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Rat hepatic uroporphyrinogen III cosynthetase

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RAT HEPATIC UROPORPHYRINOGEN III COSYNTHETASE: PURIFICATION, PROPERTIES, INHIBITION BY METAL IONS AND EVIDENCE OF A FOLATE COMPONENT

BY

Robert Peter Clement

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ABSTRACT

Synthesis of uroporphyrinogen III (URO III), the physiological precursor of heme, from porphobilinogen (PBG) requires two enzymes, uroporphyrinogen I synthetase (URO-S) and uroporphyrinogen III co-synthetase (COSYN). This reaction has been recognized as one of the enzymic steps in heme synthesis affected in patients suffering from acute intermittent porphyria (AIP) and congenital erythropoietic porphyria (CEP). A number of heavy metals including lead, cadmium, mercury, and iron are also known to alter heme biosynthesis. Patients with lead intoxication have been reported to excrete abnormally high amounts of PBG into the urine, suggesting a possible alteration in the activity of either URO-S or COSYN.

Piper and van Lier (1977) have recently isolated a pteroylpoly-glutamate factor from rat hepatic cytosol which activates URO-S and protects the enzyme from lead-mediated inhibition. Additionally, Wider de Xifra et al. (1980) have demonstrated both biochemical and clinical recovery in AIP patients following short-term treatment with folic acid. These observations suggest the possible regulation of the conversion of PBG to URO III by a folate molecule.

The objectives of the present study were to isolate, purify and characterize rat hepatic COSYN in order to study the effects of heavy metal ions on this enzyme and investigate the possible association of a folate molecule with COSYN.

Rat hepatic COSYN was isolated and purified 73-fold with a 13% yield by ammonium sulfate fractionation and sequential chromatography on DEAE-Sephacel, Sephadex G-100 superfine, and folate-AH-Sepharose 4B. Inhibition of URO III formation with increasing substrate

concentration was observed. COSYN was shown to be thermolabile, and a time-dependent loss of enzyme activity during reaction with URO-S and PBG was observed. The pH optimum for the complete system (URO-S and COSYN) was pH 7.8 in 50 mM Tris-HCl or 50 mM sodium phosphate buffer. Various metals (KCl, NaCl, $MgCl_2$, $CaCl_2$) increased formation of URO III. Heavy metals including $ZnCl_2$, $CdCl_2$, and $CuCl_2$ were shown to selectively inhibit COSYN activity, whereas other metals ($HgCl_2$, $PbCl_2$) were less selective and inhibited both URO-S and COSYN at similar concentrations.

The purified COSYN has a molecular weight of approximately 42,000 daltons, and is resolved into two bands, each possessing activity, by gel electrophoresis. A factor has been dissociated from purified COSYN. Results of both microbiological and radioassays suggest that it is a pteroylpolyglutamate. The isolated factor co-elutes with authentic N_5-methyltetrahydropteroylheptaglutamate on DEAE-Sephacel. These results suggest that a reduced pteroylpolyglutamate factor is associated with rat hepatic COSYN, and may be involved in the regulation of this step of heme biosynthesis.

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List of Abbreviations

ALA	5-Aminolevulinic acid
PBG	Porphobilinogen
URO III	Uroporphyrinogen III
URO I	Uroporphyrinogen I
URO-S	Uroporphyrinogen I synthetase
COSYN	Uroporphyrinogen III cosynthetase
AIP	Acute intermittent porphyria
CEP	Congenital erythropoietic porphyria
PBGase	Porphobilinogenase
DTT	Dithiothreitol
$5\text{-}CH_3\text{-}H_4PteGlu_5$	5-methyltetrahydropteroylpentaglutamate
$5\text{-}CH_3\text{-}H_4PteGlu_6$	5-methyltetrahydropteroylhexaglutamate
$5\text{-}CH_3\text{-}H_4PteGlu_7$	5-methyltetrahydropteroylheptaglutamate

I. INTRODUCTION

A. Review of heme biosynthesis.

Heme serves as the prosthetic group in a variety of proteins essential to metabolism and other biochemical processes. In the cells of higher animals, hemoproteins are concerned with such functions as (1) the transport and transfer of oxygen to and within cells (hemoglobin, myoglobin); (2) the protection of cells against toxic oxidizing species (catalase, peroxidase); (3) participation in electron transfer reactions necessary for ATP synthesis (mitochondrial cytochromes); (4) the microsomal metabolism of fatty acids, steroids, and foreign compounds (microsomal cytochromes P_{450} and b_5); and (5) the cytoplasmic degradation of tryptophan (tryptophan pyrrolase) (Bhagavan, 1974; DeMatteis, 1975; Bonkowsky et al., 1979). Because heme plays a central role as the prosthetic group for these important proteins, an understanding of heme biosynthesis and its control is essential to an understanding of the mechanisms whereby various drugs, hormones, or environmental contaminants may alter heme synthesis and compromise the normal biological functions of an organism.

The major steps of heme biosynthesis have been studied in preparations from a number of sources including photosynthetic bacteria, avian and mammalian erythrocytes, and avian and mammalian liver (Sassa, 1978). Although the molecular weights of the enzymes and other physical properties vary between species, the metabolic reactions of heme biosynthesis appear to be very similar throughout nature (Figure I-1).

The first reaction unique to porphyrin synthesis is the formation of 5-aminolevulinic acid (ALA) from one molecule each of

succinyl-coenzyme A and glycine. This reaction is catalyzed by the mitochondrial enzyme ALA synthetase (E.C. 2.3.1.37). The ALA formed passes out of the mitochondrion and into the cytoplasm, where two molecules of ALA are condensed to form one molecule of porphobilinogen (PBG), the monopyrrole precursor of porphyrins. This reaction is mediated by the cytoplasmic enzyme ALA dehydratase (E.C. 4.2.1.24). Four molecules of PBG are then condensed to form one molecule of uroporphyrinogen III (URO III). This step requires the sequential action of the two cytoplasmic enzymes uroporphyrinogen I synthetase (URO-S, E.C. 4.3.1.8) and uroporphyrinogen III cosynthetase (COSYN). The conversion of PBG to URO III by these two enzymes is the subject of this thesis and previous work will be reviewed later in this section. The acetic acid side-chains of URO III are next sequentially decarboxylated to form coproporphyrinogen III by the action of another cytoplasmic enzyme, uroporphyrinogen decarboxylase (E.C. 4.1.1.37). Coproporphyrinogen III then enters the mitochondrion where the enzyme coproporphyrinogen oxidase (E.C. 1.3.3.3) oxidatively decarboxylates two of the remaining four propionate side-chains to vinyl groups to form protoporphyrinogen IX. Protoporphyrinogen IX is then thought to be converted to protoporphyrin IX by the action of a mitochondrial, oxygen-dependent enzyme, protoporphyrinogen oxidase (Tait, 1978). In the final step of heme synthesis, ferrous ion is inserted into protoporphyrin IX to form heme. This reaction is mediated by the mitochondrial enzyme ferrochelatase (E.C. 4.99.1.1).

B. Disorders of heme biosynthesis affecting the conversion of porphobilinogen to uroporphyrinogen III.

Although the general outlines of heme biosynthesis and some

of the enzymatic mechanisms of the early reactions are fairly well understood, there are still many gaps in our knowledge of the details of some of the other steps and the enzymes involved. One of the most complex steps in the heme biosynthetic pathway involves the formation of URO III from PBG (Figure I-2). Several proposals for the mechanism of formation of the functional URO III have appeared in the literature, but the overall process has yet to be fully resolved. This problem is of interest not only from a scientific point of view, but also in relation to studies of the porphyrias and the effects of drugs, metals and other foreign compounds on this important step of heme synthesis.

The porphyrias are a group of disorders of porphyrin metabolism, either inherited or acquired, in which both chemical and clinical manifestations appear to be related to the tissue in which the metabolic defect occurs, as well as the site of the disorder in the heme biosynthetic pathway. Of the various porphyrias, two are known to involve the conversion of PBG to URO III.

In acute intermittent porphyria (AIP), a disease inherited as an autosomal dominant trait, the urinary excretion of ALA and PBG is significantly increased without a corresponding rise in either type I or type III porphyrinogens. The excess of precursors is known to originate in the liver, where besides an increase in ALA-synthetase activity, there is a decrease in URO-S activity (Tschudy et al., 1965; Nakao et al., 1966; Dowdle et al., 1967; Masuya, 1969; Sweeney et al., 1970; Strand et al., 1970; Miyagi et al., 1971). Diminished URO-S activity associated with AIP has also been observed in red blood cells and fibroblasts (Meyer et al., 1972; Strand et al., 1972; Sassa et al., 1973; Meyer, 1973; Sassa et al., 1974; Bonkowsky et al., 1973). These

results suggest that a URO-S deficiency is the primary genetic defect in AIP.

The second porphyria associated with a defect in the conversion of PBG to URO III is congenital erythropoietic porphyria (CEP). CEP is a rare form of marrow porphyria, inherited as an autosomal recessive trait, which is associated with deposition in the tissues and excretion in the feces and urine of large amounts of uroporphyrin I and coproporphyrin I. Major manifestations of this disease are photosensitivity, erythrodontia, hemolytic anemia, and porphyrinuria (Dean, 1971; Bhagavan, 1974).

Two models have been proposed to explain the enzymatic defect in CEP. The production of large amounts of uroporphyrinogen I (URO I) in developing erythroblasts in the bone marrow of CEP patients may be the result of either a decrease in the activity of COSYN (Levin, 1968a) or a relative increase in the activity of URO-S (Watson et al., 1964). Levin has demonstrated that the activity of COSYN in hemolysates from bovine and human subjects with CEP is lower than in hemolysates from control subjects (Levin, 1968a; Romeo and Levin, 1969), and has suggested that a defect in COSYN is the primary inherited genetic trait leading to CEP. Alternatively, Masuya (1969) observed that URO-S activity was higher in the blood of a CEP patient than in that of a normal subject, and other reports of the porphyrin excretion pattern in a patient with CEP (Rimington and With, 1973; Hofstad et al., 1973; Eriksen et al., 1973) indicated that more than 50% of the porphyrins excreted in the urine were type III isomers. These results suggest that CEP may be genetically heterogenous. The identification of the specific genetic defects responsible for this disease will

require further study of the disease process and the mechanism of action of the enzymes involved.

C. Review of past studies with uroporphyrinogen III cosynthetase.

Elucidation of the mechanism whereby PBG is enzymatically converted into URO III has been the subject of numerous investigations in recent years. The conversion of PBG into URO III has been studied in preparations from various sources, including avian and human erythrocytes, rabbit reticulocytes, mouse spleen, cow liver, Rhodopseudomonas spheroides, chlorella, and higher plants (Cornford, 1964; Levin, 1971; Batlle and Rossetti, 1977). The reaction is catalyzed by porphobilinogenase (PBGase), an enzyme complex believed to consist of two separate enzymes, URO-S, a heat-stable protein, and COSYN, a heat-labile protein (Cornford, 1964; Levin, 1971).

URO-S was first isolated from spinach leaves by Bogorad (1958 a,b, c), and has since been purified to homogeneity from a variety of sources, including Chlorella regularis (Shioi et al., 1980) and human erythrocytes (Miyagi et al., 1979; Anderson and Desnick, 1980). The mechanism whereby four molecules of PBG undergo condensation to yield one molecule of URO I has been largely defined, and involves the head-to-tail assembly of four PBG molecules to form the linear tetrapyrrole hydroxymethylbilane (Figure I-2), which is released from URO-S and cyclizes chemically to URO I (Battersby et al., 1978; Battersby et al., 1979a,b; Battersby et al., 1980; Burton et al., 1979; Jordan et al., 1979; Jordan and Seehra, 1979).

Purified preparations of URO-S have been utilized in numerous kinetic studies of the enzyme. URO-S activity is inhibited by ammonium ion (100 mM), hydroxylamine (10 mM), and the sulfhydryl

reagents N-ethylmaleimide (5 mM) and p-chloromercuribenzoate (1 mM) (Llambias and Batlle, 1970, 1971a; Sancovich et al., 1976; Russell and Rockwell, 1980). Divalent metal cations such as Hg^{+2} (0.01 mM), Cd^{+2} (1 mM), Mg^{+2} (50 mM), Ca^{+2} (50 mM), and Zn^{+2} (1 mM) have also been identified as inhibitors (Llambias and Batlle, 1971b; Frydman and Feinstein, 1974; Sancovich et al., 1976; Anderson and Desnick, 1980). Recently, Piper et al. (1976) have shown that $PbCl_2$ (5 μM) is a potent non-competitive inhibitor of purified rat hepatic and erythrocytic URO-S activities. Piper and van Lier (1977) have also isolated a pteroylpolyglutamate derivative from rat hepatic cytosol which stimulates URO-S activity and protects the enzyme from $PbCl_2$-mediated inhibition. These results suggest that a folate derivative may act as a regulator of URO-S activity, and may serve as a coenzyme in this step of heme biosynthesis.

Although the mechanism of formation of URO I from PBG is well understood, the process whereby PBG is converted into URO III in the presence of both enzymes has not been fully resolved. COSYN was initially isolated from wheat germ by Bogorad (1958b) and some properties of the enzyme examined. COSYN was shown to be heat-labile, the activity being completely lost if the enzyme was heated at 55°C for 15 minutes. In a series of preincubation experiments, Bogorad demonstrated that no detectable consumption of PBG occurs in the presence of COSYN alone, and that the formation of URO III from PBG requires the simultaneous incubation of PBG, URO-S, and COSYN. In a second series of experiments, Bogorad incubated URO-S in the presence of PBG, and then added COSYN at later times. In each case the reaction product was URO I. These results indicate that COSYN participates in the

synthesis of URO III prior to the formation of a cyclized tetrapyrrole. Bogorad proposed that COSYN required two substrates for its action; one being PBG, and the other a linear di- or tripyrrole thought to be the product of the reaction between PBG and URO-S. Bogorad's group studied the incorporation of both dipyrrylmethane and tetrapyrrylmethane intermediates into URO III, but neither was incorporated significantly into the porphyrin ring (Plusec and Bogorad, 1970; Radmer and Bogorad, 1972). These researchers concluded that free dimers and tetramers of PBG were not intermediates in the reaction.

Levin has isolated COSYN from homogenates of spleens from phenylhydrazine-treated mice and purified the enzyme 18-fold with a 5% yield by ammonium sulfate fractionation (70-95%) and chromatography on Bio-Rex 70 (Levin and Coleman, 1967; Levin, 1968b; Levin, 1971). The heat-labile, URO-S-free COSYN preparation did not affect the rate or stoichiometry of the conversion of PBG into total uroporphyrinogen, but was able to direct the synthesis of the III isomer without functioning as a uroporphyrinogen isomerase. Levin observed a time-dependent loss of COSYN activity under conditions where the enzyme was not measurably thermolabile. The inactivation of COSYN only occurred when both URO-S and the substrate PBG were included in the reaction mixture. Levin concluded that the disappearance of COSYN activity was an enzyme catalyzed, substrate-dependent reaction which might be explained by the stoichiometric participation of a substance as a cofactor.

Levin (1968b, 1971) also found that the shape of the curve formed by a plot of percent isomer III against COSYN concentration was not a straight line, but had the shape of a rectangular hyperbola. A

reciprocal plot of this data approximated a straight line which intersected the ordinate at the numeral 1.0, that is, 100% III. This result indicates that, in vitro, 100% URO III in the reaction product can only be obtained at an infinite concentration of COSYN. These observations are consistent with a mechanism in which COSYN interacts with a polypyrrole intermediate as proposed by Bogorad (1963), or they may simply reflect the inactivation of COSYN during the formation of URO III.

Sancovich, Batlle and Grinstein (1969a,b) studied COSYN isolated from bovine liver. Initially PBGase was isolated by ammonium sulfate fractionation (35-50%) and purified 182-fold by calcium phosphate and Sephadex G-100 gel chromatography. In subsequent experiments, the two components of PBGase, URO-S and COSYN, were isolated and partially purified. Ammonium ion was shown to be a non-competitive inhibitor of URO-S (K_i = 0.172 M), and a competitive inhibitor of COSYN (K_i = 0.01 M). When the activity of PBGase was plotted against PBG concentration (substrate saturation curve), sigmoid curves were obtained. These results suggest that PBGase may be an allosteric protein in which binding of the substrate PBG to the URO-S-COSYN complex might induce conformational changes in the latter, reversibly modifying the properties of the enzyme complex.

Sancovich, Batlle and Grinstein (1969a,b) found bovine liver COSYN to be heat-labile, and inhibition of COSYN activity by high substrate (PBG) concentration (100 μM) was observed. These researchers also reported that the rate of uroporphyrinogen formation was the same, whether the reaction was incubated in air or anaerobically, and the isomer composition of the product was unchanged. It was also noted

that the addition of sodium (60 mM) or magnesium (6 mM) salts at certain concentrations to the incubation mixture increased the amount of URO III formed, but did not change total porphyrin formation (I & III isomers). These investigators also provided evidence for the existence of a low molecular weight factor which seemed necessary for uroporphyrinogen formation from PBG. The factor could be stripped from PBGase preparations by ultrafiltration, resulting in a significant (50-100%) loss of activity. The activity of the treated enzyme preparations could then be restored to control levels by the addition of the ultrafiltrate back to the stripped enzyme.

COSYN has also been isolated from both human and avian erythrocytes and partially purified (Cornford, 1964; Stevens et al., 1968; Llambias and Batlle, 1971b; Frydman and Feinstein, 1974). In each case the crude homogenate of lysed erythrocytes was subjected to ammonium sulfate fractionation and one or more chromatographic techniques, including calcium phosphate, DEAE-cellulose, and Sephadex G-100 gel chromatography. In each study COSYN was separated from URO-S and partially purified, but specific activities were not calculated. Cornford (1964) observed inhibition of URO III formation in human erythrocyte preparations at substrate concentrations as low as 50 micromolar. Sodium ions had little effect on total porphyrin formation but, as observed in cow liver by Sancovich, Batlle and Grinstein (1969a,b), increased the percent formation of the III isomer. Stevens et al. (1968) obtained similar results and, in addition, estimated the COSYN:URO-S ratio in the crude extracts of human erythrocytes to be approximately 10:1. This excess of COSYN would assure the sole formation of URO III, the natural precursor

of heme, under normal conditions.

Frydman and Feinstein (1974) used a partially purified PBGase preparation from human erythrocytes to study the kinetics of the URO-S-COSYN system. A study of the effect of substrate concentration on product formation yielded classical Michaelis-Menton kinetics. These results are at variance with the observations of Sancovich, Batlle and Grinstein (1969a,b). This discrepancy may reflect actual differences in the properties of the enzymes isolated from the different tissues, or they may simply reflect differences in experimental conditions. Hydroxylamine (30 mM) totally inhibited COSYN activity, and Hg^{2+}, Zn^{2+}, and Cd^{2+} at concentrations of 1 mM inhibited both enzymes. Frydman and Feinstein (1974) also observed the inhibition of both URO-S and COSYN by sulfhydryl reagents as N-ethylmaleimide (3 mM) and p-hydroxymercuribenzoate (0.06 mM), and reported that the inhibition of URO III formation by these agents was more pronounced than the inhibition of total product formation. These results suggest that COSYN may be more sensitive to inhibition by sulfhydryl reagents than is URO-S. Frydman and Feinstein (1974) were also able to retain human erythrocyte COSYN on a Sepharose-URO-S affinity column, even in the presence of 2 M NaCl, suggesting that COSYN and URO-S form a strong complex which is not dependent on substrate.

PBGase has also been isolated from soya-bean callus tissue, and its components, URO-S and COSYN, separated and partially purified (Llambias and Batlle, 1970, 1971a). The isolated COSYN was shown to be heat-labile, and the percent isomer III formation was enhanced by certain concentrations of sodium (30 mM) and magnesium (6 mM) salts. With the soya-bean callus PBGase, the substrate saturation curve was

found to be sigmoidal. At low substrate concentration a Michaelis-Menten type portion was observed with a plateau from 50 to 125 μM; increasing PBG concentrations resulted in the addition of a second curve on top of the first. These results are similar to the observations of Sancovich, Batlle and Grinstein (1969a,b), suggesting that soya-bean PBGase may also be an allosteric protein.

Llambias and Batlle (1970, 1971a) also found that when PBGase was dialyzed at 4°C for 4-18 h there was a loss of 55 to 65% of the original PBGase activity. The loss of activity was not restored when the dialysate was added back to the treated enzyme preparation. These results suggest that a diffusible cofactor may be associated with PBGase. If the bound component is removed by dialysis and altered in some way, then it may not be able to bind to the enzyme again, and activity would not be restored.

Recent work by Battersby et al. (1978; 1979a,b; 1980) utilizing URO-S free preparations of COSYN purified from Euglena gracilis and synthesized, doubly ^{13}C-labeled forms of the linear tetrapyrrole hydroxymethylbilane suggests that the biosynthesis of the natural porphyrins occurs by the head-to-tail assembly of four PBG molecules, followed by a single intramolecular rearrangement involving the PBG unit forming the D-ring of URO III (Figure I-2). This work indicates that URO-S is not an enzyme for ring-closure and, in the absence of COSYN, its product is the unrearranged hydroxymethylbilane. This bilane serves as the substrate for COSYN, which functions as the ring-closing and rearranging enzyme. Although the nature of the rearrangement of the substrate hydroxymethylbilane to URO III is unknown, COSYN must be able to break the bond linking a methylene group to its

pyrrole nucleus for one or more of the four PBG units. This bond-breaking process may involve a one-carbon transfer by a methyl-transferase type of enzyme as suggested earlier by Frydman and Frydman (1975).

