Title

Studies on the regulation of lead inhibition of rat hepatic uroporphyrinogen I synthetase activity by pteridines

Author

Van Lier, Robert Bernard Leopold

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STUDIES ON THE REGULATION OF LEAD INHIBITION OF RAT HEPATIC UROPORPHYRINOGEN I SYNTHETASE ACTIVITY BY PTERIDINES

by

Robert Bernard Leopold van Lier
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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

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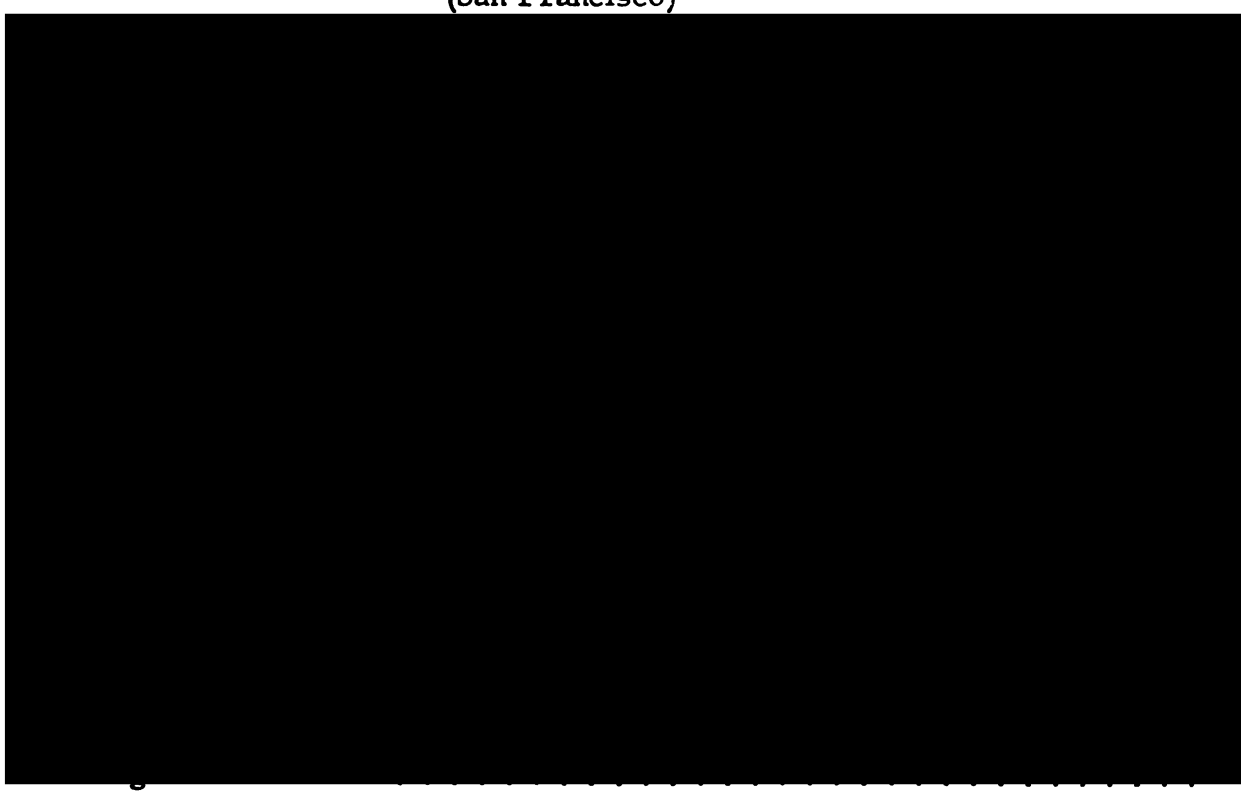

A·C·K·N·O·W·L·E·D·G·E·M·E·N·T·S .

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A B S T R A C T .

Uroporphyrinogen I synthetase (EC 4.3.1.8), an enzyme of the heme biosynthetic pathway mediates the condensation of four moles of porphobilinogen to form one mole of uroporphyrinogen I. The enzyme was purified from rat hepatic cytosol and found to be sensitive to inhibition by lead chloride. A low molecular weight, heat-stable, dialyzable factor was also isolated from rat hepatic cytosol which could protect enzymatic activity from inhibition by lead chloride. Ultraviolet and fluorescence spectra indicated that the protective factor appeared to be a pteridine derivative. Purified preparations of the factor supported the growth of the folate dependent bacteria Lactobacillus casei (ATCC 7469) and Streptococcus faecalis (ATCC 8043), and also antagonized folic acid binding to a specific folate binding protein. In addition, the factor produced stimulation of uroporphyrinogen I synthetase activity, which was antagonized by folic acid. These data suggest that the factor is a folic acid derivative. Pretreatment of the factor with γ-glutamyl carboxypeptidase destroyed the ability of the factor to protect uroporphyrinogen I synthetase activity from inhibition by lead chloride. Pteroylhexaglutamate was also able to protect enzymatic activity from inhibition by lead chloride.

Pteroyltriglutamate or various pteroylmonoglutamate derivatives were incapable of protecting against inhibition of enzymatic activity by lead chloride. These data indicate that the factor is a pteroylpolyglutamate derivative. The microbiological and competitive protein binding assays indicate that picomolar quantitites of the factor were able to protect uroporphyrinogen I synthetase activity from inhibition by 10^{-5}M lead chloride which indicates that the factor is not merely chelating lead. Factor was unable to protect against inhibition of 5-aminolevulinic acid dehydratase (EC 4.2.1.24) by lead chloride, which also indicates that the factor does not act by chelation.

It was concluded that the factor was a pteroylpolyglutamate which specifically interacts with uroporphyrinogen I synthetase to protect enzymatic activity from inhibition by lead chloride. This specific interaction and the ability of the factor to stimulate enzymatic activity suggests that the pteroylpolyglutamate may regulate uroporphyrinogen I synthetase activity and serve as a coenzyme for this enzymatic reaction of the heme biosynthetic pathway.

