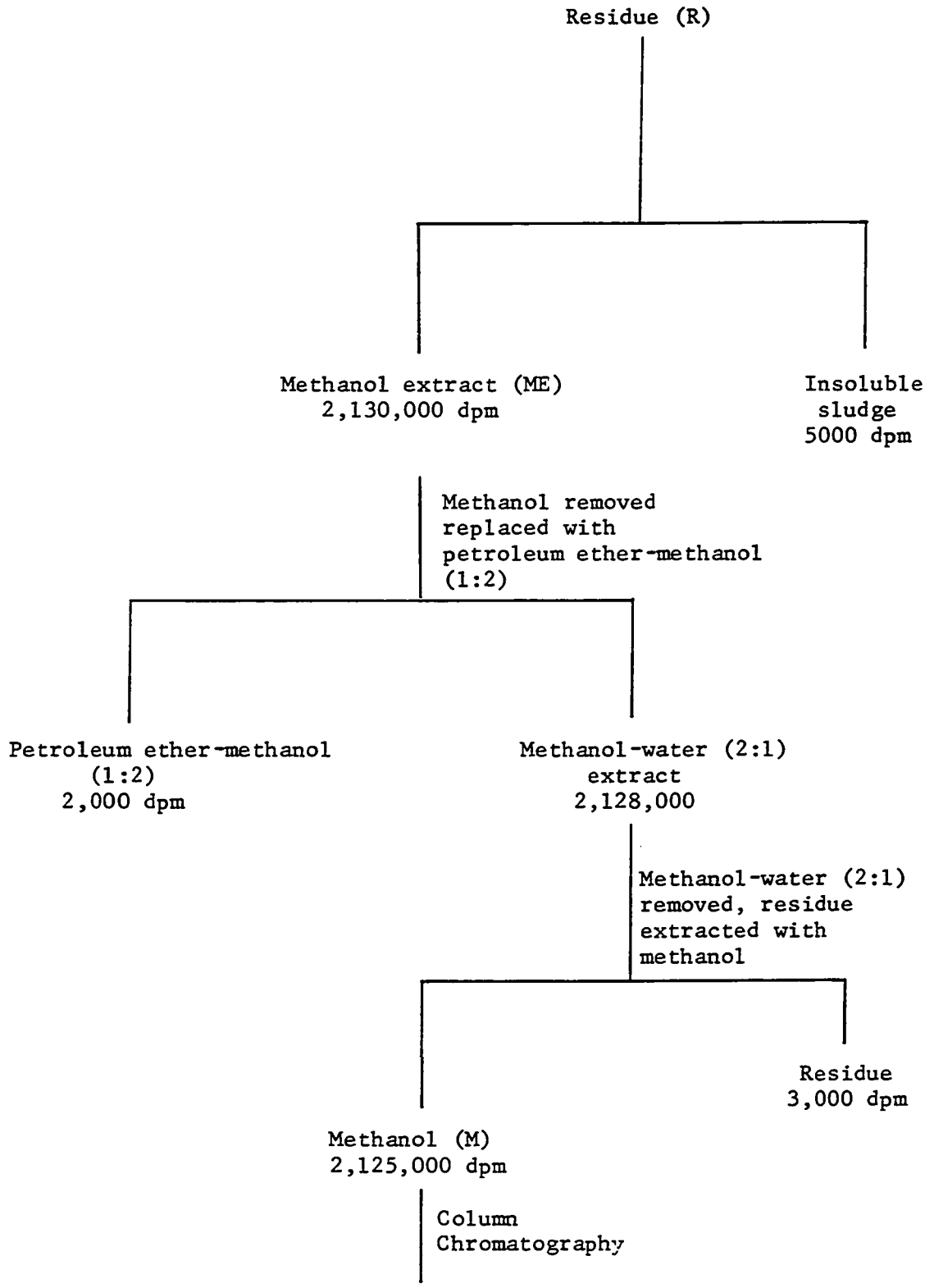


Fig. 23. Separation of DDC and its Metabolite(s)

to 10 ml and placed on top of an alumina column (2 cm x 25 cm). Details of the chromatographic separation are shown below.

Fraction no.	Eluting Solvent	Volume (ml)	Total Radioactivity (dpm)
1	petroleum ether	400	1,021,000
2	benzene-petroleum ether (1:1)	200	No activity
3	benzene-chloroform (1:1)	200	No activity
4	chloroform	200	No activity
5	chloroform-methanol (4:1; 2:1; 1:1)	200 each	No activity
6	methanol	200	463,000
7	methanol-water (2:1)	500	642,000
8	water	100	5,000

To facilitate discussion, fraction no.1 will be referred to as alumina fraction B-I and fraction no.6 and no.7, which were pooled, will be referred to as alumina fraction B-II.