The recent work of Battersby et al. (1978; 1979a,b; 1980) has been confirmed by other investigators utilizing URO-S free preparations of COSYN isolated and purified from Rhodopseudomonas spheroides (Jordan et al., 1979; Burton et al., 1979; Jordan and Seehra, 1979; Jordan and Berry, 1980; Jordan et al., 1980). Again, hydroxymethylbilane was identified as both the substrate for COSYN and the product of URO-S. In these studies, hydroxymethylbilane was observed to be very labile, being rapidly converted ($T_{\frac{1}{2}}$ = 4 min at 37°C, pH 8.2) into URO I in the absence of COSYN. Jordan et al. (1980) also found that plots of percent URO III formation against increasing COSYN concentration produced hyperbolic curves similar to those obtained by Levin in an earlier study (Levin, 1971). These data again support the idea of a coupled reaction with enzymes acting in sequence.

D. Purpose and plan of investigation.

Research during the last two decades has shown that PBG is converted into URO III by an enzyme complex, PBGase, consisting of the two enzymes URO-S and COSYN. Although the mechanism by which PBG is converted into URO I in the presence of URO-S is fairly well understood, the process whereby PBG is converted into the biologically active isomer URO III has yet to be fully resolved.

Work by Battersby et al. (1978; 1979a,b; 1980) and Jordan et al. (1979; 1980; Jordan and Seehra, 1979; Jordan and Berry, 1980) has

indicated that a one-carbon transfer may be involved in the intramolecular rearrangement whereby hydroxymethylbilane, the product of the URO-S catalyzed reaction, is rearranged to URO III in the presence of COSYN. If this is the case, then COSYN may act as a methyltransferase type of enzyme as previously suggested (Frydman and Frydman, 1975). The metabolic systems of animal tissues requiring one-carbon transfer reactions are known to involve enzymes which utilize folic acid coenzymes as the catalytic self-regenerating acceptor-donors of one-carbon units (Beck, 1974). If COSYN functions as a methyltransferase in the molecular rearrangement of hydroxymethylbilane to URO III, then it may be associated with a folate coenzyme. Recent work by Piper et al. (1979), in which a pteroylpolyglutamate factor was isolated from URO-S preparations, suggests that a folate coenzyme may be involved in this step of heme biosynthesis. Further evidence for the role of a folate in heme biosynthesis comes from the recent study of Wider de Xifra et al. (1980). These researchers demonstrated both biochemical and clinical recovery in AIP patients following short-term treatment with folic acid. These observations, together with the earlier reports of an ultrafiltrable cofactor associated with bovine liver and soya-bean PBGase preparations, suggest the possible regulation of the conversion of PBG to URO III by a folate molecule.

The purpose of the present work was to investigate the possible association of a folate component with COSYN. This was accomplished by studying the effects of folate deficiency on microsomal drug metabolism and heme content in vivo, and attempting to isolate a folate derivative from purified preparations of COSYN.

The in vivo folate deficiency studies were conducted utilizing

young, male guinea pigs. The young guinea pig appears to have a higher requirement for folic acid than most other laboratory animals (Reid et al., 1956), providing an excellent animal model for studies of folate deficiency. In contrast to the guinea pig, the rat fed normal protein levels requires no dietary folic acid for normal growth (Kornberg et al., 1946).

The relationship between folate deficiency, heme content, and microsomal drug metabolism was examined in the intestinal mucosa of guinea pigs. The gastrointestinal mucosa, like the marrow, is a rapidly proliferating tissue which is known to be adversely affected by folate depletion (Goetsch and Klipstein, 1977; Klipstein et al., 1973). The intestinal mucosa also possesses both mono-oxygenase and glucuronidation capabilities (Chhabra et al., 1974; Hartiala, 1973; Shirkey et al., 1979a,b), making it suitable for a study of the effects of folate depletion on heme content and microsomal drug metabolism. In contrast to the intestinal mucosa, the liver is the main site of folate storage in the body (Corrocher et al., 1972). In studies conducted by Thenen (1978), guinea pigs fed a folate-deficient diet for six weeks appeared to be resistant to liver folate depletion. At six weeks, the concentration of folate was not significantly reduced in the deficient guinea pig livers in comparison to livers of pair-fed control animals. These results suggest that, unlike the intestinal mucosa, the liver is an unsatisfactory tissue for the short-term study of folate depletion.

The isolation of a folate derivative from COSYN requires a purified preparation of the enzyme determined to be free of URO-S activity. The assay for COSYN activity, in turn, requires a purified,

COSYN-free preparation of URO-S isolated from the same tissue. Piper and van Lier (1977, 1979) have previously purified URO-S from rat hepatic cytosol, and provided evidence for the existence of a folate derivative associated with the enzyme. These results suggest that rat liver might be a convenient tissue for both isolation and purification of COSYN and investigation of the association of a folate derivative with the enzyme.

Because the formation of URO III from PBG has not been previously studied in rat liver, it would also be necessary to characterize the URO-S-COSYN system in order to design a suitable assay for COSYN activity. The isolation and purification of rat hepatic COSYN and investigation of its properties would also provide a convenient system for the study of the effects of metals, drugs and other foreign compounds on this important step of heme synthesis. Previous work by Frydman and Feinstein (1974) suggests that COSYN activity may be more sensitive to inhibition by sulfhydryl reagents than is URO-S. If this is true, then heavy metal ions might selectively inhibit COSYN at concentrations having little or no effect on URO-S. The development of a well characterized URO-S-COSYN system would allow investigation of this possibility.

In summary, the objectives of the present study were: (1) to isolate, purify, and characterize rat hepatic COSYN and design a suitable assay of enzyme activity; (2) to study the effects of heavy metal ions on PBG ase, URO-S, and COSYN activities; (3) to investigate the possibility that a folate derivative might be associated with COSYN; and (4) to investigate the effects of folate deficiency on microsomal drug metabolism and heme content in the intestinal mucosa of guinea pigs.

Figure I-1

Summary of the biosynthesis of heme.

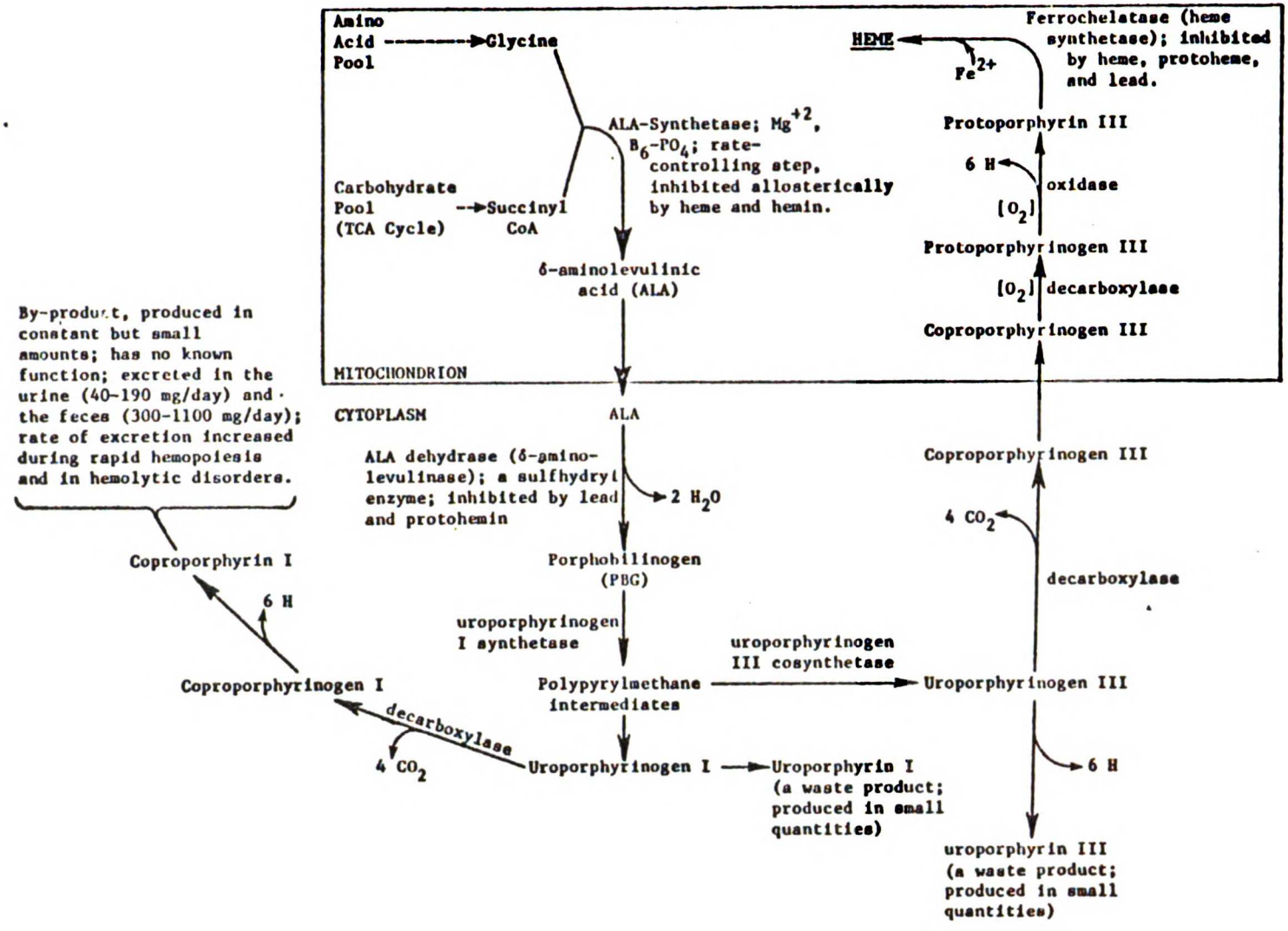
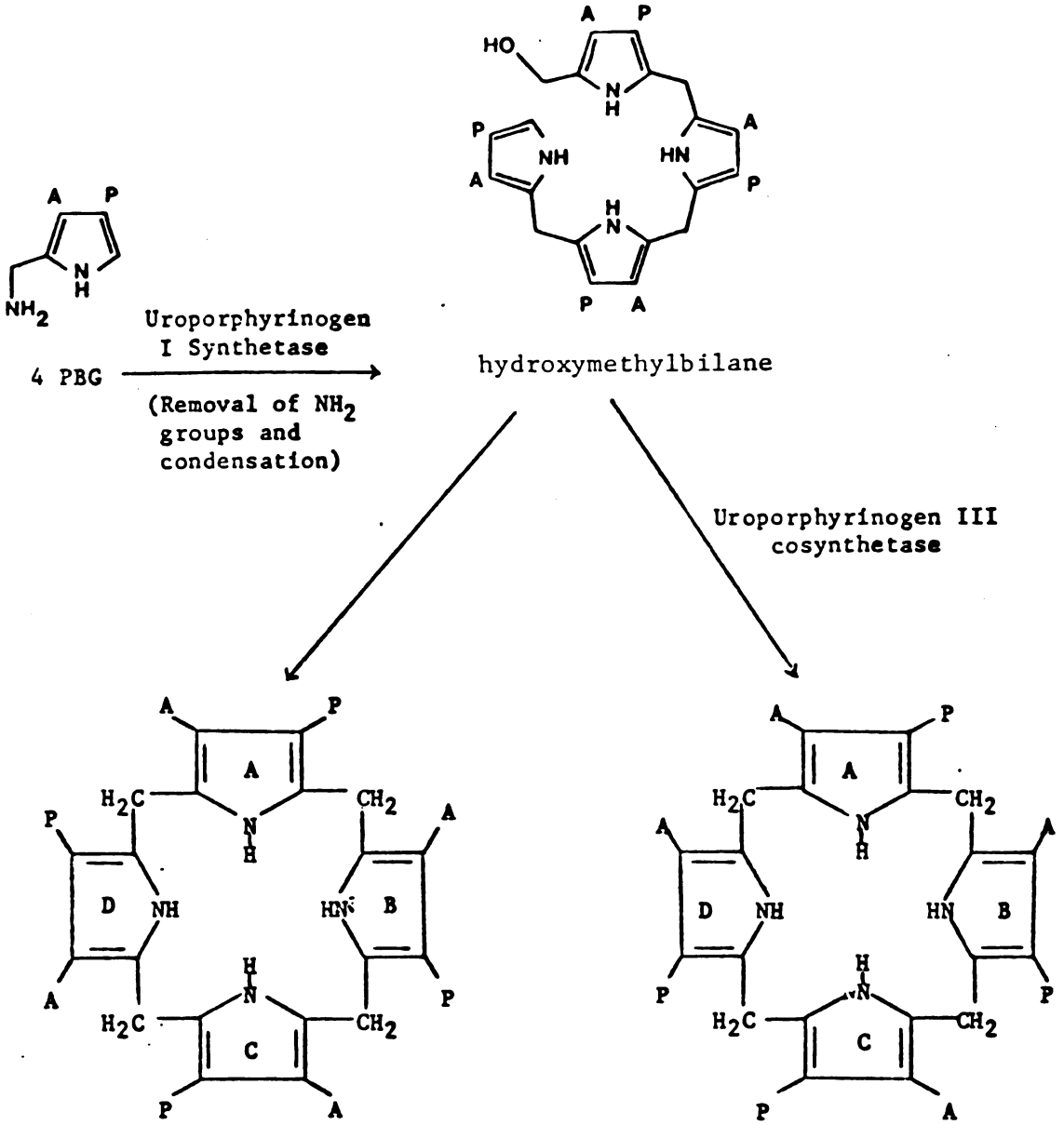

Amino Acid Pool
Glycine
HEME
Fe2+
Ferrochelatase (heme synthetase); inhibited by heme, protoheme, and lead.
ALA-Synthetase; Mg+2, B6-PO4; rate-controlling step, inhibited allosterically by heme and hemin.
Protoporphyrin III
6 H
oxidase
[O2]
Carbohydrate Pool (TCA Cycle)
Succinyl CoA
Protoporphyrinogen III
δ-aminolevulinic acid (ALA)
[O2] decarboxylase
Coproporphyrinogen III
By-product, produced in constant but small amounts; has no known function; excreted in the urine (40-190 mg/day) and the feces (300-1100 mg/day); rate of excretion increased during rapid hemopoiesis and in hemolytic disorders.
MITOCHONDRION
CYTOPLASM
ALA
Coproporphyrinogen III
ALA dehydrase (δ-amino-levulinase); a sulfhydryl enzyme; inhibited by lead and protohemin
2 H2O
4 CO2
Coproporphyrin I
Porphobilinogen (PBG)
decarboxylase
6 H
uroporphyrinogen I synthetase
uroporphyrinogen III cosynthetase
Coproporphyrinogen I
Polypyrylmethane intermediates
Uroporphyrinogen III
decarboxylase
4 CO2
Uroporphyrinogen I
Uroporphyrin I (a waste product; produced in small quantities)
6 H
uroporphyrin III (a waste product; produced in small quantities)

Figure I-2

Synthesis of uroporphyrinogens types I and III.



Note: The only difference between types I and III is the orientation of the substituents on ring D.

II. MATERIALS AND METHODS

A. Materials

1. Chemicals

Folic acid, ascorbic acid, saponin, gelatin, neutral activated charcoal, reagent grade trizma base, coomassie brilliant blue G and R, riboflavin-5'-phosphate, bromphenyl blue, 2-mercapto-ethanol, bovine serum albumin Type V, NADPH, NADH (Grade III), N-5-methyltetrahydrofolate (Grade II) - barium salt, 7-ethoxycoumarin, umbelliferone (Grade II), cytochrome C (Type III), glucose-6-phosphate, glycine, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl (EDC) and dithiothreitol (DTT) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Porphobilinogen, uroporphyrin I octamethylester, uroporphyrin III octamethylester, and uroporphyrin I dihydrochloride were purchased from Porphyrin Products, Logan, Utah. All metal salts were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri, and enzyme-grade ammonium sulfate and ultra-pure sucrose were purchased from Schwarz/Mann, Orangeburg, New York. P-nitrophenyl phosphate and p-nitrophenol were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. Bacto Lactobacilli broth AOAC, folic acid casei medium, and chicken pancreas acetone powder were purchased from DIFCO Laboratories, Detroit, Michigan, and Lactobacillus casei (ATCC 7469) was obtained from the American Type Culture Collection, Rockville, Maryland. [3',5',7-9-^{3}H]folic acid, potassium salt, (20-70 Ci/mmol), was purchased from the Amersham Corporation, Arlington Heights, Illinois. Sodium borohydride, and HPLC-grade n-heptane and acetone were obtained from MCB Manufacturing Chemists, Inc., Cincinnati, Ohio,

and HPLC-grade glacial acetic acid was purchased from J.T. Baker, Phillipsburg, New Jersey. Electrophoresis purity acrylamide, N,N'-methylene-bis-acrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), and ammonium persulfate, and Dowex 50W-X8(chloride) were obtained from Bio-Rad Laboratories, Richmond, California. Sephadex G-100 superfine, DEAE-Sephacel, AH-Sepharose 4B, and gel filtration calibration kits (low molecular weight range) were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Pteroylpenta-glutamate, pteroylhexaglutamate, and pteroylheptaglutamate standards were generous gifts from Dr. E.L.R. Stokstad. All other laboratory reagents were of analytical grade and were purchased from Mallinckrodt Chemical Works, St. Louis, Missouri, or Fisher Scientific, St. Louis, Missouri.

2. Animals

Male, albino guinea pigs (Hartley strain; 120-160 g) were obtained from the Charles River Laboratories, Baltimore, Maryland, and male, albino Sprague-Dawley rats (180-200 g) were obtained from Sasco Inc., Omaha, Nebraska.

B. Methods

1. Treatment of animals - folate deficiency study.

Forty-eight weanling male albino guinea pigs (Hartley strain, 120-160 g) were randomly divided into 2 groups of 24 animals with similar mean body weights. The experimental group received a modified Reid-Briggs diet containing 2 g/kg ascorbic acid (Bio-Mix #1278 M, Bioserv, Inc., Frenchtown, New Jersey) but without added folic acid or para-aminobenzoic acid. The control group was pair-fed an identical diet (Table II-1), but with folic acid added. Both

groups were permitted water ad libitum. Body weights were recorded at least weekly for the duration of the 5-week study. Three guinea pigs from each group were sacrificed at weekly intervals, and intestinal mucosal folate levels, heme content, and microsomal drug metabolizing activity were determined.

2. Preparation of tissues - folate deficiency study.

Animals were stunned by a blow to the head and were then sacrificed by decapitation. Heparinized blood samples were obtained and stored on ice until assay. After sacrifice, the body cavity was opened and the small intestine perfused with ice-cold isotonic saline via the hepatic portal vein. An intestinal segment consisting of the first 60 cm distal to the pylorus was immediately excised, perfused free of intestinal content with ice-cold isotonic saline, and slit open. The mucosa was removed by gently scraping the luminal surface of the small intestine with the edge of a spatula. Mucosal scrapings from each animal were homogenized in 4 ml of 1.15% KCl-50 mM Tris-HCl, pH 7.4, and centrifuged at 9,000 x g for 20 minutes. An aliquot (1.0 ml) of the 9,000 x g supernatant fraction was saved for assay of the activities of 7-ethoxycoumarin O-deethylase, NADPH-cytochrome C reductase, and alkaline phosphatase. The remaining supernatant (3.0 ml) was centrifuged for 60 minutes at 104,000 x g for isolation of the microsomes. The 104,000 x g pellet was washed by suspension in 4 ml of 1.15% KCl-50 mM Tris-HCl, pH 7.4, centrifuged at 104,000 x g for 60 minutes, and saved for the assay of microsomal heme. The 104,000 x g supernatant fraction was retained for the assay of glucose-6-phosphate dehydrogenase activity.

A 20 cm intestinal segment distal to the first 60 cm segment was

excised and washed in 1.15% KCl-50 mM Tris-HCl, pH 7.4, and the mucosal scrapings were homogenized in 2 ml of 0.1 M Tris-HCl, pH 7.8, containing 0.01 M $CaCl_2$ and 1% ascorbate. This preparation was saved for the assay of intestinal folate content.

3. Preparation of folate-AH-Sepharose 4B.

Folate-AH-Sepharose 4B was prepared by coupling with folate and AH-Sepharose 4B in the presence of carbodiimide using the method of Kamen and Caston (1975). AH-Sepharose 4B (4.7 g) was swollen and washed with 0.5 M NaCl and further with distilled water on a sintered glass-filter. The washed gel was resuspended in 20 ml of distilled water. To this suspension 50 ml of folic acid (125 mg folate, pH 7.0) and 250 mg of EDC (1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl) were added, and the mixture was incubated with gentle shaking in the dark for 18 hours. After incubation, the gel was washed with water, followed by alternating washes with 0.5 N NaOH and 0.5 N HCl, and finally by an exhaustive wash with 50 mM Tris-HCl, pH 7.6. The resulting gel-equilibrated with 50 mM Tris-HCl, pH 7.8, was packed into a column (2.0 x 5.5 cm) and used as folate-AH-Sepharose 4B for affinity chromatography.