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L I S T O F T A B L E S

L I S T O F F I G U R E S

ABBREVIATIONS:

ALA ---	5-Aminolevulinic acid
PBG ---	Porphobilinogen
Uro ---	Uroporphyrinogen
OPT ---	o-Phthalaldehyde
PteGlu ---	Pteroylmonoglutamate, folic acid, folate.
H_2PteGlu ---	7,8-Dihydropteroylmonoglutamate, dihydrofolate, dihydrofolic acid.
H_4PteGlu ---	5,6,7,8-Tetrahydropteroylmonoglutamate, tetrahydrofolic acid.
5-formyl-H_4-PteGlu ---	N^5 formyltetrahydropteroylmonoglutamate, 5- formyltetrahydrofolic acid, leucovorin, citrovorum factor, folinic acid
5,10,methylene-	N^5 ,N^{10} -methylenetetrahydropteroylmono-

H_4PteGlu ---	glutamate. 5,10. methylenetetrahydrofolic acid.
5- methyl-H_4-PteGlu ---	N^5 methyltetrahydropteroylmonoglutamate, N^5 methyltetrahydrofolic acid.
10-formyl-H_4-PteGlu ---	N^{10} formyltetrahydropteroylmonoglutamate. N^{10} formyltetrahydrofolic acid.

I. INTRODUCTION.

A. General considerations of lead intoxication.

1. History of lead use and poisoning.

The toxicity of lead compounds has been known since antiquity. The ancient Egyptians used lead compounds as homicidal agents, and some historians have claimed the Roman empire collapsed because of the ingestion of Pb^{+2} from the water in lead pipes. Physicians of the middle ages used various lead compounds therapeutically for almost every possible disease. In the 15th and 16th centuries metallic lead or lead acetate were used as sweeteners in wine which resulted in many deaths and disabilities (de Treville, 1964). This practice was later outlawed and made punishable by death. With the increasing awareness of the adverse effects of lead, it was shown that use of heated lead type by printers was responsible for the loss of coordination in their fingers. The "dry gripes" (i.e., colic - acute lower abdominal pain) in pottery and glass workers were also shown to be related to lead exposure. In the United States automobile radiators containing lead solder have been used for the distillation of illicit liquor (moonshine). Potentially dangerous concentrations of lead have been encountered in whiskey distilled through these radiators.

Currently, lead intoxication is a problem for workers during the smelting and refining of the metal, in the printing industry, and in the production of storage batteries. The National Institute of Occupational Safety and Health has recommended a threshold limit value of inorganic lead fumes and dusts of 0.15 mg/m3 for ambient working environments.

Perhaps the greatest single source of modern day environmental lead contamination has been the use of tetraethyl lead (TEL) as an antiknock agent in gasoline. In the past, gasoline has contained as much as 5.0 grams of TEL/gal. With the increasing use of motor vehicles, lead intoxication has begun to appear in non-occupationally related cases. Persons living close to freeways and other areas of heavy traffic may have substantially higher blood lead levels than those living in remote areas (Kehoe, 1961).

2. Absorption, distribution and excretion of lead:

Absorption of lead is primarily by either the gastrointestinal or respiratory tract. With the aid of human volunteers, Kehoe (1964 a) has estimated that of the normal daily intake of lead (350 ug), 310 ug are derived from food, 20 ug from water and 20 ug from air. Approximately 5-10% of orally ingested lead is absorbed via the gastrointestinal tract. Gastrointestinal absorption can

be greatly affected by dietary calcium and vitamin D levels. A high level of calcium in the diet may reduce lead absorption; whereas, either a high level of vitamin D or low level of calcium in the diet may increase absorption (Hammond, 1969).

While the intake of lead from the air is much less than that from food, the contribution toward absorption is approximately equal (20-30 ug/day). Respiratory absorption is largely dependent on particle size. If 90% of lead containing particles have a diameter between 0.01 um and 0.1 um, nearly all of the lead retained is absorbed. However, when the mean particle diameter is 0.9 um, which is more typical of urban air, only 40% of the retained lead particles is absorbed. The remaining larger particles are moved up the respiratory tree by cilia and swallowed, thus becoming a source of orally absorbed lead (Kehoe, 1961; Robinson, 1966). The volume of inhaled air can also greatly influence absorption. Thus a laborer in a lead smelter might receive a greater percentage of his daily lead exposure by respiration than would an infant confined to a crib. Under normal conditions 90% of the lead body burden in adults is contained in bone; whereas, in children under the age of six years, bone contains only 64% of the total body burden (Barry and Mossman, 1970). Although this sequestered pool is normally harmless, mobilization of lead by acidosis, alcoholism or bone fracture can produce a

significantly higher blood and soft tissue level and precipitate symptoms of lead intoxication. Although Kehoe (1961) has concluded that the normally exposed adult is in equilibrium, he has shown that with an increased exposure to lead the total body burden will tend to rise without maintaining a new plateau. In contrast, the exchangeable body burden as measured by blood lead levels seemed to reach a plateau (Kehoe, 1964 a,b). This points out the inherent fallacy of presuming blood levels of agents such as lead to be indicative of total body burden and potential toxicity.

Red blood cells have a higher affinity for lead and will contain as much as 95% of the total blood lead (Butt et al., 1964). However, this pool of lead is readily exchangeable with both soft tissue and bone. Approximately 68% (ca. 36ug) of the blood lead is excreted into the urine, principally by glomerular filtration (Rabinowitz et al., 1976). This implies a rapid equilibration between intercellular lead and the freely filterable form. When lead exposure is excessive, tubular excretion becomes an important mode of renal excretion (Vostal and Heller, 1968), but is limited to 150 ug/day (Browder et al., 1973). Although feces may contain more than 90% of the oral daily lead intake, only 1% or less of this lead is from the bile and represents excretion of previously absorbed lead. The remaining absorbed lead (32%) is secreted into the hair,

sweat, nails, saliva and sputum and accounts for the remaining 12 ug of lead. (Rabinowitz et al., 1976).

3. Clinical manifestations and diagnosis of lead intoxication.

Clinical symptoms of lead poisoning include anemia, abdominal colic, acute or chronic encephalopathy, peripheral neuropathy and nephropathy.

Lead-induced anemia is usually either hypochromic or microcytic with a reduced red cell life-span, reticulocytosis and often basophilic stippling (Waldron, 1966). Anemia due to lead poisoning has many features similar to iron deficiency anemia. However, bone-marrow preparations show a higher than normal number of sideroblasts which is the opposite of that seen in the iron-deficient state (Griggs, 1964). Signs include pallor, a waxy shallow complexion, irritability and headache. Although these signs are typical of other chronic anemias, infants and children with lead-induced anemia often also suffer from acute abdominal colic.