(a) Alumina Fraction B-I

The solvent was removed and the residue dissolved in 5 ml of methanol. A portion of the methanol solution was placed as a band on a 2 inch silica gel plate (250 μ) and the plate developed with benzene-methanol (14:1). One radioactive spot was detected with the radiochromatogram scanner, Rf 0.42. The silica gel containing the radioactive

material was removed and eluted with three 25 ml portions of methanol. A portion of the solution was diluted with ethanol and the ultraviolet spectrum recorded. Absorption maxima were located at 233 and 351.5 mμ. DDC has an Rf of 0.45 in benzene-methanol (14:1). Marks et al. (1965) reported λ max. (ethanol) 232 and 351 mμ for DDC.

(b) Alumina Fraction B-II

The solvent was removed and the residue dissolved in 5 ml of methanol. The solution was placed as a band on silica gel (750 μ) plates and the plates developed with butanol-methanol-water (9:1:1). Two radioactive spots, Rf 0.46 and 0.60 were detected. These are designated as alumina fraction B-IIa and alumina fraction B-IIb, respectively. The portions of silica gel containing the radioactive material were removed, each eluted with three 25 ml portions of methanol and the methanol solutions were concentrated to 5 ml for repetition of the chromatographic procedure. In an attempt to obtain the purest possible material for spectroscopic investigation, each fraction was subjected to repeated re-chromatography in the system described above. The fractions were diluted with ethanol and the ultraviolet spectra recorded. An absorption maximum at 251 mμ was found for alumina fraction B-IIa and 257 mμ for alumina fraction B-IIb. An attempt was made to record the infrared spectra of these fractions by the procedure described previously. No information could be obtained, due apparently to contaminating material.

xvi. Distribution Between Solvents of Radioactivity From Extracts of Chick Embryos Injected with ^{14}C-Labelled Drugs

(a) 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C

The solvent was removed from a portion of the methanol extract (M in Fig.22) and the residue dissolved in 10 ml of ether. Aliquots of

the ether solution (5 ml) were placed in each of two separatory funnels and 45 ml of ether added. One percent sodium carbonate solution (50 ml) was added to one of the funnels and 50 ml of water was added to the other. The contents of the funnels were thoroughly agitated and one ml aliquots of each layer added to vials containing 14 ml of the dioxane scintillation solution for counting. As a control, the above sequence of steps was carried out with a solution of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) in ether.

(b) DDC-^{14}C

The procedure employed for this study was identical to that used for 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) which has been described above.

Results and Discussion

The first objective of this study was to synthesize 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) in radiochemically pure form. Synthesis of the unlabelled dihydropyridine (VI) yielded a product, m.p. 158 - 163°, λ max. (ethanol) 231 and 375 mμ (ϵ, 14,800 and 6,990). Braude et al. (1960) reported m.p. 189 - 190°, λ max. 231 and 372 mμ (ϵ, 16,000 and 7,000). It was disturbing that the melting point of our compound differed so markedly from that previously reported. For this reason, a further investigation of this compound was required. Examination of the nuclear magnetic resonance spectrum (Fig. 21) revealed a peak at 7.12τ which could not be explained in terms of the proposed structure for the compound. It was suspected that this peak may have been due to ethanol which was not removed. However, recording the spectrum in the presence of D_2O indicated that

this was not the case. Examination of the dihydropyridine (VI) by gas-liquid chromatography revealed the presence of an impurity constituting approximately 5% of the sample which had a retention time identical to that of an oxidized product of the dihydropyridine (VI), viz. 3,5-diethoxycarbonyl-2,6-dimethylpyridine (XVIII). It was likely this impurity was formed by decomposition of the dihydropyridine (VI) on the hot Celite column. This was confirmed by an examination of the ultraviolet absorption spectrum of the dihydropyridine (VI) which showed clearly that no pyridine was present prior to gas-liquid chromatography. Attempts to purify the dihydropyridine (VI) on an alumina column (neutral activity grade I) or silica gel column were unsuccessful due to its instability and subsequent conversion to the pyridine (XVIII).