4. Purification of uroporphyrinogen I synthetase.

The enzyme was purified from rat liver as described previously (Piper and van Lier, 1977). This method employed heat treatment of hepatic cytosol (55°C for 5 min), ammonium sulfate fractionation (40-60%), DEAE-Sephacel chromatography with a 0-0.4 M KCl gradient, and Sephadex G-100 gel chromatography. The enzyme preparations used for this study represented 600 to 1000-fold purifications from hepatic cytosol and were stable for several weeks

when stored at -70°C. All enzyme preparations were assayed for COSYN activity before use. Only preparations free of COSYN activity were used for this study.

5. Purification of uroporphyrinogen III cosynthetase.

Rats were sacrificed by decapitation, livers were perfused in situ with 0.9% NaCl (4°C) and 25% (w/v) homogenates were prepared in 50 mM Tris-HCl buffer, pH 7.8, using a hand-operated glass (Dounce) homogenizer with the loose pestle. Tris-HCl buffer (50 mM, pH 7.8) was used throughout the enzyme purification studies. All operations were conducted in the cold room at 4°C unless otherwise stated. Homogenates were centrifuged at 9,000 x g for 20 minutes at 4°C, and the supernatants were removed and centrifuged at 105,000 x g for 1 hour to obtain the hepatic cytosol fraction. The cytosol fraction was brought to 60% ammonium sulfate saturation and centrifuged at 12,000 x g for 10 minutes, the pellet was discarded, and the remaining supernatant was brought to 90% ammonium sulfate saturation followed by centrifugation at 12,000 x g for 10 minutes. The pellet was suspended in 25 ml of buffer and dialyzed overnight against 5 liters of buffer. This enzyme preparation was subjected to chromatography on DEAE-Sephacel (2.0 x 25 cm). Columns were equilibrated with buffer, and the enzyme was eluted with a linear gradient of 0-0.25 M KCl in buffer. The eluted fractions containing the enzyme were combined and concentrated with an Amicon model 52 ultrafiltration unit, using a YM-10 filter. The concentrated enzyme preparation was then subjected to gel chromatography with Sephadex G-100 superfine (2.5 x 60 cm) which had been equilibrated with buffer. The eluted fractions containing the enzyme were combined and subjected to affinity chromatography on folate-AH-Sepharose 4B (2.0 x 5.5 cm). Columns were equilibrated at 4°C in

the dark with 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 mM DTT, and the enzyme was eluted with a linear gradient of 0-1.0 M KCl in buffer. After elution of the enzyme, columns were washed successively with 50 mM Tris-HCl buffer, pH 7.8, and 0.2 M acetic acid. The washed columns were reequilibrated with the 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 mM DTT, and stored in the dark at 4°C for subsequent use. The collected fractions were assayed for COSYN activity, and the active fractions were pooled and concentrated using Amicon CF25 Centriflo ultrafiltration membranes. Preparations at the Sephadex G-100 superfine purification step were used as the enzyme source for studies of the properties of COSYN and inhibition of enzyme activity by heavy metal ions. Preparations further purified by affinity chromatography on folate-AH-Sepharose 4B served as the enzyme source for studies of the association of a folate component with COSYN and determination of the molecular weight of the enzyme. All enzyme preparations were determined to be free of URO-S activity by fluorometric assay, and were stable for several weeks when stored at -70°C.

6. Determination of molecular weight.

The molecular weight of the purified COSYN preparation (folate-AH-Sepharose 4B step) was estimated by gel filtration utilizing a Sephadex G-100 superfine column (2.5 x 23 cm) previously equilibrated at 4°C with 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 M KCl. The marker proteins were: bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,000) and ribonuclease A (M_r 13,700). The void volume was determined with Blue Dextran 2000.

7. Polyacrylamide disc gel electrophoresis.

Polyacrylamide disc gel electrophoresis of the purified COSYN was conducted according to the procedure of Davis (1964) utilizing 7.5% polyacrylamide gels in Tris-glycine buffer, pH 8.3. This is a two step method which allows proteins to "stack" in discs between two anions in a large-pore gel prior to separation in a small-pore gel. A constant current of 2 mA per gel was applied until the protein migrated into the separation gel; then the current was increased to 3 mA per gel, and the gel was maintained at 4°C for 45 minutes. Gels were fixed in 12.5% trichloroacetic acid (TCA) for 30 minutes and stained in coomassie brilliant blue R250 (1:20 dilution of a 1% aqueous solution with 12.5% TCA) for 30 minutes.

Electrophoretic elution of protein from gel slices was carried out by the method of Braatz and McIntire (1977) with 30% glycerol in the electrophoresis buffer (25 mM Tris-HCl, 0.2 M glycine, pH 8.3). Prior to elution, gels containing protein to be eluted were aligned with duplicate stained gels and the appropriate gel sections were cut out with a clean razor blade. The appropriate gel slices were then stacked on top of support gels and dialysis tubing was fixed on top of the electrophoresis tubes. Electrophoresis buffer was added until the volume of buffer inside the dialysis bag covered the gel slices (200 µl) and the top of the bag was tied securely with string. The electrophoresis chamber electrodes were reversed so that proteins migrated toward the upper (anode) chamber. Reverse electrophoresis was conducted at 4°C at 3 mA per tube for 4 hours, the tubes were removed and inverted, and the dialysis bags containing the eluted proteins were removed.

COSYN activity on gels was detected by incubating the appropriate gel slices for 45 minutes in the presence of: 20 µmol Tris-HCl buffer pH 7.8, 5 nmol PBG, 24 µmol KCl, and 0.25 units of URO-S in a volume of 400 µl. Reaction mixtures were stopped by plunging into an ice-water bath at 0°C, gel slices were removed, and the mixture of reaction products (URO I and III) was frozen, lyophilized, esterified and analyzed by HPLC as described in section 13-f of the Methods.

8. Preparation of γ-glutamyl carboxypeptidase (conjugase).

Conjugase was isolated from chicken pancreas acetone powder and endogenous folates removed by modifications of the methods of Bird, et al. (1965) and Mims and Laskowski (1945). Ten grams of chicken pancreas acetone powder was suspended in 300 ml of 0.1 M potassium phosphate buffer, pH 7.0, and stirred for 1 hour at 25°C. The stirred mixture was incubated overnight at 37°C under toluene and then centrifuged at 1000 x g for 20 minutes at 4°C. An equal volume of calcium phosphate gel (275 ml of a 0.1 M suspension) was added to the supernatant and the mixture stirred for 30 minutes at 4°C. The mixture was then centrifuged at 1000 x g for 20 minutes at 4°C, filtered to remove any remaining calcium phosphate, and an equal volume of absolute ethanol (450 ml) was added drop-wise with stirring to the mixture at 4°C. The mixture was again centrifuged at 1000 x g for 20 minutes at 4°C, and the precipitate collected and resuspended in 100 ml of 0.1 M potassium phosphate buffer, pH 7.0. This solution was stirred for 1 hour at 4°C, centrifuged to remove any insoluble material, and the supernatant mixed with Dowex 50W-X8 (chloride) (10 g) by stirring for 1 hour at 4°C. The Dowex 50W-X8 (chloride) was removed by centrifugation, the supernatant filtered through gauze,

and the treatment with Dowex 50W-X8 (chloride) repeated. The clear supernatant obtained after the second treatment with Dowex 50W-X8 (chloride) was stored at -70°C and served as the chicken pancreas conjugase preparation. All conjugase preparations were determined to be folate-free by microbiological assay using L. casei before use.

9. Preparation of 5-methyltetrahydropteroylpolyglutamate standards.

5-methyltetrahydropteroylpentaglutamate (5-CH_3-H_4PteGlu$_5$), 5-methyltetrahydropteroylhexaglutamate (5-CH_3-H_4PteGlu$_6$), and 5-methyltetrahydropteroylheptaglutamate (5-CH_3-H_4PteGlu$_7$) were synthesized from the respective pteroylpolyglutamates by Suzuki and Wagner's (1980) modification of the method of Blair and Saunders (1970). Authentic PteGlu$_5$, PteGlu$_6$, and PteGlu$_7$ prepared by the solid phase synthetic method (Krumdieck and Baugh, 1969) were generous gifts of Dr. E.L.R. Stokstad.

10. Dissociation of folate component from uroporphyrinogen III cosynthetase.

Purified rat hepatic COSYN preparations (folate-AH-Sepharose 4B step) were heated for 10 minutes at 95°C in the presence of 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 M 2-mercaptoethanol. Heated preparations were cooled on ice, centrifuged at 12,000 x g for 10 minutes, and the supernatant stored at 4°C in the dark until analysis.

11. Hydrolysis of folate component from uroporphyrinogen III cosynthetase with conjugase.

Conjugase was prepared from chicken pancreas acetone powder as described in section B-8. The reaction mixture contained

0.10 ml of the folate component dissociated from the purified COSYN (60 µg protein), 0.05 ml of conjugase (28 µg protein), and 0.50 ml of 0.1 M Tris-HCl buffer, pH 7.8, containing 0.01 M $CaCl_2$ and 1% sodium ascorbate, in a total volume of 0.65 ml. All mixtures were incubated at 32°C for 6 hours, stopped by heating for 10 minutes at 95°C, centrifuged at 12,000 x g for 10 minutes, and the supernatants saved for assay of folate content.

12. DEAE-Sephacel chromatography of pteroylpolyglutamate standards and folate component from uroporphyrinogen III cosynthetase.

Approximately 1 pmol of each of the synthesized standards (5-CH_3-H_4PteGlu$_5$, 5-CH_3-H_4PteGlu$_6$, and 5-CH_3-H_4PteGlu$_7$) were mixed and chromatographed on a column (2.0 x 6.3 cm) of DEAE-Sephacel previously equilibrated with 10 mM potassium phosphate buffer, pH 6.0, including 10 mM 2-mercaptoethanol. The pteroylpolyglutamate standards were eluted with a linear gradient of 0-1.0 M KCl in buffer. Pteroylglutamate activity of the eluate was assayed after conjugase treatment by the microbiological method using L. casei as described in section B-16a(1). In a second experiment, identical quantities of each of the synthesized standards was mixed with 600 µl of the folate component dissociated from COSYN (160 µg protein) and the mixture was chromatographed and assayed for pteroylglutamate activity as described above.

13. Measurement of enzyme activities.

a. 7-ethoxycoumarin O-deethylase.

Guinea pig intestinal mucosal microsomal 7-ethoxycoumarin O-deethylase activity was determined in the 9,000 x g supernatant fraction by the fluorometric assay method of Greenlee and Poland (1978). The reaction mixture contained 50 µmol Tris-HCl, pH 8.0, 0.50 µmol NADPH, 0.50 µmol NADH, 5.0 µmol $MgCl_2$, 1 mg BSA,

0.50 μmol 7-ethoxycoumarin, and 0.20 ml of 9,000 x g tissue supernatant in a total volume of 1.0 ml. The reaction mixture was incubated for 15 minutes at 37°C in air with vigorous shaking. The reaction was stopped by adding in sequence 0.125 ml of 15% (w/v) trichloroacetic acid and 2 ml of chloroform. A 1.5 ml portion of the organic phase was extracted with 2.25 ml of 0.01 N NaOH/1 M NaCl solution and the concentration of 7-hydroxycoumarin in the alkaline phase was determined fluorometrically and compared with a 7-hydroxycoumarin standard using an Aminco-Bowman spectrophotofluorometer with excitation and emission wavelengths of 368 and 456 nm, respectively. Sample recovery was 94% and product formation was found to be linear for 30 minutes with up to 500 μg protein per ml reaction mixture.

b. NADPH-cytochrome c reductase.

Guinea pig intestinal mucosal microsomal NADPH-cytochrome c reductase activity was determined in the 9,000 x g supernatant fraction according to the method of Baron and Tephly (1969), by measuring the rate of increase in absorbance at 550 nm produced by the reduction of cytochrome c using a millimolar extinction coefficient of 19.1 mM^{-1} cm^{-1}. The reaction mixture in the sample and reference cuvettes contained, in a total volume of 3 ml, 1 μmol NaCN, 3 μmol cytochrome c, and 0.1 ml of 9,000 x g tissue supernatant in 0.1 M KH_2PO_4 buffer, pH 7.4. The reaction was initiated by adding 0.6 μmol NADPH to the sample cuvette. The rate of cytochrome c reduction was linear with respect to protein concentration over the range employed.

c. Alkaline phosphatase.

Guinea pig intestinal mucosal alkaline

phosphatase activity was assayed in the 9,000 x g supernatant fraction by the method of Bessey, Lowry and Brock (1946), using p-nitrophenyl phosphate as the substrate and p-nitrophenol as the standard. The reaction mixture contained, in a total volume of 2 ml, 2 μmol $ZnCl_2$, 10 μmol $MgCl_2$, 0.1 ml of a 1/200 dilution of the 9,000 x g tissue supernatant, and 0.9 μmoles p-nitrophenyl phosphate in 0.1 M glycine buffer, pH 9.2. The reaction mixture was incubated for 30 minutes at 37°C in air with vigorous shaking and then stopped by plunging into an ice-water bath at 0°C. The reaction mixture was diluted with 2.0 ml of 0.04 N NaOH, mixed and centrifuged at 1000 x g for 5 minutes to remove protein precipitates, and the absorbance of the supernatant measured at 405 nm. Product formation was found to be linear for 30 minutes, with up to 1.0 mg protein per ml reaction mixture.

d. Glucose-6-phosphate dehydrogenase.

Guinea pig intestinal mucosal glucose-6-phosphate dehydrogenase activity was determined in the 104,000 x g supernatant fraction by measuring the reduction of NADP at 340 nm, using a millimolar extinction coefficient of 6.22 mM^{-1} cm^{-1} (Olive and Levy, 1967). The reaction mixture in the sample and reference cuvettes contained, in a total volume of 3 ml, 0.6 μmol NADP and 10 μmol glucose-6-phosphate in 55 mM Tris-HCl buffer, pH 7.8, containing 3.3 mM $MgCl_2$. The reaction was initiated by adding 0.1 ml of the 104,000 x g tissue supernatant to the sample cuvette. The rate of reduction of NADP was linear with respect to protein concentration over the range employed.

e. Uroporphyrinogen I synthetase.

The activity of URO-S was measured by the method of Strand, et al. (1972). Enzyme was incubated for 45 minutes at 37°C in the presence of 20 µmol Tris-HCl buffer, pH 7.8, and 4 nmol PBG in a total volume of 400 µl. Reaction mixtures were stopped by addition of 1.5 ml 2 N $HClO_4$/95% ethanol (1:1, v/v), diluted to 3.0 ml, centrifuged to remove protein, and the uroporphyrin in the supernatant measured fluorometrically and compared with a uroporphyrin I standard using an Aminco-Bowman spectrophotofluorometer with excitation and emission wavelengths of 405 and 595 nm, respectively. Product formation was linear for 90 minutes with up to 20 µg of protein (600-1000 fold purified enzyme preparations from rat hepatic cytosol). One unit of URO-S activity was defined as the amount necessary to catalyze the formation of one nmol of uroporphyrinogen per hour.

f. Uroporphyrinogen III cosynthetase.

The activity of rat hepatic COSYN was measured by incubating the enzyme for 45 minutes at 37°C in the presence of: 20 µmol Tris-HCl buffer, pH 7.8, 2 nmol PBG, 24 µmol KCl, and 0.25 units of URO-S in a volume of 400 µl. Reaction mixtures were stopped by plunging into an ice-water bath at 0°C. Samples were then frozen in a dry ice/ethanol bath, lyophilized and esterified in 0.4 ml of 4 N methanolic-HCl. The mixtures of uroporphyrin I and III methyl esters were centrifuged at 10,000 x g for 1 minute to remove any protein precipitates, neutralized with 1 M Na_2CO_3, extracted into chloroform, washed once with 0.1 M Na_2CO_3, twice with deionized water, filtered to remove any particulate matter, and analyzed by HPLC by the method of Bommer, et al. (1979). All analyses were performed

on a Perkin-Elmer series 3 liquid chromatograph with recycle accessory utilizing a Perkin-Elmer model 204-A fluorescence spectrophotometer with excitation and emission wavelengths of 400 and 624 nm, respectively, and a Waters Associates μPorasil column (3.9 mm x 30 cm). Sample recovery following esterification, centrifugation, neutralization, extraction, washes, and filtration was 81.8 ± 2.4%. One unit of COSYN activity was defined as the amount necessary to give 50% URO III under the specified reaction conditions.

g. Porphobilinogenase.

The activity of PBGase was measured by incubating 0.25 units of URO-S and 3.5 units of COSYN for 45 minutes at 37°C in the presence of 20 μmol Tris-HCl buffer, pH 7.8, 2 nmol PBG, and 24 μmol KCl in a volume of 400 μl. Reactions were stopped by the addition of 1.5 ml of 2 N $HClO_4$/95% ethanol (1:1 v/v), diluted to 3.0 ml, and centrifuged at 2,000 x g for 20 minutes to remove any protein precipitates. The concentration of uroporphyrin in the supernatants was measured fluorometrically as described in section 13-e. One unit of PBGase activity was defined as the amount necessary to catalyze the formation of one nmol of uroporphyrinogen per hour.

14. Reversibility of inhibition of enzyme activity by heavy metal ions.

URO-S and COSYN were incubated with each metal for 45 minutes at 25°C. Preparations were then dialyzed at 4°C for 24 hours against 2 liters of 1 mM DTT - 50 mM Tris-HCl, pH 7.8, with one buffer change after 6 hours. Control enzyme preparations, devoid of any metal ions, were also examined in order to test for any loss of activity during the dialysis period. At the end of the dialysis

period, control and experimental preparations were tested for both URO-S and COSYN activity. Metal ion concentrations during incubation with enzyme were as follows: $PbCl_2$ (10 μM), $CdCl_2$ (10 μM and 100 μM), $ZnCl_2$ (10 μM and 100 μM), $CuCl_2$ (10 μM and 500 μM), $HgCl_2$ (1 μM), $CrCl_3$ (100 μM), and $FeCl_2$ (10 μM and 100 μM).

15. Kinetics of inhibition of enzyme activity by heavy metal ions.

URO-S and COSYN preparations were incubated in the presence of at least 6 different concentrations of heavy metal ions at substrate concentrations of 5 μM and 100 μM. Inhibition constants were calculated by the method of Dixon (1953).

16. Analytical procedures.

a. Quantitative estimation of folate concentration.

(1) Microbiological assay.

The microbiological assay of folate using L. casei as the test organism was modified from the methods of Waters, et al. (1961) and Herbert (1966). 0.5 ml of 50 mM sodium phosphate buffer, pH 6.1, containing 1% (w/v) sodium ascorbate was mixed with 2.5 ml of double-strength Bacto-folic acid casei medium. Various quantities of folic acid (from 0.2 to 10 pmoles) or the sample to be tested were added and the final volume adjusted to 5.0 ml with water. The tubes were loosely capped and sterilized for 30 minutes at 230°C. After cooling to room temperature, one drop of a 1:100 dilution of a 6 hour bacterial culture was added to each tube, and the tubes were mixed and incubated in a shaking water bath for 18 hours at 37°C. Bacterial growth was determined by measurement of turbidity at 660 nm. Assays of unknown folate samples were compared to a standard curve prepared from growth assays with known folic acid

concentrations.

(2) Competitive protein binding assay (radioassay).

The assay of folate by competitive protein binding was a modification of the method of Mortensen (1976). Folic acid standard or a sample of unknown folate concentration (200 µl) was mixed with 200 µl of an appropriate dilution of folate-binding protein, 100 µl of ^{3}H-folic acid (0.5 ng or 66 nCi), and 0.5 ml of 0.1 M Tris-HCl, pH 9.3. Water was added to a final volume of 1.0 ml. The reaction mixtures were incubated at 37°C for 30 minutes, cooled in an ice bath, and 1.0 ml of gelatin-coated charcoal (3.5% (w/v) charcoal - 0.75% (w/v) gelatin) was added to each tube. The tubes were mixed and incubated at 4°C for 20 minutes. The tubes were then centrifuged at 1000 x g for 20 minutes and 1.0 ml of supernatant was mixed with 3.0 ml of scintillation fluid (Merit^{R}) and counted. The percent ^{3}H-folic acid bound to the folate-binding protein is an inverse logarithmic function of the folic acid standard concentration. A standard curve prepared using folic acid concentrations of 0.16 pmole/ml to 8.0 pmole/ml bound from 69.1% to 20.4% of the H^{3}-folic acid.

The folate-binding protein was purified from milk by the method of Rothenberg, et al. (1972). Prior to the assay of unknown folate solutions, the amount of folate-binding protein necessary to bind most but not all of the labelled folic acid was determined. This was done by performing serial dilutions of the stock binding protein and assaying them with a constant amount of ^{3}H-folic acid. A dilution which bound 60-80% of the labelled folate was determined. This dilution (1:200) was used in all binding studies utlizing the purified folate-binding protein.

(3) Erythrocyte folate content.

Erythrocyte folate content was determined by the competitive protein binding radioassay preceded by extraction as described by Mortensen (1976). The assay and preparation of folate-binding protein have been described above (Section 16-a(2)). A blank without folate-binding protein was prepared for each sample to account for any interference in the analysis by erythrocyte folate-binding proteins. A standard curve prepared using N-5-methyltetrahydrofolate concentrations of 0.2 ng/ml to 8.0 ng/ml bound from 76.4% to 14.7% of the H^3-folic acid. The N-5-methyltetrahydrofolate standard was assayed spectrophotometrically at 290 nm using a molar extinction coefficient of 30,000 M^{-1} cm^{-1} (Gupta and Huennekens, 1967). Results were expressed as nanograms N-5-methyltetrahydrofolate per ml packed red cells.