Colic is manifested by acute abdominal pain, and includes headaches, generalized muscle aches and constipation. As symptoms grow more severe, vomiting, anorexia and weight loss occur. Although these symptoms may

be so severe that a laparotomy will be performed, they may abate spontaneously if exposure is eliminated (NAS, 1972).

Probably the most insidious and life-threatening symptom is acute encephalopathy. While other symptoms are relatively mild, acute encephalopathy can occur spontaneously and lead to death within a few days due to chronic intractable seizures, coma, and cardio-respiratory arrest (Chisolm, 1968; NAS, 1972). Pathologically, the most extensive neuronal injury occurs in the cerebellum, although cell damage can be observed throughout the entire brain. Direct vascular damage appears to be the underlying cause of the increased cerebral edema and increased intracranial pressure. The signs of chronic encephalopathy occur gradually and are less severe than in the acute disease. They include loss of speech and motor coordination with a poorly controlled convulsive disorder. These symptoms occur most often in children with a long history of lead absorption (Chisolm, 1968). Patients who have survived the convulsions and pains of acute encephalopathy have complained of muscle weakness and lack of coordination after the pains have subsided. This peripheral neuropathy which usually predominates in the extensor muscles of the limbs is due to demyelination and axonal degeneration of the associated motor nerves.

Nephropathy may also occur in adults with a long history of lead exposure. Symptoms associated with nephropathy may include progressive azotemia, glycosuria, hyperphosphaturia and hyperuricemia, which may produce gout. Epidemiological evidence has been presented that children who were exposed to lead at an early age developed nephritis later in life with a higher than normal incidence of deaths due to renal failure (Emmerson, 1963; Tepper, 1963). Nephrosclerosis due to lead occurs mostly in older patients.

Clinical diagnosis of lead intoxication is based largely on a proven history of industrial exposure for adults or usually by a history of pica for children. In addition, evidence of anemia with basophilic stippled cells or an abnormal blood lead level (i.e., greater than 80 ug/100 ml for adults or 40 ug/100 ml for children) can be used as clinical evidence for intoxication (Lin-Fu, 1972). Increased urinary excretion of coproporphyrin in excess of 0.2 mg/l or of 5-aminolevulinic acid (ALA) in excess of 2.0 mg/l are also indicative of excessive lead absorption. An infrequently used sensitive and specific test for diagnosis of lead poisoning is the measurement of blood ALA dehydratase (EC 4.2.1.24) activity. This enzyme is very sensitive to inhibition by lead and is ultimately responsible for the increased urinary excretion of ALA during lead poisoning. In children, X-ray examination of long bones such as the femur may also show evidence of lead

deposition around the metaphyses. This has also been used as diagnostic evidence for excessive juvenile lead exposure.

4. Lead intoxication in children.

Although lead intoxication in adults has been well studied, the adverse effects in children have gone largely unnoticed until recently. Nevertheless, children are by far more vulnerable to its harmful effects. A survey of 21 screening programs comprising 344,657 children who lived in poorly maintained urban areas showed that as many as one-third of the subjects had blood lead levels of 40 ug/100 ml or higher. Such levels are indicative of excess lead absorption and may be associated with clinical symptoms of intoxication (Gilsinn, 1972). Much of their lead absorption was due to the eating of peeling chips from walls covered with lead based paints which contained from 5-40% lead by weight.

In more recent studies, an increasing number of children who showed increased lead absorption were not from older housing in the inner cities or from houses which had lead painted walls, but were from newer suburban and rural homes. Toothpaste and toothpaste tubes can pose a threat to children if they chew on the tube or eat toothpaste which has leached lead from an improperly coated tube. Children may also chew on pencils painted with lead based paints or

newspapers which are printed with lead inks. Evaporated milk cans with lead sealed seams or improperly glazed earthenware have also been insidious sources of childhood lead exposure.

A major source of extremely high lead content which reflects the deposition of spent TEL from gasoline combustion is street dirt and surface soil. Occasional lead levels of 1.0 - 1.2% of surface soil have been reported for heavily traveled urban streets (Lin-Fu, 1973). Dust samples from Boston homes were found to contain 0.1 - 0.2% lead which is also typical in other areas. Over 90% of airborne urban lead is due to the combustion of TEL contained in gasoline.

Evidence has also been accumulating that children are more susceptible to the toxic effects of lead. The relative inefficiency of both metabolic and excretory pathways along with a less well developed blood-brain barrier may partially explain this greater susceptibility. Deficiencies in dietary iron, calcium and protein coupled with an increased gastrointestinal absorption of lead have also been cited as contributing to a greater risk in children (Lin-Fu, 1973).

B. Heme biosynthesis in relation to lead toxicity.

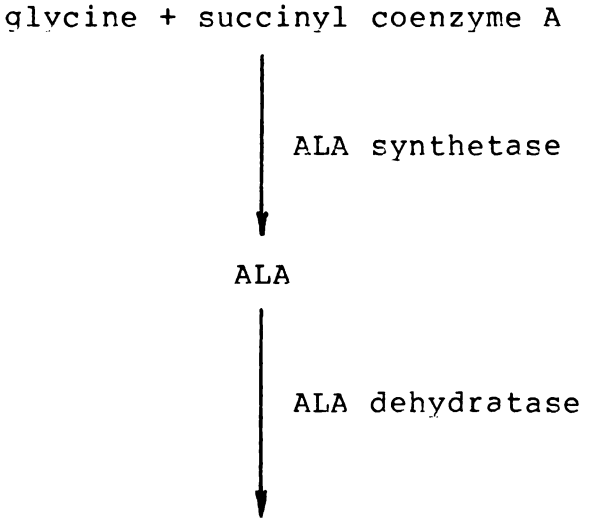
Heme is an essential prosthetic group which is present in a wide variety of proteins including hemoglobin, myoglobin, most cytochromes, peroxidase and catalase. Because of this central role as a prosthetic group for these proteins, it is apparent that the lack of heme caused by inhibition of the enzymes responsible for heme synthesis would have disastrous consequences on the metabolism and general biochemical processes of an organism.