An alternate route was explored for the preparation of the dihydropyridine (VI) in pure form. The pyridine (XVIII) was reduced to the dihydropyridine (VI) with sodium borohydride and the purity of this product examined. However, it was shown that this product was not superior to that obtained by the previous procedure. Since all attempts to obtain a product with the recorded melting point had failed, it appeared possible that the melting point was not a good criterion of purity. In particular, the ready decomposition of this product to the pyridine (XVIII) might afford an explanation for the low melting point observed. It was decided to assess the purity of the dihydropyridine (VI) as follows. 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) was crystallized to constant specific activity (0.024 mCi/mmole) and oxidized to 3,5-diethoxycarbonyl-2,6-dimethylpyridine (XVIII) (0.024 mCi/mmole). Since the specific activity of the dihydropyridine (VI) was the same as that of the PURE pyridine, it was clear that if it contained an impurity, it was present in very small quantities. For this reason, it was decided to proceed with our planned experiments

using the dihydropyridine (VI) and bearing in mind that it might contain a minor contaminant.

The second objective of this study was to measure the total amount of radioactivity and unchanged drug in the livers of 17-day old chick embryos at different time intervals after the injection of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI). The total amount of radioactive drug in the livers of chick embryos at different time intervals is shown in Table XV and is compared to results previously obtained with DDC-^{14}C in Fig. 24. The amount of radioactive drug present in the liver at any specific period in time after injection of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) was approximately one-fifth of the amount after injection of DDC-^{14}C. This observation was in accordance with the expectation that 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) would be more readily metabolized and therefore not achieve as high a concentration in the liver as DDC.

To determine whether metabolism of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) occurs in the chick embryo, the technique of reverse isotope dilution analysis was used. By this means, it was shown that the radioactive drug extracted from the chick embryo contained approximately 3.5% unchanged drug and 84% of the oxidation product, viz. 3,5-diethoxycarbonyl-2,6-dimethylpyridine (XVIII) (Table XVI). Assuming that the pyridine was formed non-enzymically from the dihydropyridine (VI), it could be calculated that of the radioactive drug extracted, approximately 12.5% of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) had been converted enzymically to a metabolite.

The total radioactive material in the livers of chick embryos

TABLE XV. AMOUNT OF RADIOACTIVE DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 3.8 MG (15,000 mμMOLES) 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYLPYRIDINE-^{14}C

Time After Injection (hours)	Amount of Radioactive Drug* mμmoles/100 mg liver†
0.5	4.7 ± 0.7 (10)
3	3.8 ± 0.4 (9)
6	5.9 ± 1.1 (8)
12	5.6 ± 1.5 (9)
24	1.9 ± 0.4 (8)
48	2.9 ± 0.7 (6)

The number of experiments is given in parentheses.

* The results in this table are calculated on the basis that all the drug in the liver is 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI).

† Mean ± SE of mean.