(4) Intestinal folate content.

Intestinal mucosal folate content was determined by both the competitive protein binding assay as described by Mortensen (1976) and the microbiological assay as described by Waters, et al. (1961) and Herbert (1966). Samples to be assayed by the microbiological method were incubated at 32°C for 60 minutes in the presence of a preparation of chicken pancreas conjugase (Mims and Laskowski, 1945; Bird, et al., 1965) which had been determined to be folate-free by microbiological assay. All samples were placed in a water bath at 95°C for 10 minutes in the presence of 1% ascorbate and were centrifuged for 10 minutes at 12,000 x g. The supernatants were saved for assay. Results were expressed as nanograms folic acid per milligram protein.

b. Microsomal heme content.

Microsomal heme was determined from the difference spectrum of the oxidized/reduced pyridine hemochromogen between 541 and 557 nm, using an extinction coefficient of 20.7 mM^{-1} cm^{-1} (Falk, 1964). The microsomal pellet was suspended in 1.5 ml of 0.1 M KH_2PO_4, pH 7.4, buffer. In a separate vial, 1 ml of microsomes was combined with 0.34 ml of water and 0.44 ml of pyridine. To this solution, 0.22 ml of 1 N NaOH was added and mixed gently. Heme degrades in aqueous alkali very rapidly, therefore, after the addition of 1 N NaOH the spectrum was measured immediately. Reaction mixtures (1.0 ml portions) were added to the sample cuvette which contained 2 mg of sodium dithionite and the reference cuvette which contained 22 µl of 3 mM potassium ferricyanide. The spectrum (500-600 nm) was then recorded on an Aminco DW-2a spectrophotometer. Measurements were based on the absorbance difference between 541 and 557 nm.

c. Protein determination.

Protein concentrations were determined either by the method of Lowry, et al. (1951), the method of Bradford (1976), or by direct absorption at 230 or 280 nm. Bovine serum albumin was used as the standard.

d. Statistics.

Data were analyzed by Student's t-test in order to determine the significance of differences between means.

TABLE II-1

COMPOSITION OF THE CONTROL DIET

Ingredient	%
Casein (vitamin free)	30.0
Corn starch	20.0
Sucrose	10.3
Dextrose	7.8
Corn oil	7.3
Fiber	1.5
Choline chloride	0.4
Vitamin mixture[1]	2.5
Salt mixture[2]	8.953

[1] Reid-Briggs Vitamin Mixture provided the following per kg diet: carrier fiber 20.154 g, Biotin .0012 g, Vit. B_{12} .00008 g, Ca. Pantothenate .08 g, Folic Acid .02 g, Inositol 4 g, Menadione .004 g, Niacin .4 g, Pyridoxine .032 g, Riboflavin .032 g, Thiamin HCl .032 g, Vit. A 34,000 I.U., Vit. D_3 3200 I.U., Vit. E 40 I.U., Vit. C 2 g.

[2] Salt mixture Briggs No. A. Chick provided the following per kg diet: $CaCO_3$ 15 g, $Ca_3(PO_4)_2$ 14 g, $CuSO_4$ .02 g, Ferric citrate .04 g, $MgSO_4$ 5 g, $MnSO_4$ .312 g, KI .04 g, K_2HPO_4 7.3 g, NaCl 8.8 g, Na_2HPO_4 7.3 g, Potassium Acetate 25 g, MgO 5 g, $ZnCO_3$ .02 g.

III. RESULTS

A. Effect of folate deficiency on microsomal drug metabolism and heme content in the intestinal mucosa of guinea pigs.

Growth of the control and experimental groups was similar for the 5-week period of pair-feeding. Table III-1 lists the mean body weight and growth rates of each group. Guinea pigs fed the folate-deficient diet gained weight until the fourth week, at which time weight gain ceased in the deficient diet group as compared to the control diet group. However, these results were not statistically significant. At week 4, diarrhea and anorexia were observed in the experimental group. Four of the 24 animals fed the folate-deficient diet died during the fourth week of the study.

The effect of folic acid deficiency on intestinal mucosal folate content is reported in Table III-2. Intestinal mucosal folate levels, determined either by microbiological or radioassay, illustrate the progressive tissue depletion of folate with time. Differences in the absolute values of folate determined by the microbiological and radioassays were noted. This discrepancy may be due to the differences in sample preparation for the microbiological assay and radioassay. Samples for microbiological assay were treated with conjugase before assay, whereas samples for radioassay were not. Intestinal mucosal folate levels were significantly depressed at week 1, and remained depressed for the duration of the study. The time course for folate depletion of the intestinal mucosa is very similar to that for folate depletion of the bone marrow as measured by erythrocyte folate content. Figure III-1 shows the parallel depletion of intestinal and erythrocytic folate content with time.

Folate deficiency of the intestinal mucosa produces a depression of intestinal mucosal microsomal heme content and drug metabolism as shown in Figure III-2. A significant decrease (78%) in intestinal mucosal microsomal drug metabolism (7-ethoxycoumarin O-deethylase activity) and a significant decrease (46%) in intestinal mucosal microsomal heme content were observed at three weeks. The intestinal mucosal microsomal heme content and drug metabolizing activity remained depressed for the duration of the 5-week study.

The observed depression in intestinal mucosal microsomal drug metabolism and heme content may be due to changes in gut morphology and/or nonspecific alterations in intestinal protein content. To test these possibilities, the activities of the nonheme-dependent enzymes NADPH-cytochrome c reductase, glucose-6-phosphate dehydrogenase, and alkaline phosphatase were measured. Webster and Harrison (1969) have shown that glucose-6-phosphate dehydrogenase activity appears to remain at a constant level throughout intestinal mucosal epithelial cell development from the crypt to the villus tip, whereas both NADPH-cytochrome c reductase and alkaline phosphatase activities show a progressive increase in activity with migration toward the villus tip from the crypt zone. As shown in Figure III-3, NADPH-cytochrome c reductase activity of the intestinal mucosa of the folate-deficient animals was depressed at 3, 4, and 5 weeks. This depression was statistically significant at both 3 and 5 weeks. Intestinal mucosal alkaline phosphatase activity was also lower in the folate-deficient animals in comparison to controls at every point in time (Table III-3). However, this decrease was not statistically significant. Glucose-6-phosphate dehydrogenase activities of the intestinal mucosas of the

control and experimental groups were comparable throughout the study (Table III-3). The ratio of the activities of alkaline phosphatase to glucose-6-phosphate dehydrogenase is also listed in Table III-3. The change in ratios between the control and experimental groups, together with the decrease in NADPH-cytochrome C reductase activity observed in the folate-deficient animals, suggests that changes in gut morphology do occur in guinea pigs fed a folate-deficient diet.

B. Purification of uroporphyrinogen III cosynthetase from rat hepatic cytosol.

Ammonium sulfate fractionation (60-90%) was initially employed in order to isolate COSYN from hepatic cytosol. This procedure separates COSYN from URO-S, making it possible to calculate the specific activity of COSYN. Chromatography on DEAE-Sephacel (Figure III-4) results in an 11-fold purification with an 87% yield, and an additional purification step using Sephadex G-100 superfine (Figure III-5) purifies COSYN 37-fold with a 39% yield. The final purification step using folate-AH-Sepharose-4B affinity chromatography (Figure III-6) purifys COSYN 73-fold with a 13% yield. The purification of COSYN is summarized in Table III-4. The purified COSYN is completely devoid of URO-S activity but retains the ability to direct the synthesis of URO III by purified URO-S.

C. Properties of uroporphyrinogen III cosynthetase.

1. Effect of cosynthetase concentration on uroporphyrinogen III formation.

The URO III produced as a function of increasing quantities of COSYN (G-100 superfine step) is shown in Figure III-7. The asymptotic relationship between URO III formed and COSYN utilized

(Figure III-7A) has been reported in mouse spleen (Levin, 1971) and Rhodopseudomonas spheroides (Jordan et al., 1980) tissue preparations, and is characteristic of a coupled reaction with enzymes acting in sequence (Jordan et al., 1980). A reciprocal plot of the data (Figure III-7B) approximates a straight line and facilitates calculation of the units of COSYN activity. The reciprocal plot intersects the ordinate at 1.0 (100% URO III), suggesting that in vitro 100% URO III can only be obtained at an infinite concentration of COSYN.

2. Effect of substrate on uroporphyrinogen III formation.

The effect of the substrate PBG on rat hepatic PBGase activity and percent URO III formation is shown in Figure III-8. The substrate saturation curve for PBGase is hyperbolic. Lineweaver-Burk plots of velocity against PBG concentration (Figure III-9) yielded a Michaelis constant of 5 μM for PBG. This value is similar to previously reported K_m values for PBGase activity in cow liver (Sancovich, Batlle, and Grinstein, 1969a) and soybean callus (Llambias and Batlle, 1970) tissue preparations.

Inhibition of URO III formation with increasing substrate concentrations was observed (Figure III-8). Similar observations have been reported by other investigators (Sancovich, Batlle, and Grinstein, 1969a; Cornford, 1964). COSYN assays were routinely conducted at a substrate concentration of 5 μM PBG. This concentration allows for sufficient product for analysis, as well as a high percentage of the III isomer.

3. Cosynthetase inactivation with time.

A plot of percent URO III formation against time under conditions allowing for constant total product formation is shown in Figure III-10. A time-dependent loss of COSYN activity during reaction with URO-S and the substrate PBG was observed. Similar observations have been reported by Levin (1968b). Incubation times of 45 minutes were chosen in order to have sufficient product formation and percent III isomer.

4. Effect of temperature on uroporphyrinogen III formation.

The effect of temperature on PBGase activity and percent URO III formation is shown in Figure III-11. PBGase activity increased with increasing temperature, whereas percent URO III formation decreased. Levin (1968b) observed COSYN activity in mouse spleen preparations to be thermolabile, and noted that the substrate dependent, URO-S catalyzed inactivation of COSYN was accelerated at higher temperatures. Our data agree with these observations. Assays were routinely conducted at physiological temperature (37°C). Although COSYN is thermolabile at 37°C, greater than 80% URO III formation can be insured by maintaining a ratio of COSYN:URO-S of at least 10 units COSYN per unit URO-S. Assays were routinely performed with a COSYN:URO-S unit ratio of 14:1.

5. Effect of pH on uroporphyrinogen III formation.

Optimal activity of PBGase was observed at pH 7.8 in 50 mM sodium phosphate buffer or 50 mM Tris-HCl buffer (Figure III-12). Increased formation of URO III was observed at more alkaline pH values. The pH optimum for rat hepatic PBGase activity is similar to values reported for human erythrocyte (Cornford, 1964) and mouse

spleen (Levin, 1971) tissue preparations. Assays were routinely conducted at pH 7.8 in 50 mM Tris-HCl buffer.

6. Effect of salt concentration on uroporphyrinogen III formation.

Certain concentrations of sodium and magnesium salts have been reported to enhance COSYN activity (Batlle and Rossetti, 1977; Sancovich, Batlle, and Grinstein, 1969a). In rat hepatic COSYN preparations the optimal salt concentration for formation of URO III was 60 mM KCl or 60 mM NaCl (Figure III-13). $MgCl_2$ and $CaCl_2$ at concentrations of 6 mM and 8 mM, respectively, also optimized formation of URO III (Figure III-14). The addition of optimal concentrations of $MgCl_2$ (6 mM) to COSYN preparations already optimal with respect to KCl (60 mM) resulted in no further increase of URO III formation. Assays were routinely conducted at a salt concentration of 60 mM KCl.

D. Heavy metal ion inhibition of uroporphyrinogen III cosynthetase activity.

PBGase and URO-S have been reported to be sulfhydryl enzymes subject to inhibition by a variety of heavy metal ions (Llambias and Batlle, 1971b; Sancovich et al., 1976). In studies of PBGase inhibition by sulfhydryl reagents such as N-ethylmaleimide and p-hydroxymercuribenzoate, inhibition of URO III formation was more pronounced than inhibition of total product formation (Frydman and Feinstein, 1974), suggesting that COSYN is more sensitive to inhibition than is URO-S. The selective inhibition of COSYN by heavy metal ions was examined by assessing the effect of various heavy metal ions on the activity of purified preparations of URO-S, COSYN, and PBGase.

$CdCl_2$, $ZnCl_2$, and $CuCl_2$ selectively inhibited COSYN activity at concentrations having little or no effect on URO-S or PBGase activity (Table III-5). $PbCl_2$ and $HgCl_2$ were less selective, inhibiting PBGase, URO-S, and COSYN activities at similar concentrations. $FeCl_2$ also inhibited all three enzymes, but the inhibition of URO-S and PBGase activity was more pronounced than was the inhibition of COSYN activity. $CrCl_3$ inhibited URO-S and PBGase activity to the same extent, but no change in isomer composition of the reaction products was observed, suggesting the selective inhibition of URO-S by this metal ion. $NiCl_2$ and $CoCl_2$ had a negligible effect on the activity of the enzymes at metal ion concentrations up to 100 μM.

In order to determine whether inhibition was reversible, enzyme aliquots were dialyzed in the presence of each inhibitor at concentrations known to inhibit enzymatic activity by at least 50 percent. Dialysis restored the activity of each enzyme preparation to 90 percent of control activity. These findings indicate that inhibition of URO-S, PBGase, and COSYN activity by heavy metal ions is reversible. From Dixon plots the inhibition constants for each metal were calculated (Table III-6) and it was determined that each heavy metal ion is a non-competitive inhibitor. A representative Dixon plot for $CdCl_2$ inhibition of COSYN activity is shown in Figure III-15.

E. Molecular weight determination of uroporphyrinogen III cosynthetase.

The purified COSYN resolved by the final affinity chromatography step was subjected to chromatography on a calibrated column of Sephadex G-100 superfine to determine the approximate molecular weight. As illustrated in Figure III-16, the purified COSYN eluted as a single peak corresponding to a molecular weight of approximately

42,000 daltons.

F. Electrophoresis of uroporphyrinogen III cosynthetase.

The 37-fold purified COSYN preparation (Sephadex G-100 superfine step) showed five bands on polyacrylamide gel electrophoresis at pH 8.3 (Figure III-17-1). This enzyme preparation was divided into two major protein peaks, designated A and B, by further purification utilizing folate-AH-Sepharose 4B affinity chromatography (Figure III-6). After concentration using Centriflo CF25 ultrafiltration membranes, peak A (very little COSYN activity) and the pooled active fractions of peak B (73-fold purified COSYN preparation) were subjected to polyacrylamide gel electrophoresis. Peak A was resolved into two protein bands corresponding to the two lower bands of the Sephadex G-100 superfine COSYN preparation (Figure III-17-2), and the pooled, concentrated active fractions of peak B were resolved into two bands corresponding to two of the upper, slower migrating bands of the Sephadex G-100 superfine COSYN preparation (Figure III-17-3).

The two bands associated with the pooled, concentrated active fractions of peak B, designated B_1 and B_2 (Figure III-17-3), were each assayed for COSYN activity by incubating the appropriate gel slices with the substrate PBG and URO-S as described in section B-7 of the Methods. Both bands had COSYN activity (expressed as percent URO III formation), and the sum of the activities of bands B_1 and B_2 was similar to the activity of an uncut gel containing both bands and a similar amount of protein (Table III-7).

G. Folate content of uroporphyrinogen III cosynthetase.

Preparations of the 73-fold purified COSYN (folate-AH-Sepharose 4B step) were heated in the presence of 2-mercaptoethanol as

described in the Methods (section B-10) in an attempt to dissociate any coenzymes or cofactors from the native protein. The supernatant obtained from this procedure, after centrifugation to remove any protein precipitates, was assayed for folate content, both before and after conjugase treatment, by the microbiological method using L. casei as the test organism and the competitive protein binding assay. Growth of the folate-dependent organisms and antagonism of [^{3}H]-folate binding to folate-binding protein by the dissociated factor indicated that it was a pteroylglutamate derivative (Table III-7). Estimated values of ng factor (as pteroylglutamate) per mg protein (folate-AH-Sepharose 4B step) by radioassay were in agreement with values obtained by microbiological assay. The enhanced growth response of the test organisms after treatment of the factor with conjugase (Table III-7) indicates that the factor dissociated from COSYN is a pteroylpolyglutamate.

To test further the association of a pteroylpolyglutamate factor with COSYN, the individual bands B_1 and B_2, obtained by gel electrophoresis of the 73-fold purified COSYN preparation, were eluted from the polyacrylamide gels as described in the Methods (section B-7) and subjected to heat treatment in the presence of 2-mercaptoethanol to dissociate the factor. The supernatants obtained by centrifugation of the heated, eluted bands were assayed by the microbiological method, both before and after conjugase treatment, for folate content. Both bands B_1 and B_2 were associated with a pteroylglutamate factor (Table III-7), and the enhanced growth of the test organism (L. casei) after treatment of the dissociated factor from bands B_1 and B_2 with conjugase, again suggests that the factor isolated from COSYN is a

pteroylpolyglutamate.

H. Characterization of the pteroylpolyglutamate associated with uroporphyrinogen III cosynthetase.

Because most of the pteroylglutamate isolated from tissues exists in pteroylpolyglutamate forms (Reed et al., 1977; Shin et al., 1974), with reduced pteroylpenta- and hexaglutamates being the predominant forms isolated from rat liver (Houlihan and Scott, 1972; Shin et al., 1972; Brown et al., 1973; Brown et al., 1974), the isolated pteroylpolyglutamate factor associated with rat hepatic COSYN was chromatographed with the N_5-methyl derivatives of tetrahydropenta-, tetrahydrohexa-, and tetrahydroheptaglutamate standards. The N_5-methyl derivatives were chosen because of their stability (Leslie and Baugh, 1974) and predominance in rat liver (Beck, 1974; Bird et al., 1965).

Each standard was individually chromatographed on the same DEAE-Sephacel column (2.0 x 6.3 cm) and the conductivity at the point of elution determined. These standard conductivity values were subsequently utilized to identify the respective standards in a mixture of all three. As shown in Figure III-18A, chromatography on DEAE-Sephacel with a linear gradient of 0 to 1.0 M KCl successfully separates the 5-CH_3-$H_4PteGlu_5$, 5-CH_3-$H_4PteGlu_6$, and 5-CH_3-$H_4PteGlu_7$ standards. When the pteroylpolyglutamate factor dissociated from COSYN is chromatographed with the pteroylpolyglutamate standards (Figure III-18B) it co-chromatographs with the 5-CH_3-$H_4PteGlu_7$, increasing the growth response of the test organisms approximately three-fold. These results suggest that the isolated pteroylpolyglutamate factor may be either 5-CH_3-$H_4PteGlu_7$ or a closely related $H_4PteGlu_n$ ($n \geq 6$) derivative with similar elution characteristics on a DEAE-Sephacel

anion exchange column.

The pteroylpolyglutamate factor isolated from rat hepatic COSYN did not stimulate URO-S activity or protect the enzyme from $PbCl_2$-mediated inhibition. These results suggest that the folate derivative isolated from COSYN is either different from the pteroylpolyglutamate associated with URO-S or has been structurally altered and inactivated during the isolation procedures. Attempts to enhance URO III formation by addition of the isolated pteroylpolyglutamate factor to purified preparations of COSYN were unsuccessful. These results may reflect the inability of the isolated factor to bind to the enzyme again, and suggest that either the folate derivative is altered in some way during the isolation procedure, or that all available binding sites on the enzyme are occupied.

TABLE III-1

A COMPARISON OF BODY WEIGHTS AND GROWTH RATES IN GUINEA PIGS FED THE CONTROL AND FOLATE-DEFICIENT DIETS[a]

Weeks on diet	Body weight		Rate of gain	
	Control	Experimental	Control	Experiment
	gm	gm	gm/wk	gm/wk
0	145 ± 3	157 ± 4	--	--
1	186 ± 4	203 ± 4	40.4 ± 2.8	45.7 ± 1.6
2	216 ± 4	229 ± 6	29.6 ± 2.4	26.2 ± 3.0
3	241 ± 7	248 ± 9	24.1 ± 4.8	20.4 ± 4.8
4	248 ± 11	246 ± 11	6.1 ± 7.6	-9.6 ± 7.4
5	282 ± 14	273 ± 16	24.6 ± 4.6	14.5 ± 5.5

[a] Values represent the mean body weight or mean weight gain per week in grams ± S.E.M. of at least 8 determinations.

TABLE III-2

EFFECT OF FOLATE DEFICIENCY ON FOLATE CONTENT
IN THE INTESTINAL MUCOSA OF GUINEA PIGS[a]

Weeks on diet	Control folate content, ng/mg protein		Experimental folate content, ng/mg protein	
	Microbiological assay	Radioassay	Microbiological assay	Radioassay
0	11.33 ± 1.36	18.13 ± 1.71	9.40 ± 1.81	26.85 ± 8.80
1	6.27 ± 0.88	---	2.39 ± 0.30	---
2	10.63 ± 1.50	14.75 ± 2.09	1.02 ± 0.05	1.11 ± 0.74
3	13.96 ± 1.35	20.26 ± 5.18	1.30 ± 0.18	6.77 ± 1.21
4	6.73 ± 1.41	26.50 ± 3.35	0.78 ± 0.05	3.01 ± 0.88
5	---	19.37 ± 0.35	---	3.51 ± 0.42

[a] Values represent the mean ± S.E.M. of 3 determinations.