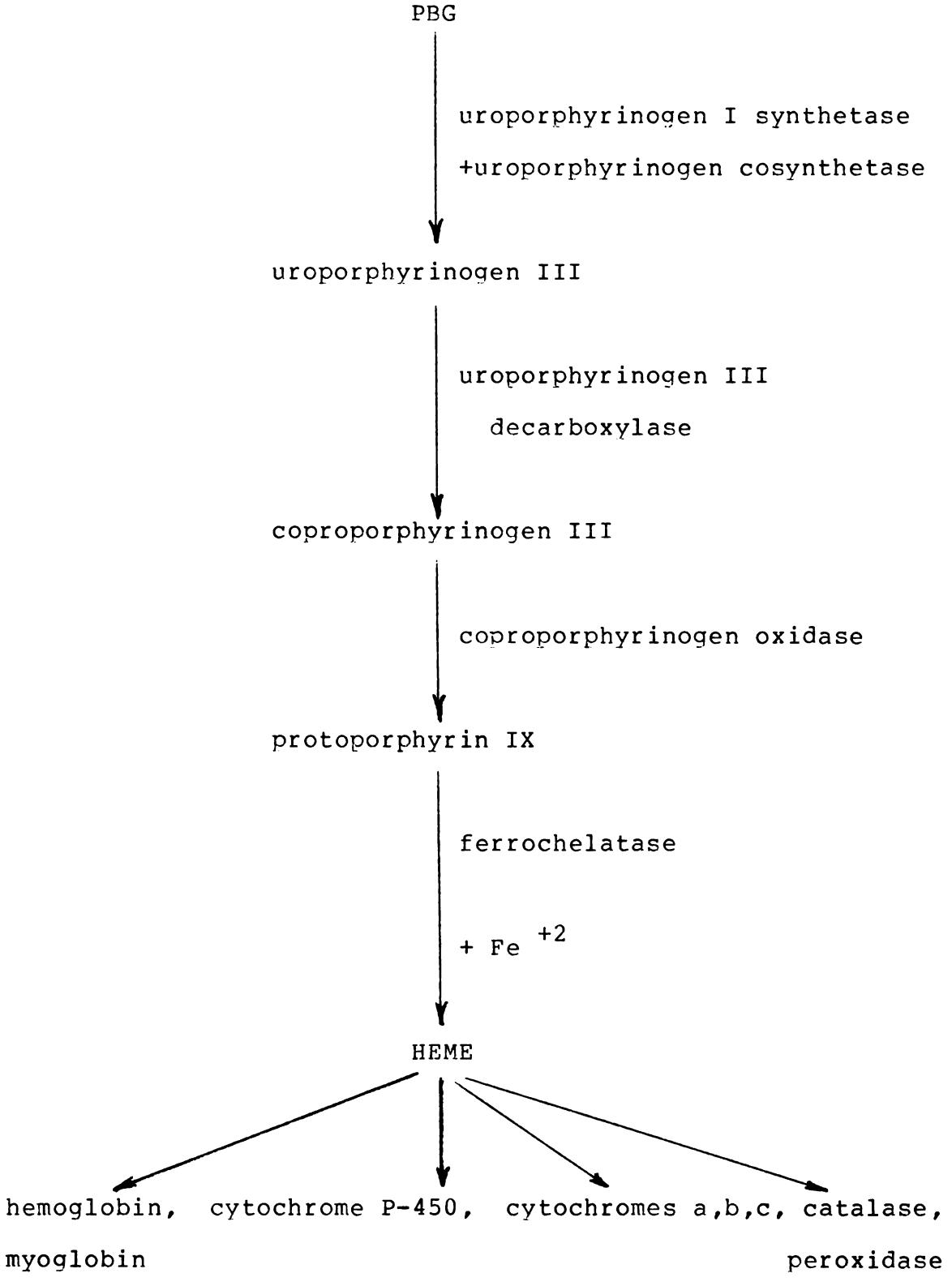
1. Review of heme biosynthesis

The relationship between the work to be presented and lead toxicity can best be understood by a brief review of the heme biosynthetic pathway. It is generally accepted that because of the ubiquitous presence of heme in a wide variety of organisms, both plant and animal, that its synthesis must be very similar throughout nature. Although the molecular weights of the enzymes and other physical properties may vary between species, the overall reactions have proven to be similar. A diagram of the heme biosynthetic pathway is illustrated below.

The first reaction whose product is committed to the synthesis of heme is the condensation of glycine and succinyl coenzyme-A to form 5-aminolevulinic acid (ALA). The reaction is mediated by the rate-limiting mitochondrial enzyme ALA synthetase (EC 2.3.1.37).

The ALA passes out of the mitochondrion and two molecules are combined to form one molecule of porphobilinogen (PBG) by the enzyme ALA dehydratase. Four molecules of PBG condense under the action of uroporphyrinogen I synthetase (EC 4.3.1.8) and uroporphyrinogen cosynthetase to form one molecule of uroporphyrinogen III (uro III). It is this reaction which is the subject of this thesis and past work will be reviewed later in this section. Uroporphyrinogen III is then sequentially decarboxylated to form coproporphyrinogen III by uroporphyrinogen decarboxylase (EC 4.1.1.37). Coproporphyrinogen III then enters the mitrochondrion where the enzyme coproporphyrinogen III oxidase (EC 1.3.3.3) converts two of the remaining four propionic acid side-chains to vinyl groups to form protoporphyrin IX. Ferrochelatase (EC 4.99.1.1) then mediates the insertion of the ferrous ion into protoporphyrin IX to form the final product, heme.




PBG
uroporphyrinogen I synthetase
+uroporphyrinogen cosynthetase
uroporphyrinogen III
uroporphyrinogen III
decarboxylase
coproporphyrinogen III
coproporphyrinogen oxidase
protoporphyrin IX
ferrochelatase
+ Fe +2
HEME
hemoglobin, myoglobin
cytochrome P-450,
cytochromes a,b,c,
catalase, peroxidase

The underlying basis of lead-induced anemia is almost certainly the direct biochemical inhibition of several of the heme biosynthetic enzymes. Of the six enzymatic steps involved in heme biosynthesis, two have been shown to be affected by lead. Inhibition of ALA dehydratase is responsible for the increased ALA found in both urine and blood. Similarly inhibition of ferrochelatase is responsible for the increased protoporphyrin IX found in the red blood cells and in urine (Tephly et al., 1971). Excretion of coproporphyrin has long been recognized as an early warning of lead intoxication. However, it is uncertain whether this is directly due to inhibition of coproporphyrinogen oxidase.