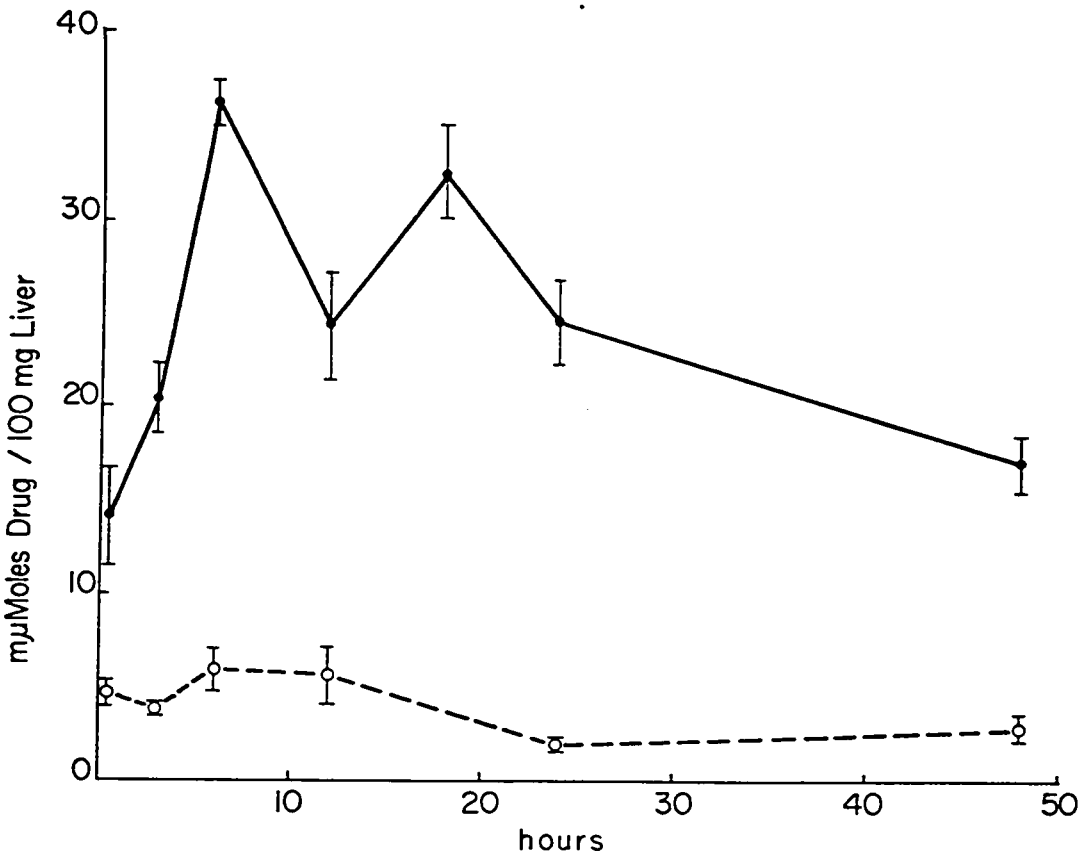
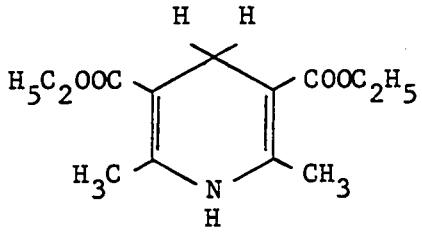
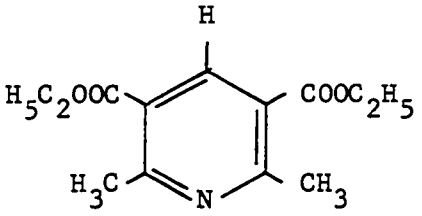


Fig. 24. Amount of Radioactive Drug in Livers of 17-Day Old Chick Embryos at Different Time Intervals After Injection of DDC-^{14}C (o————o) and 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (O----O). Each Point Represents Mean ± S E of Mea

TABLE XVI. ISOTOPE DILUTION ANALYSIS OF RADIOACTIVE EXTRACTS FROM EMBRYOS INJECTED WITH 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYLPYRIDINE-^{14}C

Diluting Agent	Unchanged Drug % of Total Radioactivity Extracted*
	4
	3
	84
	85

* Duplicate analyses were performed.

was extracted at various time intervals and separated by means of thin-layer chromatography using benzene-methanol (14:1) as the developing solvent. By means of the radiochromatogram scanner, radioactive material could only be detected at the origin of the chromatogram. This radioactive material could not have been 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) or its oxidation product (XVIII) since these compounds have Rf values of 0.38 and 0.71 respectively in this system. Since the amount of radioactive material which had been placed on the chromatogram was small, it was possible that the radiochromatogram scanner was not sufficiently sensitive to detect small amounts of radioactivity contributed by the unchanged drug (VI) or by its oxidation product (XVIII). For this reason, the areas of silica gel corresponding to the Rf values of these compounds were removed and counted(Table XVII).

The results show that at 6 hours only 3.7% of the total activity in the liver was represented by unchanged drug (VI) while at 24 hours, this drops to a level which could not be detected. These results may be too low in view of the following considerations. When an aliquot of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) is added to a control liver, extracted and chromatographed by our usual procedure, the material on the chromatogram is shown to be approximately 40% 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) and approximately 60% 3,5-diethoxycarbonyl-2,6-dimethylpyridine-^{14}C (XVIII). Clearly, oxidation occurs during extraction and it is therefore possible that in previous experiments (Table XVII), the liver contained 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) which was converted to the oxidation product (XVIII) during the extraction procedure. For purposes of comparison with the results previously

TABLE XVII. AMOUNT OF UNCHANGED DRUG IN LIVERS OF 17-DAY OLD CHICK EMBRYOS FOLLOWING INJECTION OF 3.8 MG (15,000 mμMOLES) 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO 2,6-DIMETHYLPYRIDINE-^{14}C

Time After Injection (Hours)	Amount Recovered as Dihydropyridine (VI)		Amount Recovered as Pyridine (XVIII)		Amount Recovered as Pyridine (XVIII) and Dihydropyridine (VI)	
	% of Total Activity in Liver*	mμmoles/100 mg Liver*	% of Total Activity in Liver*	mμmoles/100 mg Liver*	% of Total Activity in Liver*	mμmoles/100 mg Liver as Dihydropyridine* (VI)
6	3.7 ± 0.9	0.19 ± 0.09	14.3 ± 6.9	0.98 ± 0.41	18.0 ± 7.8	1.17 ± 0.50
24	†	†	†	†	†	†

* Mean ± SE of mean.