TABLE III-3

EFFECT OF FOLATE DEFICIENCY ON ENZYME ACTIVITY IN THE INTESTINAL MUCOSA OF GUINEA PIGS[a]

Weeks on diet	Control			Experimental		
	Alkaline phosphatase activity (AP) nmols p-nitrophenol formed/mg protein/hr	Glucose-6-phosphate dehydrogenase activity (G-6-P) nmols NADP reduced/mg protein/min	AP/G-6-P ratio	Alkaline phosphatase activity (AP) nmols p-nitrophenol formed/mg protein/hr	Glucose-6-phosphate dehydrogenase activity (G-6-P) nmols NADP reduced/mg protein/min	AP/G-6-P ratio
1	687 ± 119	30.8 ± 1.6	22.3	594 ± 71	36.9 ± 3.9	16.1
2	783 ± 104	37.7 ± 2.2	20.8	509 ± 14	45.7 ± 4.1	16.9
3	808 ± 107	55.3 ± 4.5	14.6	608 ± 54	47.9 ± 9.0	12.7
4	824 ± 140	32.0 ± 1.3	25.8	509 ± 32	34.9 ± 2.0	14.6

[a] Values represent the mean ± S.E.M. of 3 determinations.

TABLE III-4

PURIFICATION OF UROPORPHYRINOGEN III
COSYNTHETASE FROM RAT LIVER

FRACTION	PROTEIN CONCENTRATION	PROTEIN TOTAL	TOTAL ACTIVITY[a]	SPECIFIC ACTIVITY[a]	YIELD	PURIFICATION
	mg/ml	mg	units	units/mg	%	-fold
Ammonium Sulfate, 60-90%	13.8	622.0	18,598	30	100	1
DEAE-Sephacel	5.5	49.2	16,285	331	87.6	11.1
Sephadex G-100 superfine	0.6	6.6	7,296	1099	39.2	36.8
Folate-AH-Sepharose 4B	0.4	1.2	2,500	2174	13.4	72.7

[a] One unit is defined as the amount of COSYN necessary to give 50% URO III under the specified reaction conditions.

TABLE III-5

INHIBITION OF RAT HEPATIC PORPHOBILINOGENASE, UROPORPHYRINOGEN I SYNTHETASE AND UROPORPHYRINOGEN III COSYNTHETASE ACTIVITIES BY VARIOUS HEAVY METAL IONS[a]

Metal Additive	PBGase Inhibition (%)	URO-S Inhibition (%)	COSYN Inhibition (%)
$PbCl_2$ (10 μM)	67	73	75
$HgCl_2$ (1 μM)	45	80	57
$CdCl_2$ (10 μM)	0	0	82
$ZnCl_2$ (10 μM)	10	0	47
$CuCl_2$ (10 μM)	0	0	49
$FeCl_2$ (10 μM)	77	74	22
$NiCl_2$ (100 μM)	3	0	0
$CoCl_2$ (100 μM)	11	4	3
$CrCl_3$ (100 μM)	46	37	0

[a] Activities were determined as described in Methods.

TABLE III-6

INHIBITION CONSTANTS FOR RAT HEPATIC PORPHOBILINOGENASE, UROPORPHYRINOGEN I SYNTHETASE AND UROPORPHYRINOGEN III COSYNTHETASE INHIBITION BY VARIOUS HEAVY METAL IONS

Metal Additive	PBGase Ki (μM)	URO-S Ki (μM)	COSYN Ki (μM)
$PbCl_2$	2.2	2.0	0.9
$HgCl_2$	1.1	0.4	0.8
$CdCl_2$	67.3	61.9	2.9
$ZnCl_2$	58.9	62.5	8.5
$CuCl_2$	576.0	596.0	8.9
$FeCl_2$	4.9	3.1	40.3
$NiCl_2$	> 100.0	> 100.0	> 100.0
$CoCl_2$	> 100.0	> 100.0	> 100.0
$CrCl_3$	116.0	132.0	> 100.0

TABLE III-7

FOLATE CONTENT AND ENZYME ACTIVITY OF UROPORPHYRINOGEN III COSYNTHETASE SEPARATED FROM RAT LIVER ON ANALYTICAL POLY-ACRYLAMIDE DISC GEL ELECTROPHORESIS

Fraction	Folate Content				COSYN Activity[b]
	Microbiological Assay		Radioassay		
	Before[a]	After[a]	Before[a]	After[a]	% formation URO III
	ng/mg Protein		ng/mg Protein		
Folate-Sepharose B	14	50	14	15	83.3
Folate-Sepharose B_1	6	36	--	--	64.3
Folate-Sepharose B_2	4	30	--	--	24.7

[a] Microbiological and radioassay were carried out before and after conjugase treatment as described in Methods.

[b] Cosynthetase activity was assayed in gels after electrophoresis as described in Methods and expressed as percent formation URO III under specified reaction conditions.

Figure III-1

The time-course for intestinal and erythrocyte folate depletion in guinea pigs fed a folate-deficient diet.

Each point represents the mean ± S.E.M. for three determinations. The intestinal folate content was determined by microbiological assay and the erythrocyte folate content was determined by radioassay as described in Methods.

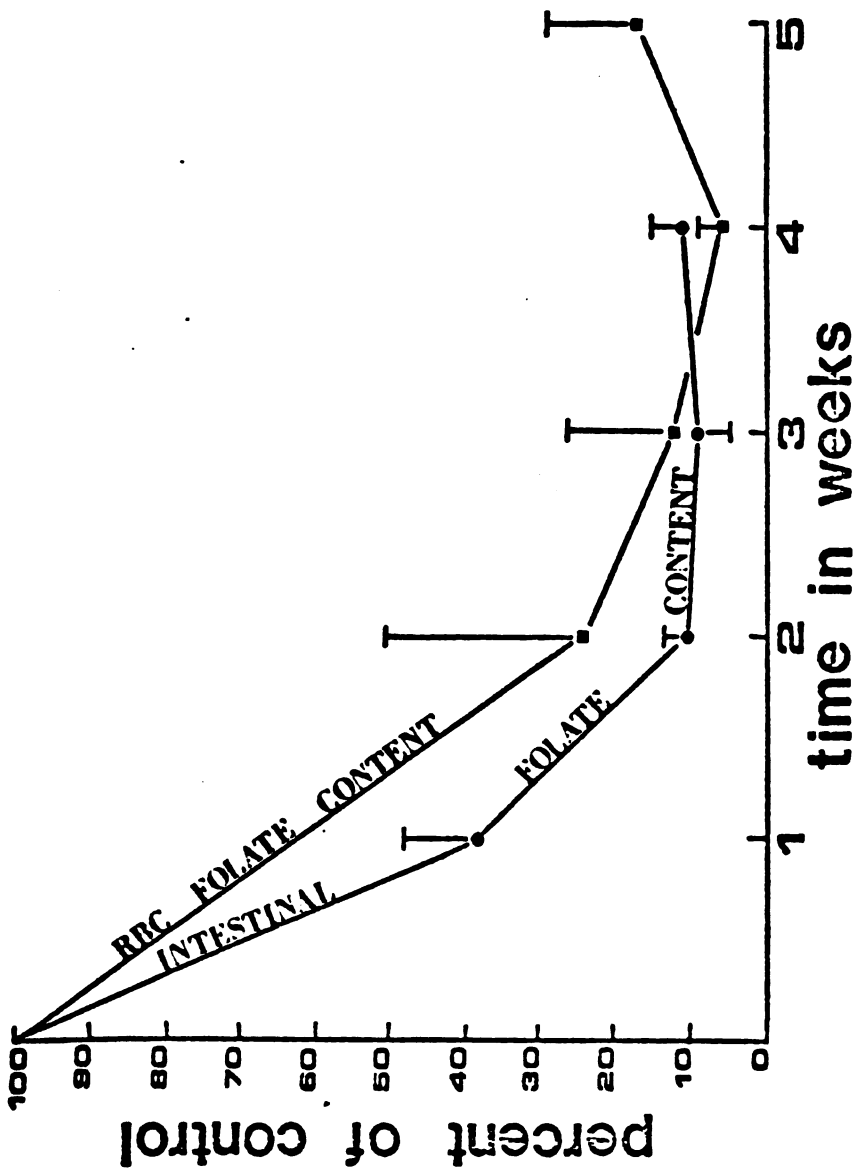

percent of control
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
time in weeks
RBC FOLATE CONTENT
INTESTINAL FOLATE CONTENT

Figure III-2

The effect of folate deficiency on intestinal microsomal heme content and drug metabolism (7-ethoxycoumarin O-deethylase activity).

Each point represents the mean ± S.E.M. for three determinations. An asterisk denotes a significant difference ($p < 0.05$) between folate-deficient and control animals. The mean control value for microsomal heme was 0.273 nmol heme/mg protein. The mean control value for 7-ethoxycoumarin O-deethylase activity was 38.5 pmol /mg protein/hr.

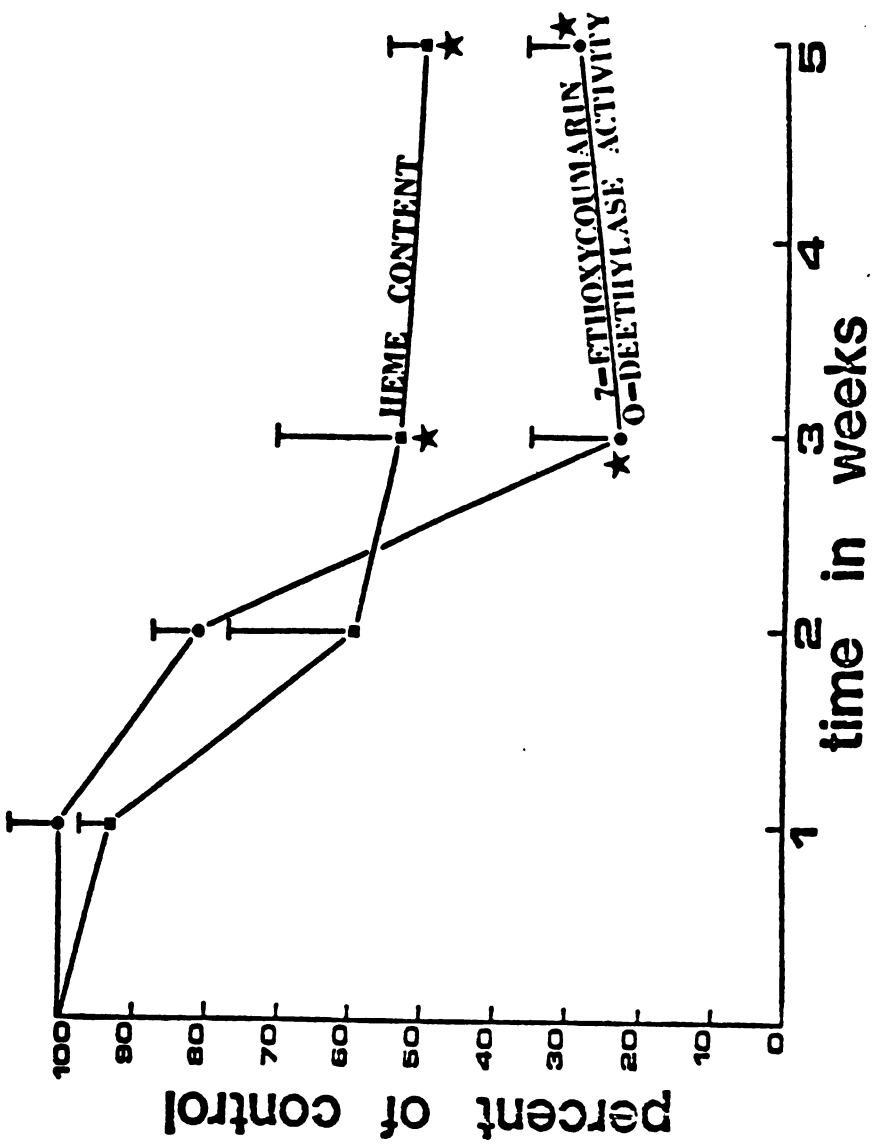

HEME CONTENT
7-ETHOXYCOUMARIN
O-DEETHYLASE ACTIVITY
percent of control
time in weeks
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5

Figure III-3

The effect of folate deficiency on intestinal microsomal cytochrome c reductase activity.

Each value represents the mean ± S.E.M. for three determinations. An asterisk denotes a significant difference ($p < 0.05$) between folate-deficient (E) and control (C) animals.

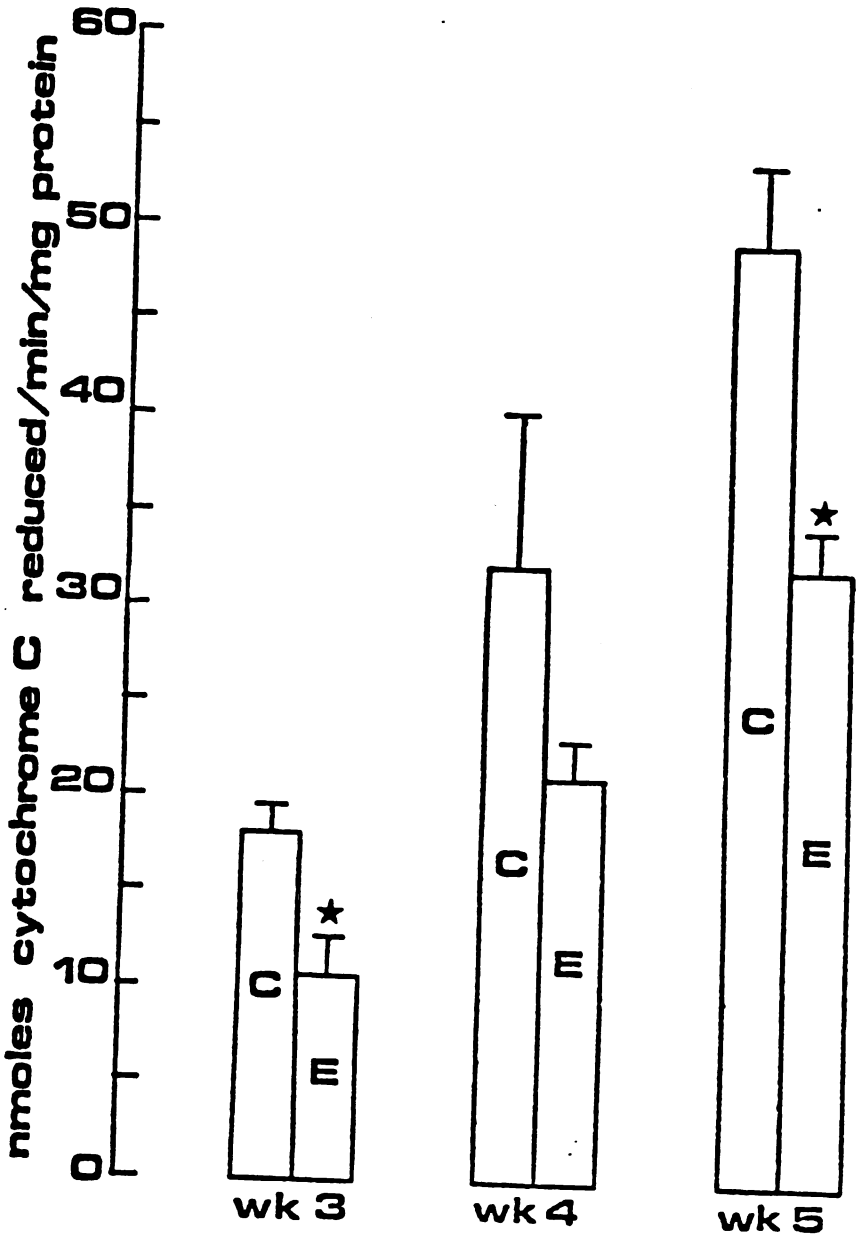

nmoles cytochrome C reduced/min/mg protein
60
50
40
30
20
10
0
C
E
★
wk 3
wk 4
wk 5

Figure III-4

Purification of rat hepatic uroporphyrinogen III cosynthetase on DEAE-Sephacel.

Concentrated enzyme from ammonium sulfate fractionation was applied to a column (2.0 x 25 cm) of DEAE-Sephacel and eluted with a 0-0.25 M KCl gradient in 50 mM Tris-HCl, pH 7.8, buffer. One-hundred 3 ml fractions were collected. ●——●, A_{280}; o——o, uroporphyrinogen III cosynthetase activity expressed as percent of total uropoprhyrin as the III isomer. The linear gradient of potassium chloride is superimposed on the elution pattern. Activities were determined as described in Methods.

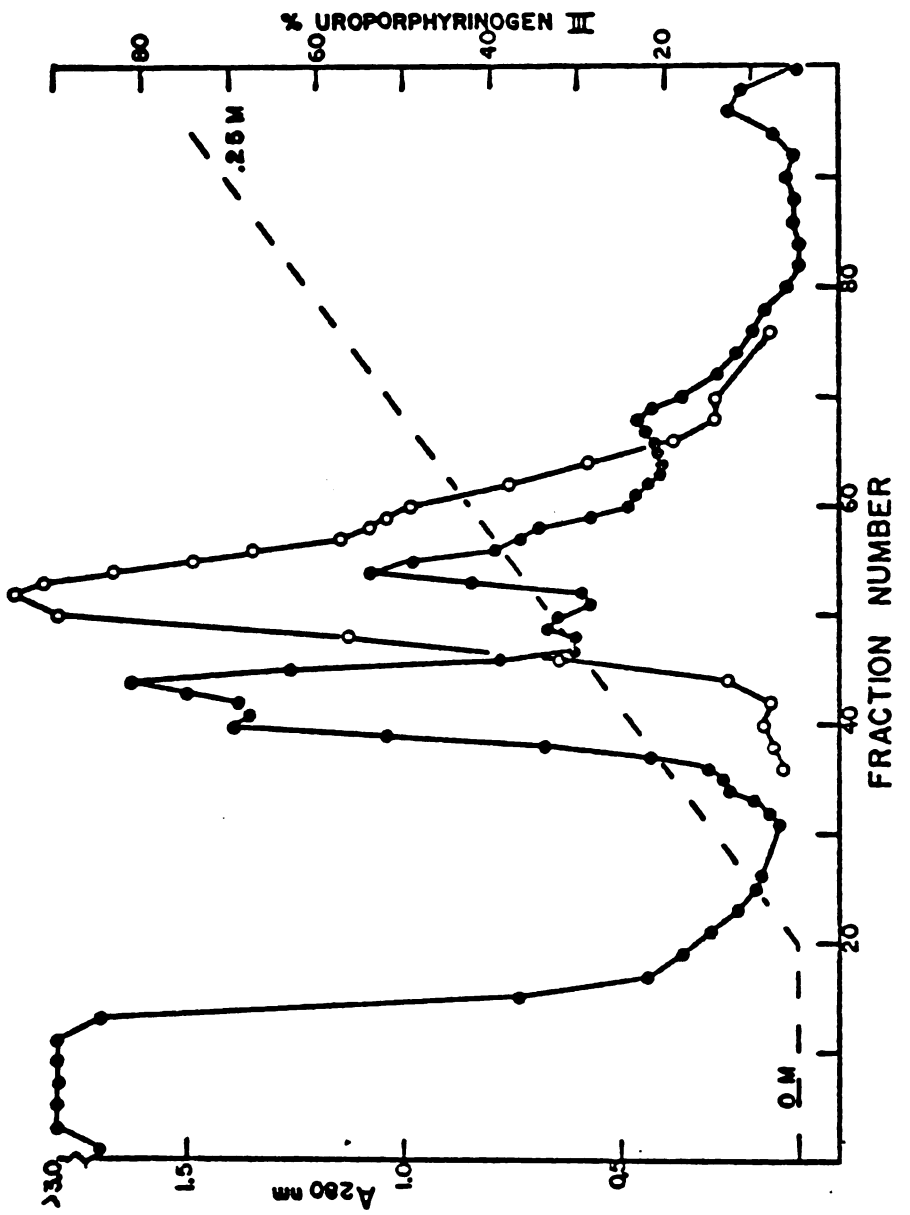

% UROPORPHYRINOGEN III
80
60
40
20
.25 M
0 M
A280 nm
>3.0
1.5
1.0
0.5
20
40
60
80
FRACTION NUMBER

Figure III-5

Purification of rat hepatic uroporphyrinogen III cosynthetase on Sephadex G-100 superfine.

A concentrated solution of enzyme from DEAE-Sephacel chromatography was applied to a column (2.5 x 60 cm) of Sephadex G-100 superfine. One-hundred 2 ml fractions were eluted with 50 mM Tris-HCl, pH 7.8, buffer. ●——●, A_{280}; O——O, uroporphyrinogen III cosynthetase activity expressed as percent of total uroporphyrin as the III isomer. Activities were determined as described in Methods.