2. Evidence for increased PBG excretion during lead intoxication.

In severe cases of lead intoxication there is an increased urinary excretion of PBG associated with its accumulation in tissues. PBG excretion in non-lead exposed adults is less than 1.0 mg/l, and values greater than 1.5 mg/l are considered the upper limit of normal. Evidence for increased excretion of PBG was first presented by Watson, Hawkinson and Bossenmair (1953) and Bashour (1954). Haeger (1957) studied 20 industrial lead workers and reported that four showed elevated urinary excretion of PBG. de Kretser and Waldron (1963) studied 100 lead pottery workers and

reported that 20 had urinary PBG excretion values exceeding 1.0 mg/l and two above 2.0 mg/l.

Gibson, MacKenzie and Goldberg (1968) studied three groups of lead workers. The first group contained 15 workers with a "presymptomatic state of lead exposure". A second group of 30 workers had "mild symptoms such as mild anemia", and a third group of 16 workers had "severe signs and symptoms" of lead intoxication. Five of their patients in group 2 had PBG excretion levels greater than 1.5 mg/l as did 13 of the 16 in group 3. None of the workers in group one had PBG excretion levels exceeding 1.5 mg/l.

None of these studies reported procedures for storage of urine samples. This is important since PBG in urine is unstable unless frozen and may spontaneously form porphyrins leading to an underestimation of PBG excretion. These studies also did not report the daily urinary PBG excretion. Daily urine volumes differ among various individuals (Diem and Lentorrer, 1972) and may also change during lead intoxication as well as physical exercise.

Gibson and Goldberg (1970) studied the excretion of heme precursors in lead intoxicated rabbits. Urinary coproporphyrin levels became elevated within 48 hours after the first dose of lead. ALA levels were not elevated until after the seventh day, while PBG levels were not elevated

until after the 20th day. The mean urinary excretion level of PBG was 9.90 $\pm$ 1.30 mg/l in the lead poisoned group as compared to 1.20 $\pm$ 0.20 mg/l in the control group. Both coproporphyrin and ALA excretion were also elevated. Brain, liver, kidney and bone-marrow were also examined for increased levels of heme precursors. While there was only a statistical elevation of kidney PBG content, the other tissues also appeared to have elevated levels. These authors should have reported their percent recovery of PBG from tissue. Since it is quite difficult to extract PBG from tissue samples, it is likely that the tissue PBG concentrations reported are low. ALA levels were also elevated in liver, kidney and bone-marrow. Tissue lead levels were reported for brain, liver and kidney. When expressed in equivalent molarity for wet tissue, the tissue lead concentrations were 1.13×10^{-5} M, 6.17×10^{-5} M and 1.45×10^{-4} M for brain, liver and kidney, respectively.

Although these studies do not indicate that there is a massive excretion of PBG during lead intoxication, they do suggest that uroporphyrinogen I synthetase, the enzyme which utilizes PBG may be inhibited. The Km of uro I synthetase is the second lowest (ca. 10^{-6}M) of the six heme synthetic enzymes (De Matteis, 1975). During periods of increased heme demand, ALA synthetase levels increase in an attempt to compensate for the heme deficit. However, if a second enzyme such as uro I synthetase is inhibited to a rate

slower than any of the other enzymes of the heme pathway, it will become the new rate-limiting enzyme. Thus, inhibition of uro I synthetase activity in periods of increased heme demand would be expected to result in enhanced urinary PBG excretion and possible impairment of heme production.

C. Review of past studies with uroporphyrinogen I synthetase:

Uroporphyrinogen I synthetase is responsible for the condensation of four moles of PBG to yield one mole of uroporphyrinogen I (uro I) and was first isolated from spinach leaves by Bogorad (1958 a,b,c). Since then numerous reports have appeared describing a possible mechanism for the reaction (see figure 1). While it is relatively easy to depict a mechanism for isomer I formation, it is much more difficult to visualize how the biologically active isomer III is formed. Bogorad (1958b) isolated from wheat germ a specific protein, uroporphyrinogen cosynthetase, which promoted formation of the correct uro III isomer when incubated with uro I synthetase. Uro III cannot be formed by incubation of uro I with the cosynthetase. Uro I synthetase has been isolated from a number of different sources and the enzyme from human erythrocytes has a reported molecular weight of 25,000 $\pm$ 5000. In the course of product formation, 4 moles of ammonium ion are produced which at high concentrations (0.01M) can act as a non-

competitive inhibitor. Divalent metal cations such as Hg^{+2}, Cd^{+2}, Sr^{+2}, Mg^{+2} and Ca^{+2} have also been identified as inhibitors (Frydman and Frydman, 1970; Frydman, 1973). Frydman (1973) reported that 25mM lead was not an inhibitor. However, phosphate buffer was used in the reaction mixture which is known to form insoluble lead phosphate. Piper et al., (1976) demonstrated that $PbCl_2$ was a very potent non-competitive inhibitor for purified rat hepatic and erythrocytic uro I synthetase activities.

In order to study the mechanism of the conversion of PBG to uroporphyrinogen, previous investigators have synthesized possible intermediates and measured their incorporation into the porphyrin ring. Bogorad's group (Plusec and Bogorad, 1970; Radmer and Bogorad, 1972) have studied the incorporation of a dipyrrylmethane and a tetrapyrrylmethane into uro III. Although both of these substances seemed to be appropriate substrates, neither produced significant incorporation into the porpyrin ring. These data suggest that free dimers and tetramers of PBG are not intermediates of the reaction. Several other groups (Battersby et al., 1977 a,b,c; Frydman et al., 1976; Scott et al., 1976; Frydman and Frydman, 1975; Frydman et al., 1975) have also studied the incorporation of bilanes (straight chained PBG tetramers). However, none of these derivatives has convincingly explained the ring rearrangement to form the uro III isomer.