† No radioactivity detected.

obtained with DDC, it was assumed that the 3,5-diethoxycarbonyl-2,6-dimethylpyridine (XVIII) recovered (Table XVII) from livers represented unchanged 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) in the liver which had been oxidized in the course of the extraction procedure. The total unchanged drug in the liver was calculated by adding the amounts of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) and 3,5-diethoxycarbonyl-2,6-dimethylpyridine (XVIII). This amount of unchanged drug is plotted in Fig. 25, and compared with results previously obtained with DDC. Clearly, there is far less unchanged 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) in the liver than unchanged DDC. This observation is in accordance with the expectation that 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine would be more readily metabolized and therefore not achieve as high a concentration in the liver as DDC. However, it is unlikely that the inactivity of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) can be attributed solely to the low level in the liver for the following reason. When 0.1 mg (325 mμmoles) of DDC-^{14}C was injected into the fluids surrounding 17-day old chick embryos, a level of 1.1 ± 0.1 mμmoles/100 mg liver (± SE of mean, n = 9) of total radioactive drug was attained 6 hours later, which was sufficient to induce porphyrin biosynthesis. This level is comparable to the level of unchanged 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) attained 6 hours after injection.

A third objective of our studies was to investigate the nature of the metabolite(s) of DDC and 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI). Our first studies were directed to an examination of the metabolite(s) of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-

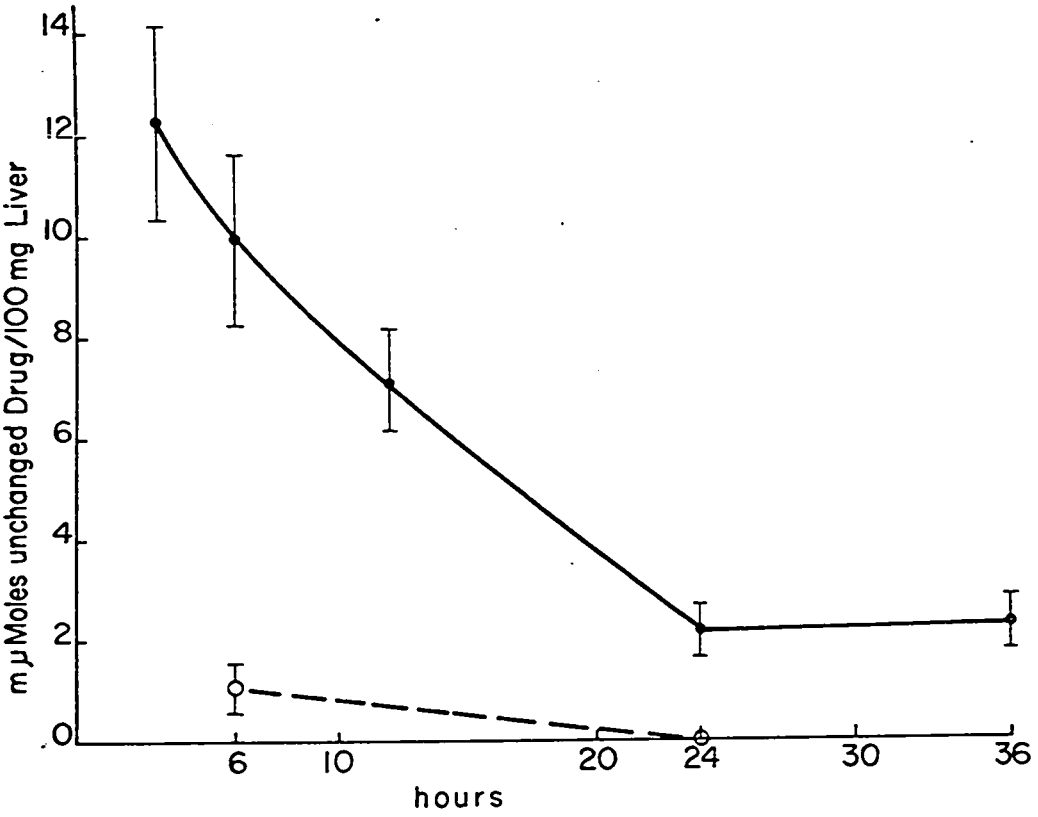


Fig. 25. Amount of Unchanged Drug in Livers of 17-Day Old Chick Embryos at Different Time Intervals After Injection of DDC-^{14}C (o———o) and 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (O- - - -O).