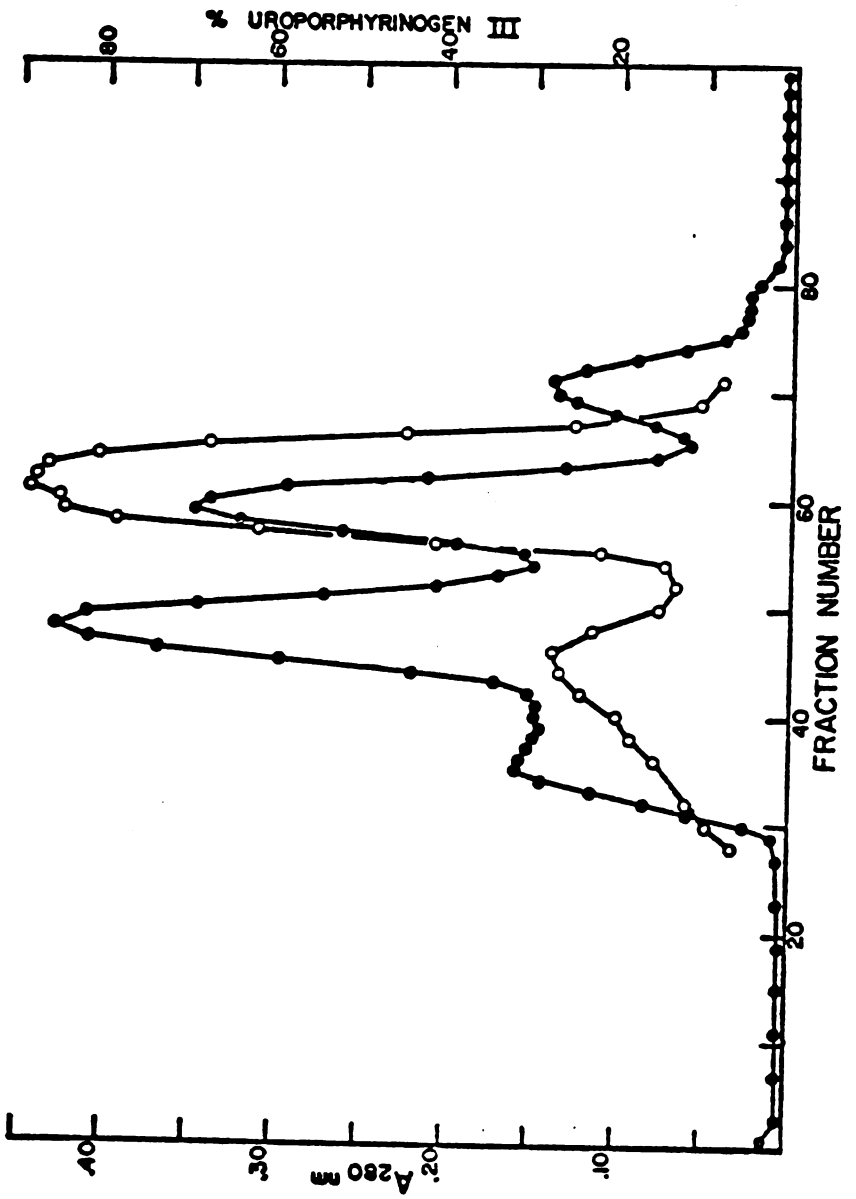

% UROPORPHYRINOGEN III
80
60
40
20
A280 nm
.40
.30
.20
.10
FRACTION NUMBER
80
60
40
20

Figure III-6

Purification of rat hepatic uroporphyrinogen III cosynthetase on folate-AH-Sepharose 4B.

Enzyme from the Sephadex G-100 superfine purification step was applied to a folate-AH-Sepharose 4B column (2.0 x 5.5 cm) and eluted with a linear gradient of 0-1.0 M KCl in 50 mM Tris-HCl, pH 7.8, buffer containing 0.2 mM DTT. The active fractions (⊢—⊣) were pooled and concentrated as described in Methods. Fractions of 1.0 ml were collected. ●——●, A_{280}; O——O, uroporphyrinogen III cosynthetase activity expressed as percent of total uroporphyrin as the III isomer. The linear gradient of potassium chloride is superimposed on the elution pattern. Activities were determined as described in Methods.

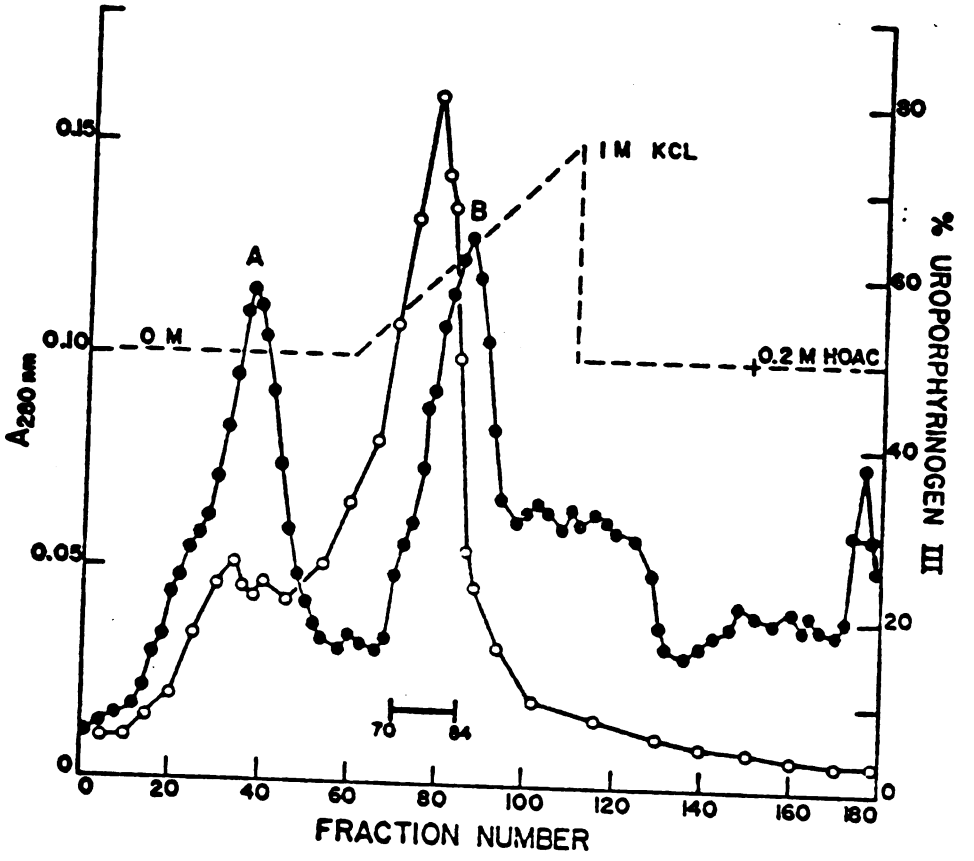

0.15
0.10
0.05
0
A280 nm
0 M
1 M KCL
+0.2 M HOAC
A
B
70
84
80
60
40
20
0
% UROPORPHYRINOGEN III
0
20
40
60
80
100
120
140
160
180
FRACTION NUMBER

Figure III-7

The effect of uroporphyrinogen III cosynthetase concentration on uroporphyrinogen III formation.

(A) Plot of percent uroporphyrinogen III formation against the amount of uroporphyrinogen III cosynthetase (Sephadex G-100 superfine step) added. (B) Reciprocal plot of A. Activities were determined as described in Methods. Similar plots were obtained for the ammonium sulfate (60-90%), DEAE-Sephacel, and folate-AH-Sepharose 4B preparations of cosynthetase.

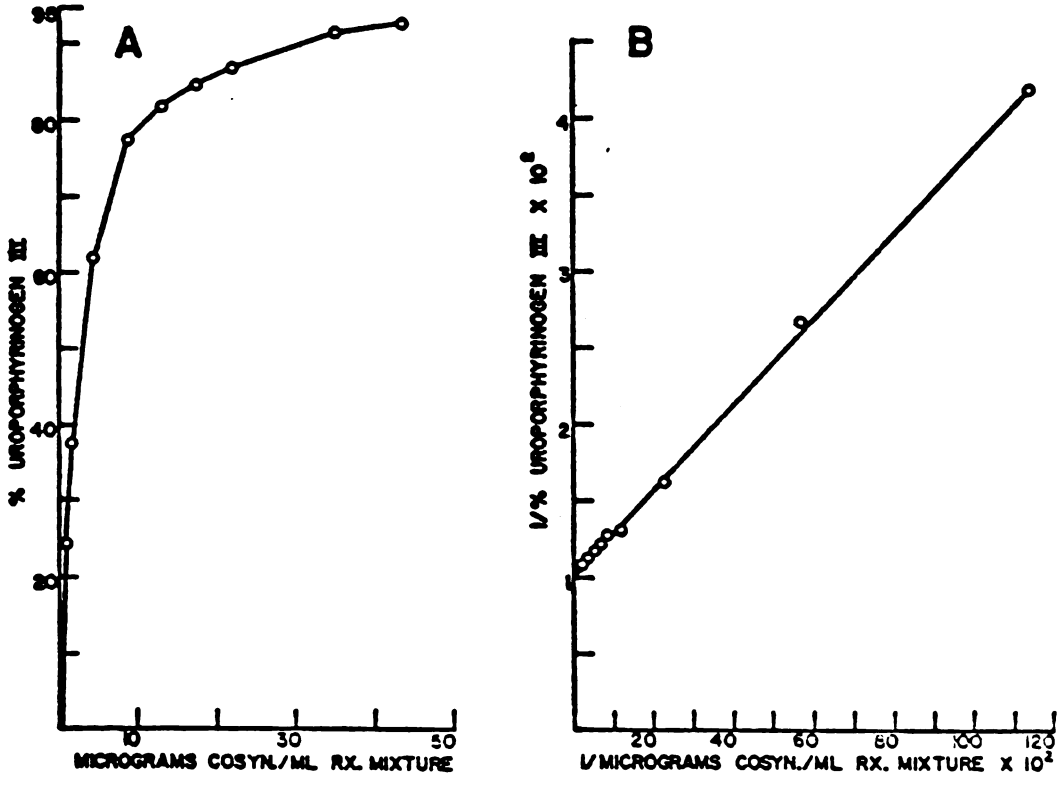

A
% UROPORPHYRINOGEN III
40
60
10
30
50
MICROGRAMS COSYN./ML RX. MIXTURE
B
1/% UROPORPHYRINOGEN III X 10²
2
4
20
40
60
80
100
120
1/MICROGRAMS COSYN./ML RX. MIXTURE X 10²

Figure III-8

The effect of the substrate porphobilinogen on rat hepatic porphobilinogenase activity and percent uroporphyrinogen III formation. ●——●, porphobilinogenase activity; ●---●, percent uroporphyrinogen III formed by porphobilinogenase. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

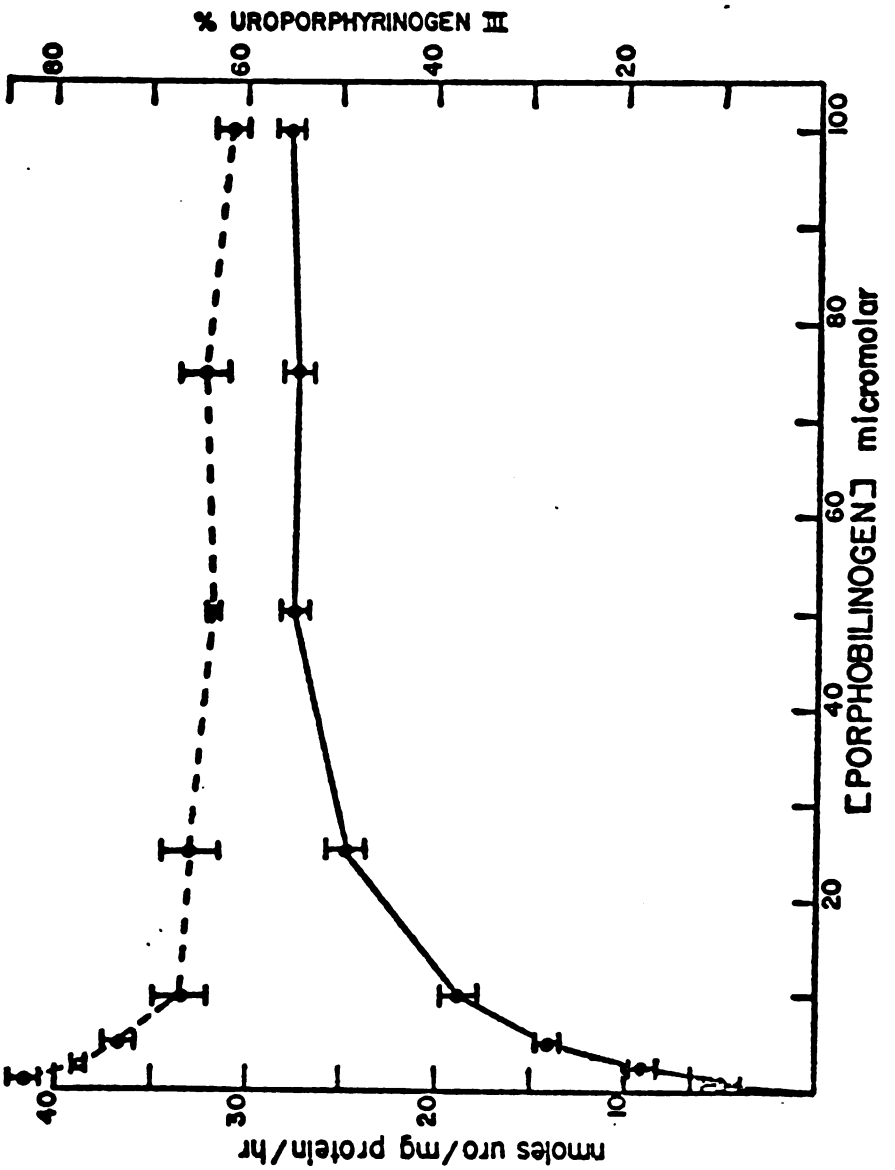

% UROPORPHYRINOGEN III
80
60
40
20
100
80
60
40
20
[PORPHOBILINOGEN] micromolar
nmoles uro/mg protein/hr
40
30
20
10

Figure III-9

Lineweaver-Burk plot for rat hepatic porphobilinogenase activity.

Each point represents the mean value of three determinations. Activities were determined as described in Methods. Reaction velocity (V) is in units of nmol uroporphyrinogen per mg protein per hour.

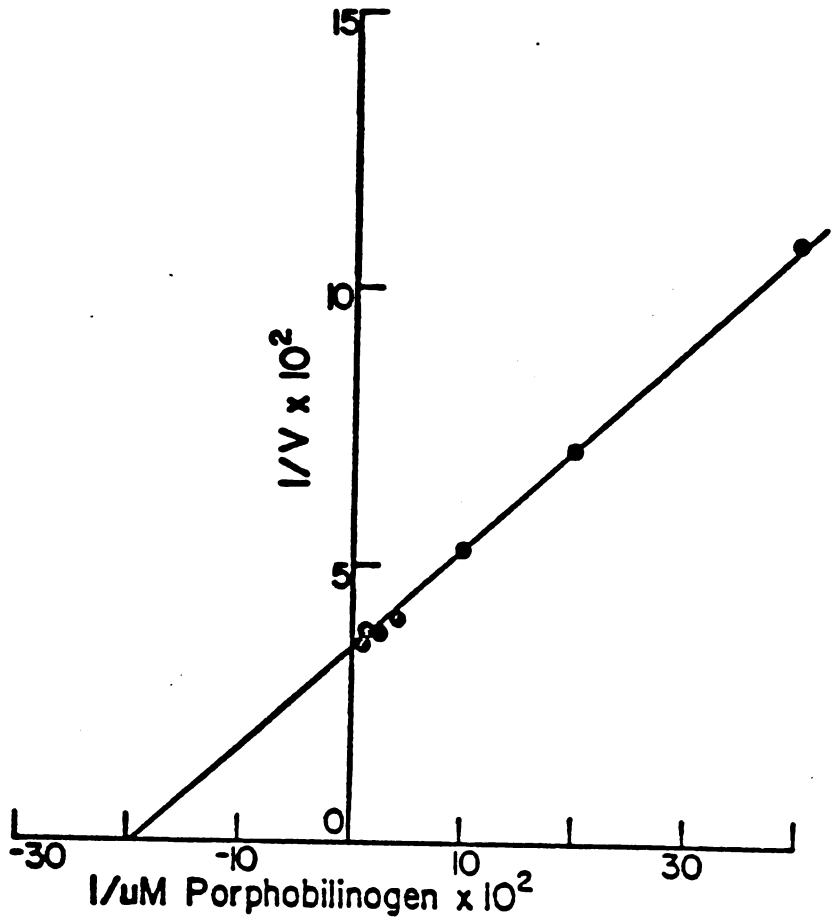

15
10
5
0
1/V x 10²
-30
-10
10
30
1/uM Porphobilinogen x 10²

Figure III-10

Time course of uroporphyrinogen III formation.

O——O, percent uroporphyrinogen III formed by porphobilinogenase. All reaction mixtures contained uroporphyrinogen III cosynthetase and uroporphyrinogen I synthetase in a ratio of 14 units COSYN per unit URO-S. Absolute amounts of enzyme were adjusted to allow for constant total product formation at each time point. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

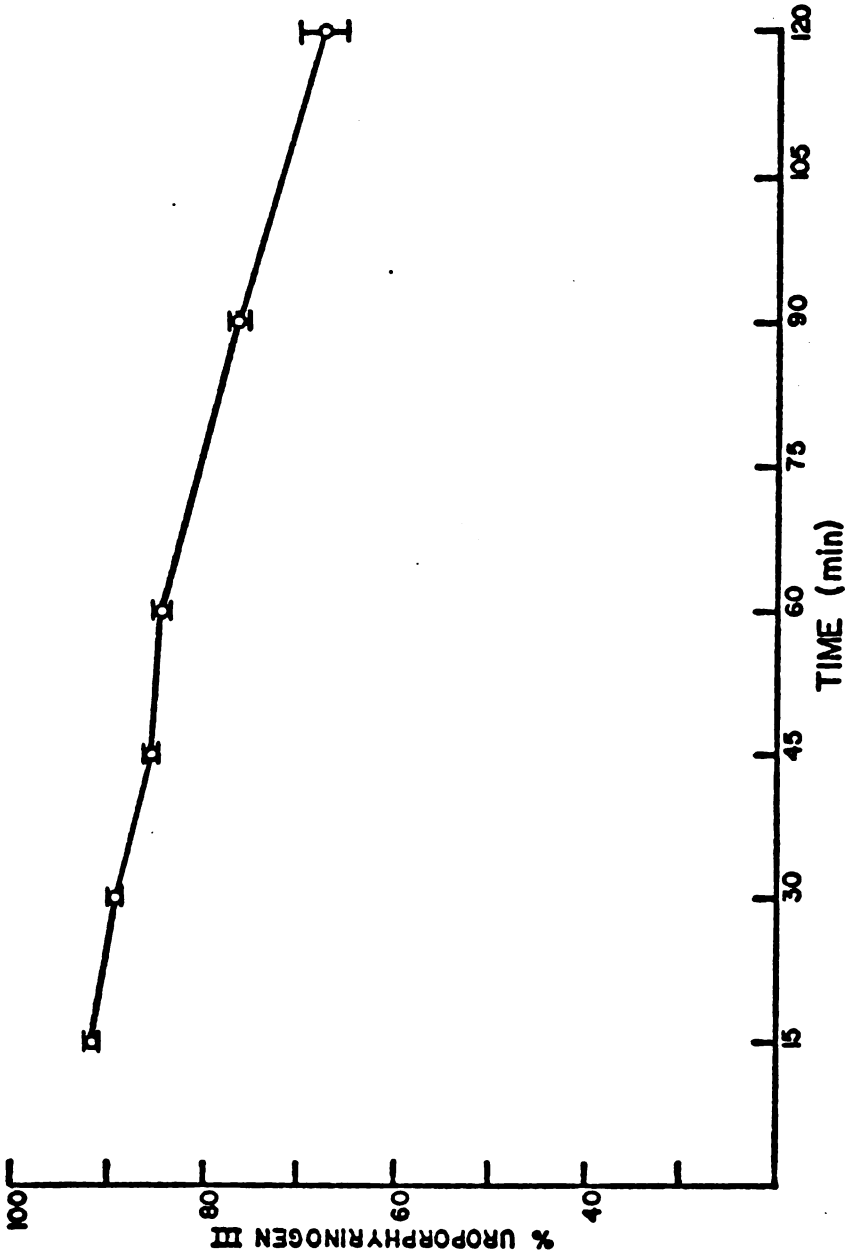

% UROPORPHYRINOGEN III
100
80
60
40
TIME (min)
15
30
45
60
75
90
105
120

Figure III-11

The effect of temperature on rat hepatic porphobilinogenase activity and percent uroporphyrinogen III formation.