Ammonium ion, hydroxylamine and methoxamine have been used extensively in the study of uroporphyrinogen formation. Both ammonium ion and hydroxylamine can be incorporated into the nascent uroporphyrinogen molecule and will be released upon cyclization and formation of the tetrapyrrole ring (Frydman and Frydman, 1975)

Sancovich, Battle and Grinstein (1969 a,b) studied uro I synthetase from bovine liver. They showed that ammonium ion could act as a non-competitive inhibitor (Ki=0.172 M) for uroporphyrinogen I synthetase but was a competitive inhibitor for the cosynthetase. The combined effects yielded sigmoidal kinetics indicative of an allosteric protein. Physiological levels of ammonium ion are, however, three orders of magnitude lower than the concentrations used in these experiments. Another interesting finding of this work was the demonstration of an utrafiltrable factor which could stimulate uroporphyrinogen production from PBG. Unfortunately, these workers have not reported any further work on this factor.

Piper and Tephly (1974) studied uro I synthetase and the direct effect of lead chloride on enzymatic activity <u>in vitro</u>. Lead chloride produced significant inhibition of human and rat hemolysate enzyme. However, the enzyme derived from either rat or human liver cytosol was not inhibited by $PbCl_2$ concentrations as high as 10^{-4}M. These

workers concluded that a plausible explanation for the excess PBG excretion seen in severely poisoned patients is inhibition of the enzyme in blood or bone-marrow but not in liver.

Some insight into this differential inhibition of uro I synthetase activity was obtained when it was realized that dialysis of hepatic cytosol rendered the enzyme sensitive to inhibition by $PbCl_2$ (Piper et al., 1976; van Lier and Piper, 1976). Evidence for a heat-stable dialyzable factor was obtained when non-dialyzed rat hepatic cytosol was fractionated on Bio-Gel P-30 (Piper et al., 1976). Fractions were assayed for their ability to reverse inhibition of uro I synthetase activity by $PbCl_2$ in dialyzed hepatic cytosol and fractions containing the protective factor were pooled and studied. When factor samples were heated for 30 minutes in a boiling water bath, no change in protective ability was observed. Treatment with either concentrated nitric acid or sodium hydroxide for 30 minutes and subsequent neutralization resulted in a 50% destruction of the factor. Approximately 80% destruction of the protective ability of the factor occurred when aliquots were ashed at high temperatures. This indicated that the protective factor was an organic substance and not a metal ion. Neither deoxyribonuclease (deoxyribonucleate 5'-oligonucleotido-hydrolase, EC 3.1.4.5) nor ribonuclease (ribonucleate 3'-pyrimidino-oligonucleotidohydrolase, EC

3.1.4.22) pretreatment of the factor had any effect on its ability to protect against inhibition of uro I synthetase activity by $PbCl_2$. However, pretreatment with a non-specific protease (Sigma Type VI) resulted in a significant destruction of protective capacity which indicates that the protective factor might be a protein or might contain a peptide chain as a portion of its structure (Piper et al., 1976; van Lier and Piper, 1976).

The purpose of the present work was to identify this factor and to study its regulation of inhibition of uroporphyrinogen I synthetase activity by lead chloride.

II. M-A-T-E-R-I-A-L-S--A-N-D--M-E-T-H-O-D-S:

A. Materials:

1. Chemicals:

Porphobilinogen was purchased from Porphyrin Products, Salt Lake City, Utah, ammonium sulfate, ultra-pure enzyme grade from Swartz-Mann, Orangeburg, New Jersey, and biopterin from Regis Chemical Company, Morton Grove, Illinois. Biopterin was reduced to dihydrobiopterin by the addition of sodium dithionite. Pterine, 6,7-dimethyl-5,6,7,8-tetrahydropterine, D,L-6-methyl-5,6,7,8-tetrahydropterine and crystalline bovine serum albumin were obtained from Calbiochem, La Jolla, California. Folic acid, Ecteola cellulose, 5-methyl tetrahydrofolic acid and o-phthalaldehyde were bought from Sigma Chemical Company, St. Louis, Mo. Folic acid [3', 5',9(n) -^{3}H] (63 Ci/mmole) was obtained from Amersham-Radiochemical Center, Arlington Heights, Illinois. Dihydrofolic acid was prepared by sodium dithionite reduction according to Futterman (1963). Tetrahydrofolic acid was prepared by platinum dioxide catalytic reduction in 0.1 M tris - HCl, pH 7.0 (Blakley, 1957). N^{5} -formyltetrahydrofolic acid was obtained from Lederle Laboratories, Pearl River, New York and was used to prepare both N^{5}, N^{10}-methenyltetrahydrofolate and N^{10}-

formyltetrahydrofolate by adjusting the pH (Rabinowitz, 1963). Pteroyltri- and pteroylhexaglutamate were generous gifts from Dr. E.L.R. Stokstad, University of California, Berkeley, California. Lactobacillus casei (ATCC 7469) and Streptococcus faecalis (ATCC 8043) were obtained from American Type Culture Collection, Rockville, Maryland. Chick pancreas acetone powder, and all bacteria growth and assay media were supplied by Difco Laboratories, Detroit, Michigan. DEAE cellulose and Bio-Gel P-30 (100-200 mesh) were purchased from Bio-Rad Laboratories, Richmond, California, and Sephadex G-100 was obtained from Pharmacia, Piscataway, New Jersey.

2. Animals

Male Sprague-Dawley rats, 180-220 grams from Simonsen Laboratories, Gilroy, California were used throughout this study.