pyridine (VI) separated as shown in Fig. 22. Examination of the 1 N HCl solution containing 270,000 dpm and alumina fraction A-I, revealed that they contained a product whose chromatographic and ultraviolet spectral characteristics were the same as 3,5-diethoxycarbonyl-2,6-dimethyl-pyridine (XVIII). Investigation of alumina fraction A-II and A-III showed that neither migrated from the origin on a thin-layer chromatographic system in which both the dihydropyridine (VI) and pyridine (XVIII) migrate. These substances are clearly metabolites. Alumina fraction A-II has an absorption band at 241 mμ and alumina fraction A-III has an absorption band at 251 mμ indicating that these metabolites contain an aromatic ring rather than a dihydropyridine ring.

Our next studies were directed to an examination of the metabolite(s) of DDC (Fig. 23). Investigation of alumina fraction B-I revealed that it contained a product whose ultraviolet spectral and chromatographic characteristics were the same as those of DDC. Neither alumina fraction B-IIa or B-IIb migrated on a thin-layer chromatographic system in which DDC moved. Neither of these fractions had an absorption band in the 350 mμ region in which DDC and other dihydropyridines absorb strongly. The absorption band in the 250 mμ region indicated that these compounds might contain aromatic rings.

Since we could not deduce from these studies whether 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) had been metabolized by a hydrolytic mechanism and DDC oxidatively metabolized, further information was sought as follows. The distribution of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) between equal volumes of ether and water and between equal volumes of ether and dilute sodium carbonate was studied (Table XVIII). It was shown

TABLE XVIII. DISTRIBUTION BETWEEN SOLVENTS OF RADIOACTIVITY FROM PURE DRUGS AND FROM EXTRACTS OF CHICK EMBRYO INJECTED WITH ^{14}C-LABELLED DRUGS

	Ether:Base	Ether:Water
Extract of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine injected chick embryo	15:1	10:1
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C	75:1	65:1
Extract of DDC-^{14}C injected embryos	3:1	3.5:1
DDC-^{14}C	18:1	23:1

that 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine-^{14}C (VI) greatly favored the ether layer over the neutral aqueous or alkaline aqueous layers. The radioactive material from 3,5-diethoxycarbonyl-1, 4-dihydro-2,6-dimethylpyridine-^{14}C (VI) injected chick embryos distributed in such a manner that a larger amount of radioactivity passed into the neutral and alkaline aqueous phases than in the case of the pure drug. Had all the di-ester (VI) been hydrolyzed, it would have distributed in such a way that all the radioactivity would have passed into the alkaline aqueous phase. Clearly this is not the case. If a portion of the di-ester (VI) had been hydrolyzed, the resulting acid(s) would pass from ether into the alkaline aqueous phase but only partially from ether into the neutral aqueous phase. No evidence for this has been obtained (Table XVIII). By means of this technique, it was shown that the radioactive material from DDC-^{14}C injected chick embryos contained a metabolite but no evidence on the nature of this metabolite could be deduced from the distribution ratios.

Thus we were unable to obtain evidence for the nature of the metabolites of DDC or 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (VI) from these studies. There are two factors which have complicated these studies. The first is the conversion of the dihydropyridine (VI) to the pyridine (XVIII) by a non-enzymic mechanism. The second complicating factor is the small amount of drug that can be administered to chick embryos. In further studies, it is planned to circumvent these problems as follows. Instead of the unstable dihydropyridine (VI), the stable pyridine derivative (XVIII) will be employed and the metabolism of this inactive, sterically unhindered compound compared with the metabolism of the active, sterically hindered pyridine

(VII) in monolayer cultures of chick embryo liver cells.

Furthermore, it is planned to study the metabolism of ^{14}C-labeled porphyria-inducing drugs and related inactive analogues in species such as the rat, rabbit or guinea pig. By this means, large quantities of drug could be administered and the isolation of metabolites from urine would be more readily achieved.

BIBLIOGRAPHY

BARNES, H.D. (1955). Porphyria in the Bantu races on the Witwatersrand. S. Afr. Med. J. 29, 781.