●——●, porphobilinogenase activity; O——O, percent uroporphyrinogen III formed by porphobilinogenase. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

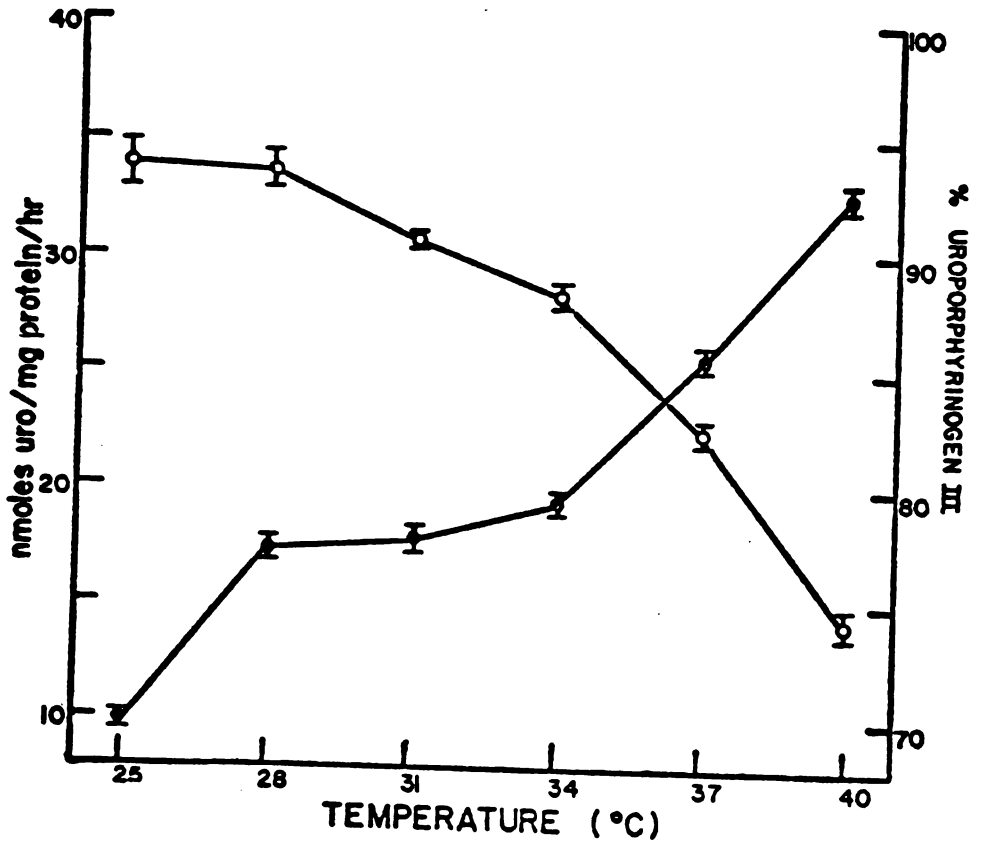

40
30
20
10
nmoles uro/mg protein/hr
100
90
80
70
% UROPORPHYRINOGEN III
25
28
31
34
37
40
TEMPERATURE (°C)

Figure III-12

The effect of pH on rat hepatic porphobilinogenase activity and percent uroporphyrinogen III formation.

●——● , porphobilinogenase activity; O——O, percent uroporphyrinogen III formed by porphobilinogenase. (A) Activities measured in 50 mM sodium phosphate buffer. (B) Activities measured in 50 mM Tris-HCl buffer. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

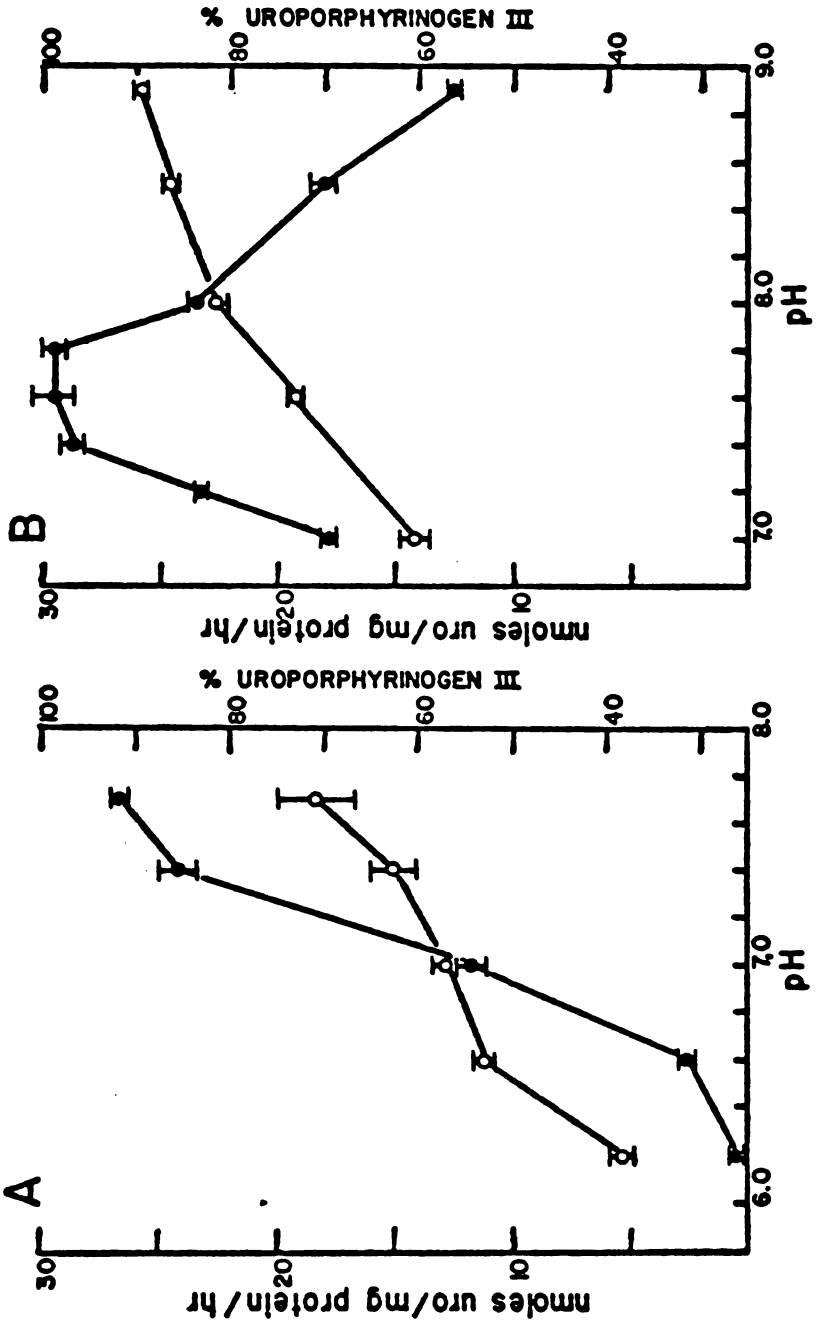

% UROPORPHYRINOGEN III
100
80
60
40
9.0
8.0
7.0
pH
B
30
20
10
nmoles uro/mg protein/hr
% UROPORPHYRINOGEN III
100
80
60
40
8.0
7.0
6.0
pH
A
30
20
10
nmoles uro/mg protein/hr

Figure III-13

The effect of potassium chloride and sodium chloride on percent uroporphyrinogen III formation.

●——●, percent uroporphyrinogen III formed by porphobilinogenase in the presence of varying concentrations of potassium chloride;

○——○, percent uroporphyrinogen III formed by porphobilinogenase in the presence of varying concentrations of sodium chloride. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

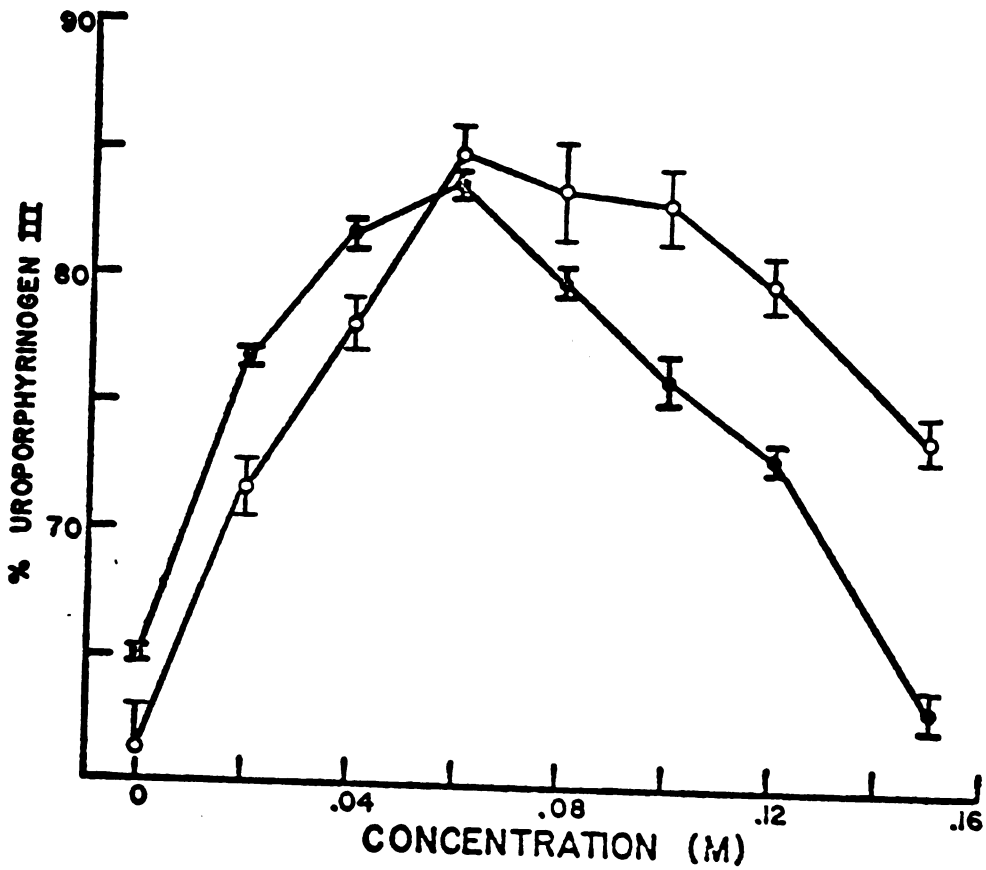

90
80
70
% UROPORPHYRINOGEN III
0
.04
.08
.12
.16
CONCENTRATION (M)

Figure III-14

The effect of magnesium chloride and calcium chloride on percent uroporphyrinogen III formation.

●——●, percent uroporphyrinogen III formed by porphobilinogenase in the presence of varying concentrations of magnesium chloride;

O——O, percent uroporphyrinogen III formed by porphobilinogenase in the presence of varying concentrations of calcium chloride. Activities were determined as described in Methods. Values are the mean ± S.E.M. of three determinations.

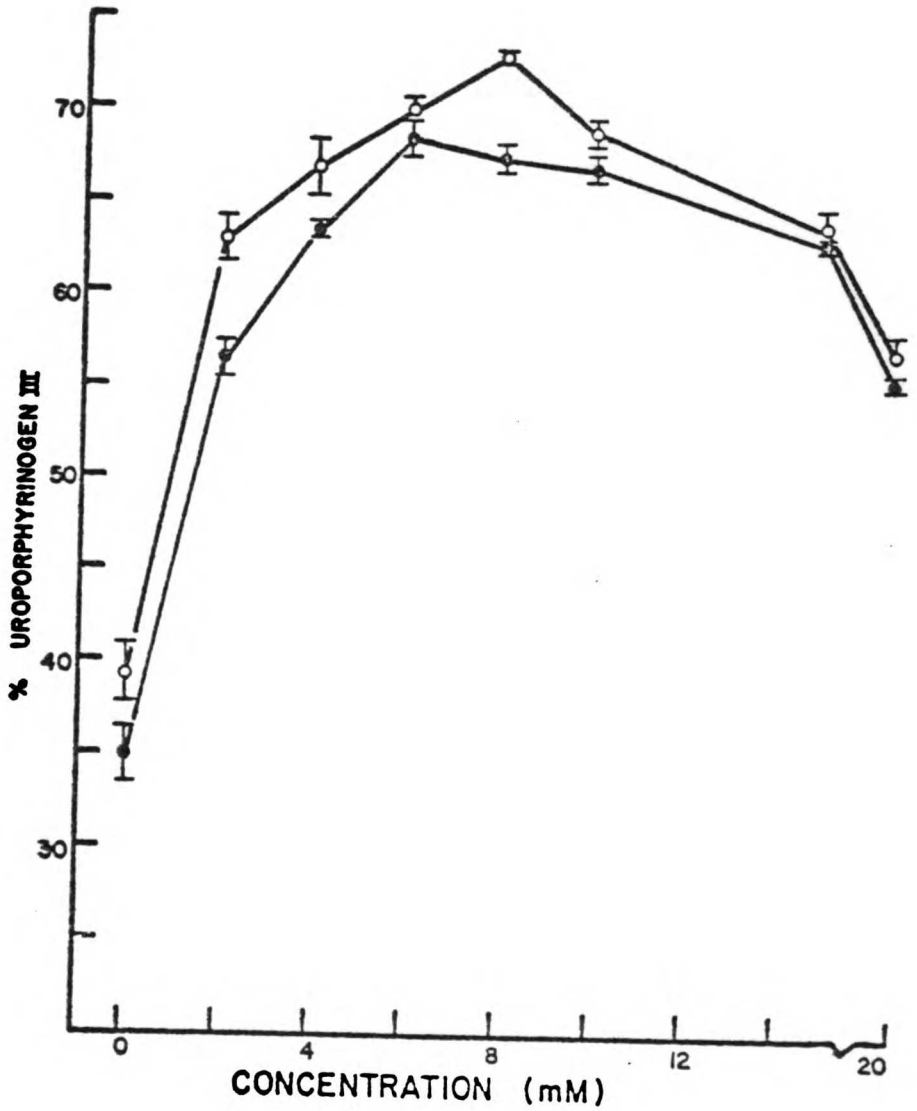

% UROPORPHYRINOGEN III
70
60
50
40
30
0
4
8
12
20
CONCENTRATION (mM)

Figure III-15

Dixon plot for determining the inhibition constant for cadmium chloride inhibition of uroporphyrinogen III cosynthetase activity.

Activities were determined as described in Methods. Each point represents the mean value of three determinations.

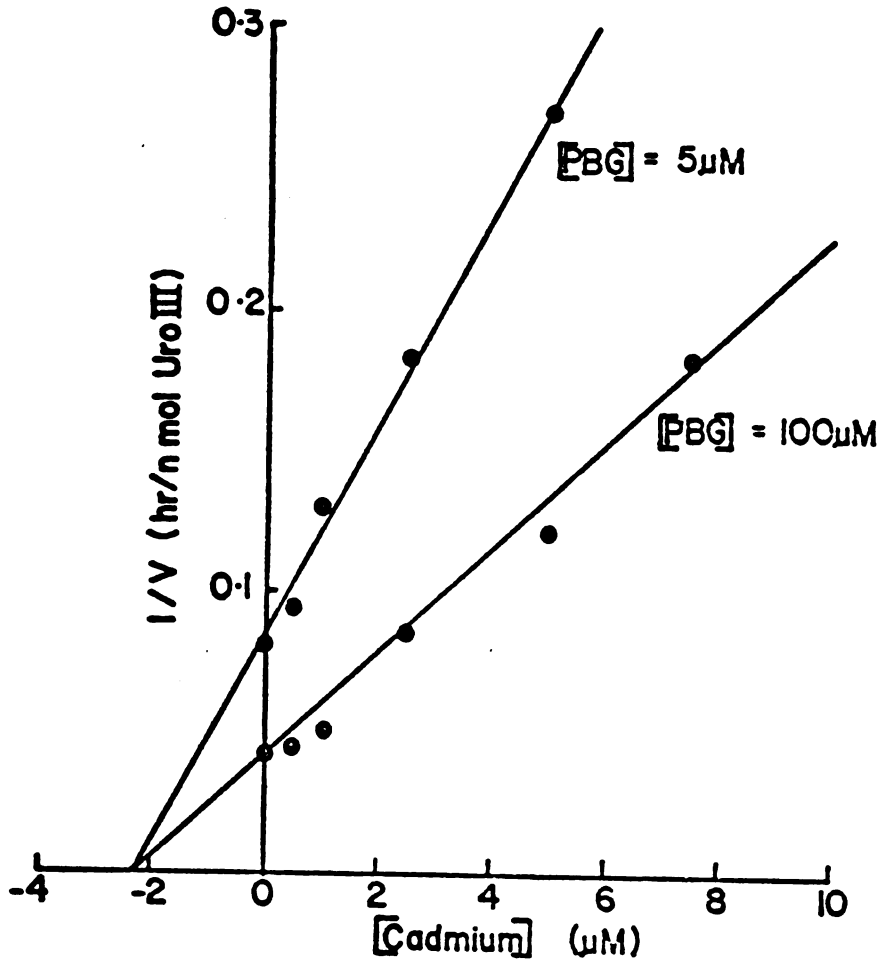

0·3
0·2
0·1
1/V (hr/n mol UroIII)
[PBG] = 5μM
[PBG] = 100μM
-4
-2
0
2
4
6
8
10
[Cadmium] (μM)

Figure III-16

Determination of the molecular weight of rat hepatic uroporphyrinogen III cosynthetase.

(A) Chromatography of purified uroporphyrinogen III cosynthetase on a calibrated column of Sephadex G-100 superfine. 400 μg of the concentrated enzyme preparation was applied to a Sephadex G-100 superfine column (2.5 x 23 cm) and eluted with 50 mM Tris-HCl, pH 7.8, buffer containing 0.2 M KCl. Fractions of 1 ml were collected. ●——●, A_{230}; O——O, uroporphyrinogen III cosynthetase activity expressed as percent of total uroporphyrin as the III isomer. Activities were determined as described in Methods. (B) Plot of relative mobility against log molecular weight for determination of uroporphyrinogen III cosynthetase molecular weight. Purified enzyme (400 μg protein) and 5 mg of each protein standard were applied to a column (2.5 x 23 cm) of Sephadex G-100 superfine. Elution of enzyme and standards was by the same procedure as described in Figure III-16(A).

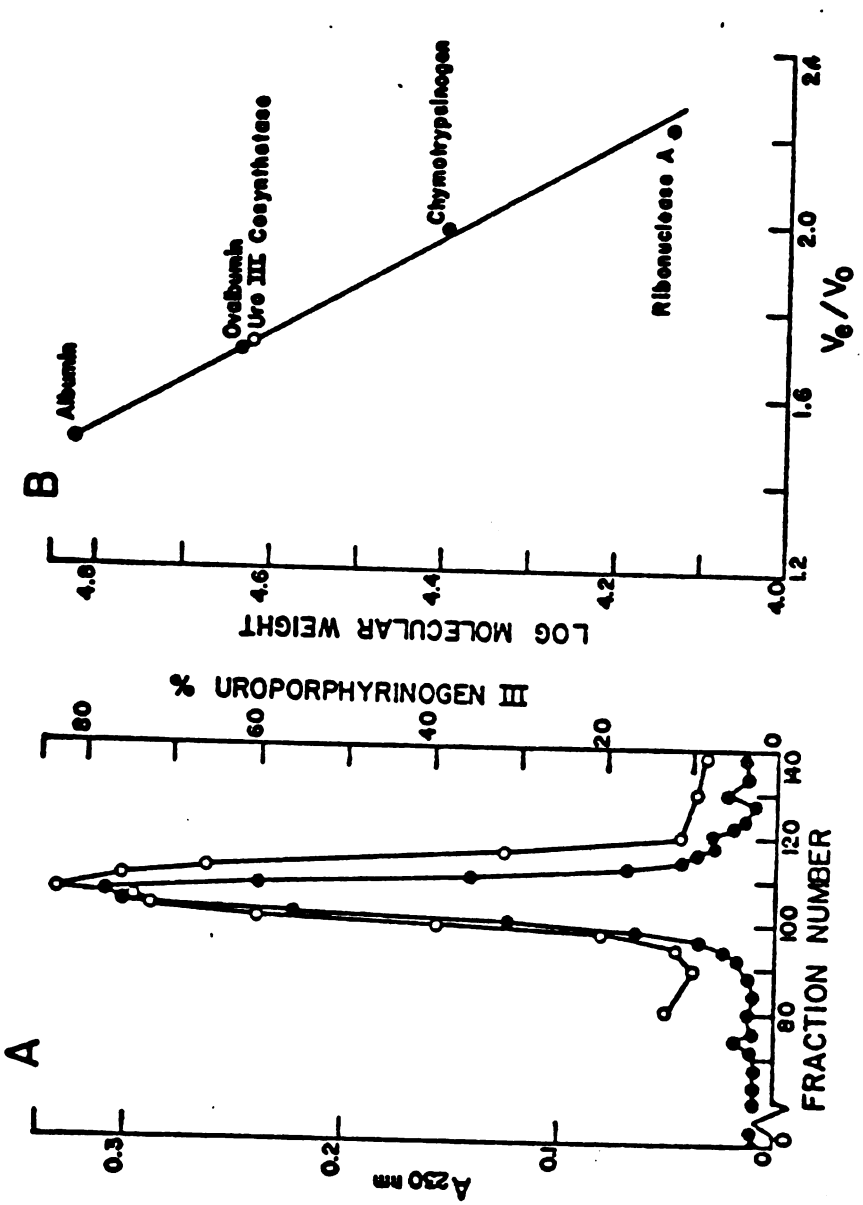

A
A280 nm
0.3
0.2
0.1
0
% UROPORPHYRINOGEN III
80
60
40
20
0
FRACTION NUMBER
20
40
60
80
100
120
140
B
LOG MOLECULAR WEIGHT
4.8
4.6
4.4
4.2
4.0
Albumin
Ovalbumin
Uro III Cosynthetase
Chymotrypsinogen
Ribonuclease A
1.2
1.6
2.0
2.4
Ve/Vo

Figure III-17

Separation of rat hepatic uroporphyrinogen III cosynthetase on analytical polyacrylamide disc gel electrophoresis.

(1) 30 µg of the Sephadex G-100 superfine preparation was applied and electrophoresed as described in Methods.

(2) 16 µg of the folate-AH-Sepharose 4B - Peak A concentrated preparation was applied and electrophoresed as described in Methods.