B. Methods

1. Enzyme Assays

a. Uroporphyrinogen I synthetase

Uro I synthetase activity was measured by the method of Strand et al., (1972). The enzyme was incubated aerobically

in the presence of 50 umoles tris-HCl buffer, pH 8.0 and 150 nmoles PBG in a total volume of 1.5 ml for 1 hour at 37°C in the dark. The reaction was terminated by the addition of an equal volume of a 1:1 mixture of 2N $HClO_4$ and 95% ethanol. The extracted material was measured fluorometrically at excitation and emission wavelengths of 405 and 595 nm, respectively and compared to a uroporphyrin I standard curve. Product formation was a linear function of time for 80 minutes; however, assays were conducted for 60 minutes.

b. ALA-dehydratase

This assay was a slight modification of that described by Gibson and others (Gibson et al., 1955; Berlin and Schuller, 1974; Weissburg and Vovtek, 1974). A 9000 x g supernate (0.25 ml) was incubated at 37°C in the presence of 0.75 umoles ALA and 100 umoles of sodium phosphate, pH 6.3 in a total volume of 1.5 ml. The incubation mixtures were maintained under nitrogen gas for 1 hour. The reaction was then stopped by the addition of 0.5 ml of 50 mM $HgCl_2$ in 5% (w/v) trichloroacetic acid and the extracted material reacted with a 2% acidic solution of dimethyl-aminobenzaldehyde. Product formation was estimated by the absorption at 553 nm. An extinction coefficient of 61 $mMolar^{-1}$ cm^{-1} was used to convert absorption to enzymatic activity.

2. Quantitative protein determinations.

Protein concentrations were measured either by the method of Lowry et al (1951), the microbiuret method (Albro, 1975; Itzhaki and Gill, 1964; Zamenhof 1957), or by direct absorption at 280 nm.

A fluorometric method utilizing o-phthalaldehyde (OPT) was also used for the detection of protein, primary amines and amino acids (Torres et al., 1976; Benson and Hare, 1975; Weidekamm et al., 1973). Samples (1.0 ml) were added to 1.0 ml of 0.1 N sodium phosphate, pH 8.5. 2-Mercaptoethanol (5 ul) and 5 ul of a 1% (w/v) solution of OPT in methanol were then added. The mixtures were maintained in the dark at room temperature for 45 minutes. Samples were then analyzed fluorometrically (λ ex 340; λ em 445) and compared to a standard solution of bovine serum albumin similarly treated.

3. Purification of rat hepatic uroporphyrinogen I synthetase.

Male rats were decapitated with a guillotine, the blood allowed to drain and their livers perfused in situ with 0.9% (w/v) NaCl (at 5^{o}C) via the inferior vena cava. A 33 1/3% (w/v) liver homogenate was prepared in cold 50 mM tris-HCl, pH 8.0 at 5^{o}C using a Dounce glass homogenizer. The homogenate was centrifuged at 25,000 x g at 5^{o}C for 20

minutes. The supernate was removed and recentrifuged at 105,000 x g (5°C) for 60 minutes to yield a clear hepatic cytosol. The cytosol was heated in a hot water bath for 5 minutes at 60°C. The suspension which formed was cooled to 5°C, rehomogenized and centrifuged to yield the heated cytosol fraction. This material was subjected to ammonium sulfate fraction, and the material which precipitated between 40 and 60% saturation was collected, redissolved in 10 ml of tris buffer, and dialyzed against 4 liters of buffer. The enzyme was then applied to a 1.7 x 24 cm column of DEAE cellulose which had been equilibrated in 50 mM tris-HCl, pH 8.0 buffer. The enzyme was eluted with a 0-0.4 M KCl linear gradient prepared in tris buffer. Fractions which contained uro I synthetase activity were pooled and concentrated with an Amicon model 52 stirred cell concentrator fitted with a PM-10 membrane. This material was then applied to a Sephadex G-100 column (2.5 x 66 cm). The enzyme which eluted from this column was pooled and store at -20°C and was stable for several months.

4. Purification of the factor protecting against inhibition of uroporphyrinogen I synthetase activity by $PbCl_2$.

Rats were decapitated and their livers perfused with cold saline as described above. Liver homogenates (33 1/3% (w/v)) were prepared in water rather than tris buffer. A

9000 x g supernatant fraction was prepared and recentrifuged at 105,000 x g at 5°C for 90 minutes. Cytosol (30 ml) was applied to a 2.5 x 60 cm Bio-Gel P-30 column which had been equilibrated in water. Fractions were assayed for the factor by incubating 0.1 ml aliquots of every third fraction in the presence of dialyzed hepatic cytosol and 10^{-4} M $PbCl_2$. Fractions containing the factor were pooled and lyophilized. The resulting freeze-dried powder was reconstituted with 1.0 ml water and applied to an Ecteola cellulose column, pH 7.0 (0.9 x 45 cm) which had been equilibrated with water (Fukushima and Akino, 1968; Piper and van Lier, 1977). The factor was then eluted with a linear gradient of 0-0.1 M NH_4COOH, pH 7.0. Fractions which contained the factor were pooled, lyophilized and reconstituted with a small volume of water.

5. Preparation of metallothionein

Metallothionein was prepared by the methods of Kagi et al., (1974) and Shaikh and Lucis (1971). Rats received 5 daily injections of $CdCl_2$ (2.0 mg/kg). On the sixth day livers were removed and homogenized in 0.25 M sucrose (20% (w/v)). Homogenates were centrifuged at 32,000 x g for 20 minutes at 5°C and the resulting supernate was recentrifuged for 90 minutes at 105,000 x g. Cytosol (30 ml) was chromatographed on a Sephadex G-75 (2.5 x 135 cm) column which had been equilibrated in 6 mM borate - 50 mM NaCl, pH

8.5. Fractions which had an increased absorption at 250 nm compared to control cytosol preparations were collected, pooled and lyophilized.

6. Quantitative estimation of folate concentration

a. Bacterial growth assays

The assay procedure was essentially that described by Herbert (1966), Waters and Mollin (1961), and Difco (1971). Sodium phosphate (50 mM) - 1% (w/v) sodium ascorbate, (0.5 ml) buffer at pH 6.1 for *L. casei* or pH 6.8 for *S. faecalis* was mixed with 2.5 ml of the appropriate double strength assay media. Various quantities of folic acid (from 0.4 to 10 picomoles for *L. casei* or 1.0 to 8.0 picomoles for *S. faecalis*) or the sample to be tested were added and the final volume adjusted to 5.0 ml with water. The tubes were capped and sterilized for 30 minutes at 230°C. After being cooled to room temperature, one drop of a 1:100 dilution of a 6 hour bacterial culture was added to each tube and mixed. The tubes were incubated in a shaking water bath for 18 hours at 37°C. Bacterial growth was estimated by measuring the turbidity at 660 nm. Assays of unknown folate samples were compared to a standard curve prepared from growth assays with known folic acid concentrations.

b. Folate competitive protein binding radioassay

1. Preparation of folate binding protein

Folate binding protein is a B-lactoglobulin found in milk and was prepared by the method of Rothenberg et al., (1972).