BARNES, H.D. (1959). The excretion of porphyrin and porphyrin precursors by Bantu cases of porphyria. S. Afr. Med. J. 33, 274.

BECKETT, A.H. & CASY, A.F. (1954). Synthetic analgesics: stereochemical considerations. J. Pharm. Pharmac. 6, 986.

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.

BRAUDE, E.E., HANNAH, J. & LINSTEAD, SIR R. (1960). Hydrogen transfer, Part XVI. Dihydrides of nitrogenous heterocycles as hydrogen donors. J. Chem. Soc. 3249.

BRIGNELL, P.J., EINSER, W. & FARRELL, P.G. (1966). Substituent effects in the ultraviolet spectra of 1,4-dihydropyridines. J. Chem. Soc. (B), 1083.

BRODIE, B.B. & HOGBEN, A.M. (1957). Some physico-chemical factors in drug action. J. Pharm. Pharmac. 9, 345.

BROWN, E.G. (1958). The relationship of the tricarboxylic acid cycle to the synthesis of δ-aminolaevulinic acid in avian erythrocyte preparations. Biochem. J. 70, 313.

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 dehydratase from Rhodopseudomonas spheroides. Biochem. J. 87, 462.

CAM, C. (1959). Cutaneous porphyria related to intoxication. Dirim. (Istanbul) 34, 11.

CASE, J.D., ALDRICH, R.A. & NEVÉ, R.A. (1953). Sedormid-induced porphyria in the rat. Proc. Soc. Exp. Biol. Med. 83, 566.

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

CORNFORD, P. (1964). Transformation of porphobilinogen into porphyrins by preparations from human erythrocytes. Biochem. J. 91, 64.

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

DAVIDSON, J.D. & OLIVERIO, V.T. (1967). Tritium and carbon-14 by oxygen flask combustion. Atomlight, No.60, 1.

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

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

DE MATTEIS, F., PRIOR, B.E. & RIMINGTON, C. (1961). Nervous and biochemical disturbances following hexachlorobenzene intoxication. Nature 191, 363.

DE MATTEIS, F. & RIMINGTON, C. (1963). Disturbances of porphyrin metabolism caused by griseofulvin in mice. Brit. J. Dermatol. 75, 91.

DUJOUNE, C.A., LAVELLE, G., WEISS, P., BIANCHIVE, J. & LASAGNA, L. (1969). Effect of hepatotoxic drugs on liver tissue culture. Pharmacologist 11, 282.

EASSON, L.H. & STEDMAN, E. (1933). Studies on the relationship between chemical constitution and physiological action. V. Molecular dissymmetry and physiological activity. Biochem. J. 27, 1257.

FALK, J.E. (1964). Porphyrins and Metalloporphyrins, pp.1-29. Elsevier Publishing Co., New York.

FIESER, L.F. (1957). Experiments in Organic Chemistry. 3rd Ed., p. 303. D.C. Heath & Co., Boston.

FISCHER, H. & ORTH, H. (1937). Die Chemie des Pyrrols. Vol. II, part 1, p. 579. Verlagsgesellschaft, Leipzig.

FOLCH, J., LEES, M. & STANLEY, G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497.

GIBSON, K.D., MATTHEW, M., NEUBERGER, A. & TAIT, G.H. (1961). Biosynthesis of porphyrins and chlorophylls. Nature 192, 204.

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.

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

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

GOLDBERG, A. & RIMINGTON, C. (1962). Diseases of Porphyrin Metabolism (ed. KUGLELMASS, I.N.) p. 175. Charles C. Thomas, Springfield.

GRANICK, S. (1964). A test for detection of porphyria-inducing drugs. J. Am. Med. Ass. 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. & LEVERE, R.D. (1964). Heme synthesis in erythroid cells. In Progress in Hematology, Vol. IV, p.1. Grune & Stratton.

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

HIRSCH, G.H., BUBBAR, G.L. & MARKS, G.S. (1967). Studies of the relationship between chemical structure and porphyria-inducing activity. III. Biochem. Pharmac. 16, 1455.

HIRSCH, G.J., GILLIS, J.D. & MARKS, G.S. (1966). Studies on the relationship between chemical structure and porphyria-inducing activity. II. Biochem. Pharmac. 15, 1006.

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

KADESCH, R.G. (1944). Some new aspects of the ortho effects. Cyclic ketones related to acetophenone. J. Am. Chem. Soc. 66, 1207.

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. U.S.A. 61, 509.