(3) 11 µg of the folate-AH-Sepharose 4B cosynthetase preparation (pooled, concentrated active fractions of peak B) was applied and electrophoresed as described in Methods. Both bands B_1 and B_2 had cosynthetase activity when assayed as described in Methods.

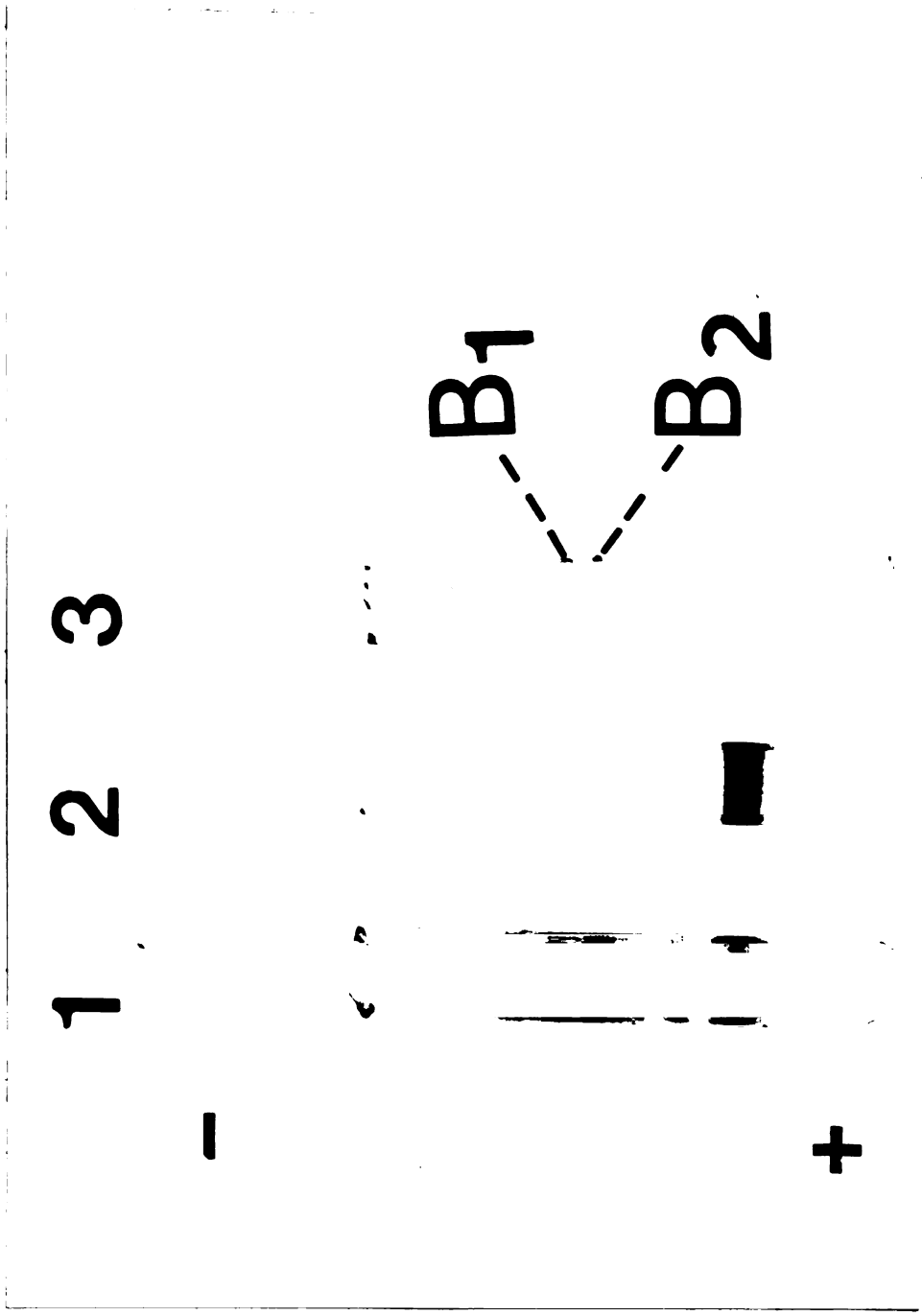
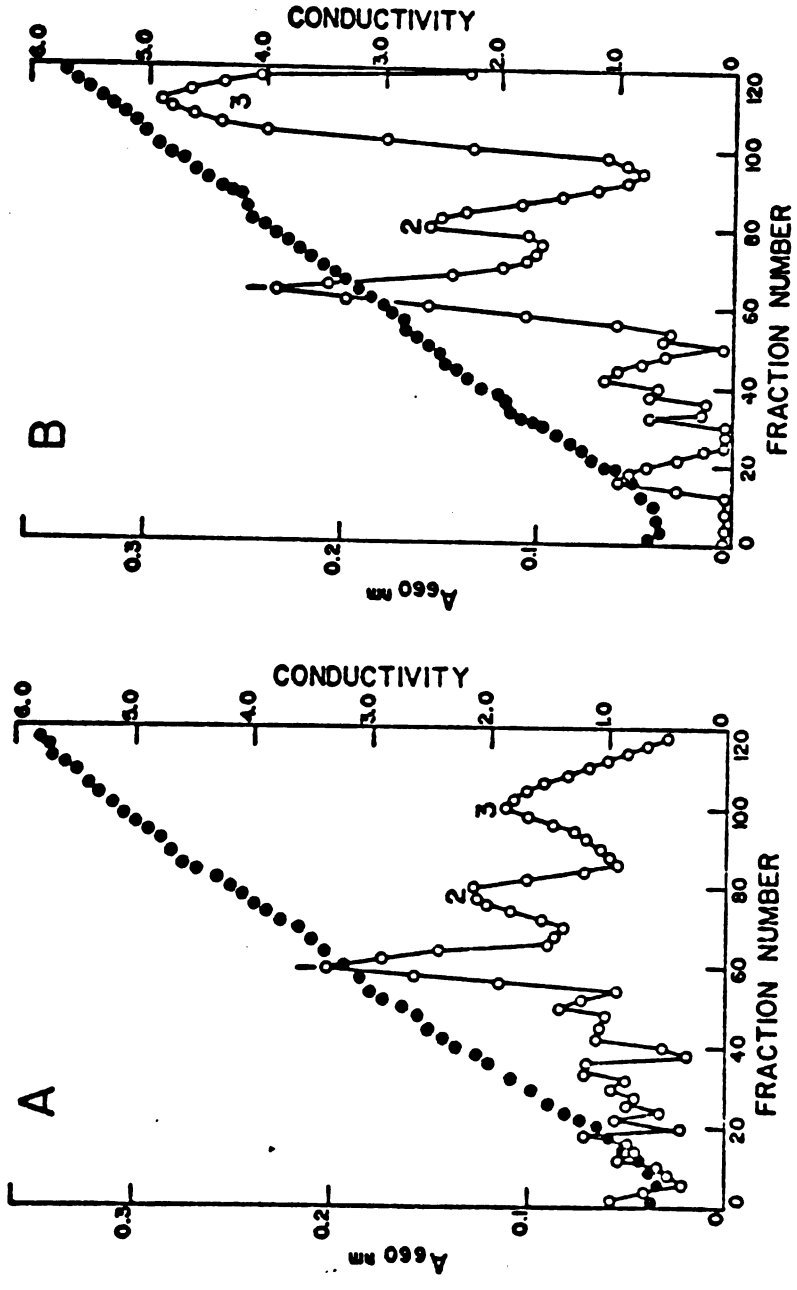

B1
B2
3
2
1
I
+

Figure III-18

Characterization of folate component of rat hepatic uroporphyrinogen III cosynthetase.

(A) Chromatography of pteroylpolyglutamate standards on a DEAE-Sephacel column (2.0 x 6.3 cm). A mixture of approximately one pmol of each standard was applied to the column and eluted with a linear gradient of 0-1.0 M KCl in 10 mM potassium phosphate buffer, pH 6.0, including 10 mM 2-mercaptoethanol. Peak 1 is the 5-CH_3-H_4PteGlu$_5$; Peak 2 is the 5-CH_3-H_4PteGlu$_6$; and Peak 3 is the 5-CH_3-H_4PteGlu$_7$. Fractions of 2 ml were collected. ● , conductivity in μmho x 10^{-4}; O——O, A_{660}. Pteroylglutamate activity of the eluted fractions was assayed after conjugase treatment by the microbiological method using L. casei as described in Methods. (B) Chromatography of pteroylpolyglutamate standards and folate component of uroporphyrinogen III cosynthetase on a DEAE-Sephacel column (2.0 x 6.3 cm). A mixture of approximately one pmol of each standard and 600 μl of the folate component dissociated from 160 μg of cosynthetase was applied to the column and eluted and assayed for pteroylglutamate activity as described under Figure III-18 (A). Peak 1 is the 5-CH_3-H_4PteGlu$_5$; Peak 2 is the 5-CH_3-H_4PteGlu$_6$; and Peak 3 is a mixture of the 5-CH_3-H_4PteGlu$_7$ and the folate component isolated from uroporphyrinogen III cosynthetase. ● , conductivity in μmho x 10^{-4}; O——O, A_{660}.


B
CONDUCTIVITY
6.0
5.0
4.0
3.0
2.0
1.0
0
3
2
1
0.3
0.2
0.1
$A_{660\ m\mu}$
0
20
40
60
80
100
120
FRACTION NUMBER
A
CONDUCTIVITY
6.0
5.0
4.0
3.0
2.0
1.0
0
3
2
1
0.3
0.2
0.1
$A_{660\ m\mu}$
0
20
40
60
80
100
120
FRACTION NUMBER

IV. DISCUSSION

Uroporphyrinogen III is the universal precursor of heme, the prosthetic group for various hemoproteins such as the mitochondrial cytochromes, and cytochrome P-450. The formation of URO III from PBG is one of the most complex steps in the heme biosynthetic pathway and has not been previously studied in rat liver. In the present study rat hepatic COSYN has been separated from URO-S and purified 73-fold with a 13% yield, providing a convenient system for the study of this important step of heme synthesis. COSYN from mouse spleen has previously been purified 18-fold with a 5% yield (Levin, 1971). COSYN has also been partially purified from bovine liver (Sancovich, Batlle, and Grinstein, 1969a), however, specific activities were not calculated.

The kinetics of rat hepatic COSYN are quite similar to those reported for COSYN isolated from mouse spleen, cow liver, Rhodopseudomonas spheroides, human erythrocyte, and soybean callus tissue preparations. The asymptote obtained from a plot of percent URO III formation against COSYN utilized supports previous data obtained in both mouse spleen (Levin, 1971) and Rhodopseudomonas spheroides (Jordan et al., 1980) tissue preparations, and suggests that PBG is converted to URO III via a coupled reaction with two enzymes, URO-S and COSYN, acting in sequence. The intermediate in this coupled reaction has, in fact, recently been isolated and characterized (Battersby et al., 1979b; Jordan and Berry, 1980). The observed K_m (5 μM) and pH optimum (pH 7.8) for PBGase, as well as the inhibition of URO III formation by increasing substrate concentration, are consistent with experimental data recorded by other investigators (Sancovich, Batlle, and Grinstein,

1969a; Llambias and Batlle, 1970; Cornford, 1964).

The decrease in percent URO III formation associated with the time-dependent loss of COSYN activity and relative instability of COSYN at higher temperatures can be minimized in the in vitro reaction mixture by maintaining a ratio of COSYN:URO-S of at least 10:1. Such a ratio of COSYN to URO-S may, in fact, approximate tissue levels of the two enzymes. An excess of COSYN has been reported to be present in crude preparations of PBGase (Batlle and Rossetti, 1977), and it may be that under physiological conditions several units of COSYN are combined with one unit of URO-S in an active complex. Stevens et al. (1968) proposed a COSYN:URO-S ratio in crude extracts of human erythrocytes of approximately 10:1. The excess of COSYN would be important in assuring the exclusive formation of URO III.

The observed activation of rat hepatic COSYN activity by NaCl, KCl, $CaCl_2$, and $MgCl_2$ may be due to the production of an enzyme complex with a more favorable structural arrangement for optimal activity as previously suggested (Batlle and Rossetti, 1977). Divalent cations as $MgCl_2$ or $CaCl_2$ might also enhance URO III formation by serving at the catalytic site of COSYN. Such a mechanism has been recently proposed to account for the effect of Mg^{+2} ion on the soybean enzyme system (Scott et al., 1976).

Studies on the inhibition of URO-S, PBGase, and COSYN by various heavy metal ions support previous data suggesting that COSYN is more sensitive to inhibition by metals than URO-S. The selective inhibition of COSYN by $CdCl_2$, $ZnCl_2$, and $CuCl_2$ at 10 μmolar concentrations may be due to metal ion interference with the formation of an active URO-S-COSYN complex. Frydman and Feinstein (1974) were able to retain

human erythrocyte COSYN on a Sepharose-URO-S affinity column, suggesting that COSYN and URO-S form a complex which is not dependent on substrate. Alternatively, COSYN may possess monothiol groups which are more exposed than are similar thiol groups of URO-S. Because the inhibition by metals is reversed by thiol compounds and is non-competitive with PBG, it is probable that the thiol groups on the enzyme are not at the active site, but are involved in maintaining the correct conformation.

The purified COSYN (folate-AH-Sepharose 4B step) eluted as a single peak on a calibrated Sephadex G-100 superfine column, and comparison with standard proteins allowed calculation of an approximate molecular weight of 42,000 daltons. The calculated molecular weight of rat hepatic COSYN (42,000) is higher than previously reported values for COSYN isolated from bovine liver (30,000) or Euglena gracilis (30,000) (Rossetti et al., 1980). These differences may reflect actual differences in the enzymes isolated from different sources, or they may result from differences in experimental conditions. Alternatively, the difference in molecular weights may be due to the degree of aggregation of COSYN as has been recently suggested by Rossetti et al. (1980). Attempts to further purify rat hepatic COSYN utilizing both anion and cation exchange resins and PBG-AH-Sepharose 4B affinity columns were unsuccessful. In each instance the protein eluted as a single peak and no further purification over that obtained using the folate-AH-Sepharose 4B affinity gel was noted.

Electrophoresis of the purified rat hepatic COSYN (folate-AH-Sepharose 4B step) showed two bands (B_1 and B_2). Each band was shown to possess COSYN activity, and the sum of the individual

activities was approximately equal to the activity of an uncut gel containing both bands. These results suggest that COSYN may exist in at least two different forms, either as two isoenzymes or as two different aggregates of lower molecular weight subunits. Alternatively, the two bands may reflect differences in the net charge of a single protein due to differences in the degree of saturation of charged cofactor binding sites. Further investigation of the subunit structure of rat hepatic COSYN will be necessary to resolve this issue.

The factor dissociated from rat hepatic COSYN stimulated the growth of folate-dependent organisms and antagonized [^{3}H]-folate binding to folate-binding protein. Treatment of the isolated factor with conjugase enhanced the growth response of the test organisms. These results suggest that a pteroylpolyglutamate derivative is associated with COSYN. It is unlikely that the isolated pteroylpolyglutamate is an impurity associated with the partially purified enzyme preparation, as it was isolated from single bands shown to possess COSYN activity which had been eluted from polyacrylamide gels. The identification of the pteroylpolyglutamate factor associated with COSYN as 5-CH_3-H_4PteGlu$_7$, based on anion-exchange elution characteristics, is tentative, and must await more rigorous methods of structure elucidation. Shin et al. (1972) have shown that the unsubstituted tetrahydropteroylpentaglutamate elutes from DEAE-cellulose at a higher ionic strength then does the N_5-methyl derivative. If the unsubstituted tetrahydropteroylhexaglutamate behaves in a similar manner, it might be expected to elute after the N_5-methyl derivative, and may even co-elute with 5-CH_3-H_4PteGlu$_7$.

Results reported here indicate that a pteroylpolyglutamate is associated with rat hepatic COSYN. If this factor is either the N_5-methyl- or other one-carbon substituted pteroylpolyglutamate, then it may function as a coenzyme in the one-carbon transfer reaction thought to occur during the intramolecular rearrangement whereby the intermediate hydroxymethylbilane is converted into URO III in the presence of COSYN (Battersby et al., 1978, 1979b, 1980; Jordan et al., 1979; Jordan and Berry, 1980). If a folate derivative is necessary for the conversion of PBG to URO III, then a depletion of tissue folate content might result in decreased heme synthesis, lowered levels of heme, and an associated decrease in drug metabolism. Folate deficiency has, in fact, been associated with decreased drug metabolism in individuals on chronic anticonvulsant therapy (Labadarios et al., 1978) and in pregnant women (Blake et al., 1978).

In the present study guinea pigs fed a folate-deficient diet showed a marked reduction in intestinal mucosal microsomal heme content and drug metabolizing activity as early as two weeks after initiation of the study. Intestinal mucosal folate levels were significantly depressed at this time. If the pteroylpolyglutamate isolated from COSYN functions as a coenzyme for URO III synthesis, then the observed depletion in tissue folate content may have led to a reduction in coenzyme concentration, diminished uroporphyrinogen formation, and depressed heme levels.

L-serine is the most efficient known source of glycine for 5-aminolevulinic acid synthetase, the first step in the synthesis of heme (Shemin et al., 1950). The conversion of serine to glycine requires folic acid in the reduced form as a coenzyme (Beck, 1974).

Therefore, folic acid deficiency may also lead to decreased heme synthesis by limiting supplies of glycine.

Alternatively, the observed decreases in drug metabolism and heme content may be due to changes in gut morphology. Hoensch et al. (1975) have shown that mature villus tip cells contain 6-10 times more cytochrome P-450 and microsomal drug metabolizing activity per milligram microsomal protein than epithelial crypt cells. Studies of folate deficiency of the intestinal mucosa in rats have shown that a folate-deficient diet leads to abnormalities of the intestinal morphology, such as elongation of the villus crypts and megalocytic changes of the surface epithelium (Goetsch and Klipstein, 1977). These observations, as well as the changes in intestinal mucosal alkaline phosphatase and NADPH-cytochrome C reductase activites noted in the present study, suggest that changes in gut morphology may be partially responsible for the depressed intestinal mucosal microsomal heme content and drug metabolism.

The decreased intestinal mucosal microsomal heme content and drug metabolizing activity associated with folate deficiency observed in this study may be extremely important from a clinical standpoint. Nutritional folate deficiency is one of the most common hypovitaminosis disorders (Blakley, 1969). Widespread folate deficiency has been detected both in elderly persons from urban low-income households (Bailey et al., 1979) and in preterm infants (Strelling et al., 1979). Folate deficiency has been associated with chronic anticonvulsant therapy, pregnancy, malignancy, hemolytic anemia, inflammatory disease, and chronic alcoholism (Eichner and Hillman, 1973; Herbert et al., 1963; Hoffbrand, 1971; Strauss, 1973). Within the last decade, folate

deficiency has been identified as the most prevalent nutrient deficiency in Canada (Nutrition Canada, 1973).

The intestinal mucosal microsomal drug-metabolizing system appears to play an important role in the metabolism of orally ingested foreign chemicals. When this system is induced, enzyme activities are comparable to those seen in the liver on a per milligram basis (Stohs et al., 1976). K_m values for biphenyl-4-hydroxylase and benzpyrene hydroxylase activity in intestinal microsomes are lower than corresponding K_m values in the liver, and may indicate a particular ability of the intestinal tissue to metabolize aromatic hydrocarbons occurring at low concentrations in the diet (Zampaglione and Mannering, 1973). Therefore, biotransformations by intestinal aryl hydrocarbon hydroxylase may constitute a protective mechanism against dietary carcinogens.

Any changes in the intestinal mucosa, whether morphological or physiological, which influence drug metabolism may compromise the ability to detoxify foreign compounds. Malnourished individuals are often more susceptible to disease than are healthy individuals, and are often in need of medication. Any change in normal gut function which alters metabolism of orally ingested medications would present another problem to the malnourished patient. Therefore, the alterations in intestinal mucosal microsomal drug metabolism associated with folate deficiency may represent a problem of potentially widespread occurrence which should be considered by the clinician or researcher in any study involving malnourished or folate-deficient individuals.

In view of the prevalence of folate deficiency today, the role of a folate derivative in the regulation of heme synthesis is of

central importance. Although the regulation of heme biosynthesis is normally governed by the activity of 5-amino-levulinic acid synthetase, the first and rate-limiting enzyme, under certain conditions other enzymes in the pathway may become rate-limiting (De Matteis, 1975). In acute intermittent porphyria, for example, URO-S is thought to become the rate-limiting enzyme in the biosynthesis of heme (Strand et al., 1972; Meyer et al., 1972), and it is also believed that COSYN activity may become rate-limiting in congenital erythropoietic porphyria (Levin, 1968a). The isolation of a pteroyl-polyglutamate factor from rat hepatic cytosol which activates URO-S (Piper and van Lier, 1977; Piper et al., 1979), and the reversal of both biochemical and clinical symptoms in AIP patients following short-term treatment sith folic acid (Wider de Xifra et al., 1980) suggest the possible regulation of the conversion of PBG to uroporphyrinogens by a folate molecule. Results of the present study suggest that a pteroylpolyglutamate is associated with COSYN. Further work will be necessary to clarify the mechanism whereby PBG is converted to URO III and the possible regulation of this step by a folate derivative. Although this issue has not been resolved, the procedures described herein for the isolation and purification of rat hepatic COSYN provide a convenient system for the study of this important step of heme biosynthesis. This in vitro system will allow future studies to be conducted in order to learn more about the regulation of COSYN and URO-S activity and their interaction with compounds of pharmacological and environmental importance.

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