KARIBIAN, D. & LONDON, I.M. (1965). Control of heme synthesis by feedback inhibition. Biochem. Biophys. Res. Comm. 18, 243.

KEBERLE, H., HOFFMANN, K. & BERNHARD, K. (1962). The metabolism of glutethimide (Doriden). Experentia. 18, 105.

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

LASCELLES, J. (1956). The synthesis of porphyrins and bacteriochlorophyll by cell suspensions of Rhodopseudomonas Spheroides. Biochem. J. 62, 78.

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

LOEV, B. & SNADER, K.M. (1965). The Hantzsch reaction. I. Oxidative dealkylation of certain dihydropyridines. J. Org. Chem. 30, 1914.

MAGNUS, I.A., JARRETT, A., PRANKERD, T.A.J. & RIMINGTON, C. (1961). Erythropoietic protoporphyria. A new porphyria syndrome with solar uticaria due to protoporphyrinaemia. Lancet 2, 448.

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

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

MORRISON, R.T. & BOYD, R.N. (1966). Organic Chemistry. 2nd ed. pp. 675-679. Allyn & Bacon Inc., Boston.

NAKAO, K., WADA, O., TAKAKU, F., SASSA, S., YANO, Y. & URATA, G. (1967). The origin of the increased protoporphyrin in erythocytes of mice with experimentally induced porphyria. J. Lab Clin. Med. 70, 923.

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

NEWMAN, M.S. (1956). Steric Effects in Organic Chemistry (ed., NEWMAN, M.S.), p.216. John Wiley & Sons, New York.

NOUJAIM, A. (1969). Personal communication.

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

OMURA, T., SATO, R., COOPER, D.Y., ROSENTHAL, O. & ESTABROOK, R.W. (1965). Function of cytochrome P-450 of microsomes. Fed. Proc. 24, 1181.

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. Comm. 16, 60.

REMMER, H. & MERKER, 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. & ZIEGLER, G. (1963). Experimental porphyria in rats induced by chlorinated benzenes. Biochem. Pharmac. 12, 1387.

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

SCHINDLER, R. (1969). Use of cell culture in pharmacology. A. Rev. Pharmac. 9, 393.

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

SCHMID, K., REISS, W. & KEBERLE, H. (1965). Studies with racemic drugs containing different labels in the two enantiomorphs. In Isotopes in Experimental Pharmacology (ed., ROTH, L.J.), p. 383. Univ. of Chicago Press, Chicago.

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., RACZ, W.J., HIRSCH, G.H., BUBBAR, G.L. & MARKS, G.S. (1968). Studies on the relationship between chemical structure and porphyria-inducing activity. IV. Biochem. Pharmac. 17, 1385.

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. & KUMIN, S. (1952). The mechanism of porphyrin formation. The formation of a succinyl intermediate from succinate. J. Biol. Chem. 198, 827.

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

STAHL, E. (1965a). Thin-Layer Chromatography: A Laboratory Handbook (ed., STAHL, E.), pp. 7-34. Academic Press, New York.

STAHL, E. (1965b). Thin-Layer Chromatography: A Laboratory Handbook (ed., STAHL, E.), p. 304. Academic Press, New York.

STICH, W. & DECKER, P. (1955). Studies on the mechanism of porphyrin biosynthesis with the aid of inhibitors. In Ciba Foundation Symposium on Porphyrin Biosynthesis and Metabolism (ed., WOLSTENHOLME, G.E.W. & MILLAR, E.C.P.), p. 254. J. & A. Churchill, London.

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

TALMAN, E.L., LABBE, R.F. & ALDRICH, R.A. (1957). Porphyrin metabolism. IV. Molecular Structure of acetamide derivatives affecting porphyrin metabolism. Arch. Biochem. Biophys. 66, 289.

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

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

WALDENSTRÖM, J. (1939). Neurological symptoms caused by so called acute porphyria. Acta. Psychiat. Neurol. Scand. 14, 375.

WANG, C.H. & WILLIS, D.L. (1965). Radiotracer Methodology in Biological Science, pp. 165-170. Prentice-Hall, Englewood Cliffs, New Jersey.

WATSON, C.J. (1966). Pursuit of the purple. J. Am. Med. Ass. 197, 1074.

WESTHEIMER, F.H. (1962). Advances in Enzymology (ed., NORD, F.F.), p. 441. Interscience, New York.

WOODS, J.S. & DIXON, R.L. (1969). Perinatal differences in activity and inducibility of hepatic delta-aminolevulinic acid (ALA) synthetase. Pharmacologist 11, 249.