2. Assay procedure

The assay was modified from the method of Mortensen (1976). Folic acid or a sample of unknown (200 ul) was mixed with 200 ul of an appropriate dilution of binding protein (see below), 100 ul of ^{3}H-folate (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 mixtures were incubated at 37^{o}C for 30 minutes. The tubes were then 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 and the tubes mixed and incubated at 4^{o}C for 20 minutes. The tubes were then centrifuged (2000 rpm) and 1.0 ml of supernate was mixed with scintillation fluid (Merit ^{R}) and counted. The percent ^{3}H-folic acid bound to the binding protein is an inverse logarithmic function of the folic acid standard concentration. A standard curve was prepared using folic acid.

Prior to the assay of unknown folate solutions, it was necessary to determine the amount of folate binding protein

to bind most but not all of the labelled folic acid. This was done by performing serial dilutions of the stock protein and assaying them with a constant amount of ^{3}H-folic acid. In this manner a dilution which bound 60-80% of the labelled folate was determined. For this study a dilution of 1:200 was adequate.

7. Treatment of factor samples with γ-glutamyl carboxypeptidase.

Chick pancreas acetone powder served as the source of γ-glutamyl carboxypeptidase. The powder was dissolved (20 mg/ml) in water, and any insoluble material was removed by centrifugation. This preparation was dialyzed against water and frozen at -20°C until used (Bird et al., 1965). Aliquots of factor (20 ul) were mixed with 0.1 ml of γ-glutamyl carboxypeptidase and 0.1 ml of 0.1 M NH_4COOH, pH 7.0 in a 3 ml centrifuge tube. The tubes were incubated for 2 hours at 37°C. The reactions were stopped by placing the tubes in a boiling water bath for 10 minutes. The precipitate was removed by centrifugation and the supernate was concentrated by lyophilization.

8. Polyacrylamide gel electrophoresis.

Disc-gel electrophoresis was performed using the method of Davis (1964). This is a two step method which allows

proteins to "stack" in discs between two anions in a large-pore gel. Separation of these discs occurs in a small-pore gel at pH 8.9. Electrophoresis was conducted at 4 ma/gel. After the run, gels were fixed in 12.5% (w/v) trichloroacetic acid and then stained in a 0.05% (w/v) Coomassie blue R-250 in 10% (w/v) trichloroacetic acid.

9. Sulfhydryl analysis:

Reduced sulfhydryl groups were estimated using the colorimetric reaction 5',5'-dithiobis (2-nitrobenzoic acid) (DTNB) by the procedure of Ellman (1959). An unknown sample contained in 1.5 ml is mixed with 0.5 ml of 0.1 M sodium phosphate, pH 8.0 and 20 ul of 0.01 M DTNB. The absorption was measured at 412 mM. An extinction coefficient of 13,600 M^{-1} cm^{-1} was used to calculate the reduced sulfhydryl concentration.

Oxidized sulfhydryl groups were assayed by the method of Zahler and Cleland (1968). Disulfides were first reduced by the addition of dithiothreitol or dithioerythritol. The excess reducing agent was then complexed by the addition of AsO_2^- and the resulting sulfhydryl groups reacted with DTNB. Since the dithiothreitol-AsO_2^- complex will react with DTNB, but at a slower rate, the reaction is followed with time on a Gilford 2000 recording spectrophotometer and the

absorption extrapolated to zero time is used to measure the amount of disulfide initially present.

III. RESULTS:

A. Purification of the factor from rat hepatic cytosol.

The factor which protects uro I synthetase activity from inhibition by $PbCl_2$ was purified as described in Materials and Methods. Hepatic cytosol was prepared from livers that were homogenized in water in order to avoid the isolation of factor preparations having high concentrations of the buffer salts, which interfered with subsequent identification procedures. Elution profiles of the protective factor from Bio-Gel P-30 and Ecteola Cellulose are shown in figures 2 and 3, respectively.

Aliquots of the factor were subjected to thin-layer chromatography on MN 300 cellulose, and chromatographed with 5% (w/v) NH_4HCO_3, pH 7.8. The cellulose was scraped from the plates in 0.5 cm segments. Scrapings were extracted with 0.5 ml water, and 0.4 ml aliquots were tested for their ability to protect against inhibition of purified rat hepatic uro I synthetase by 10^{-5} M $PbCl_2$. Protective factor was located at only one position with an Rf of 0.74. Two other additional solvent systems, 5% (w/v) ammonium carbamate, pH 9.0, and 95% ethanol - 3% (w/v) NH_4Cl (70:30), pH 6.0 were also used. These systems yield single spots of

protective factor with Rf values of 0.77 and 0.46 for the two solvent systems, respectively.

It soon became evident that a means to quantitate the amount of factor in a sample was necessary. Hence, one unit of factor was defined as "that amount of factor necessary to cause a ten fold increased requirement of $PbCl_2$ concentration in order to maintain a level of 50% inhibition of uro I synthetase activity." (Piper and van Lier, 1977). This definition is demonstrated in figure 4. o-Pthtalaldehyde (OPT), which reacts with primary amines and is used in the micro-analysis of proteins at 0.1 ug/ml concentrations, was used for estimation of the degree of purification of the factor from hepatic cytosol. A 1792 fold purification of the factor from hepatic cytosol was obtained (table 1). Factor preparations from the Ecteola cellulose step were not likely to have contained protein, since factor could be recovered by ultra-filtration with a Amicon UM-2 membrane which has a molecular weight cutoff of 1000. The small amount of OPT reactive material might be due to the presence of a primary amino group as part of the chemical structure of the factor.

B. <u>A comparison of the properties of the factor to metallothionein.</u>

Metallothionein, a sulfhydryl rich protein, has been reported to bind cadmium, zinc, and mercury (Pulido et al., 1966; Webb, 1972). It was possible that the factor was a substance similar to metallothionein which could bind lead and protect against inhibition of uro I synthetase activity by $PbCl_2$. Therefore, metallothionein was purified from rat liver and tested for its ability to protect against inhibition of uro I synthetase activity by $PbCl_2$. As shown in table 2. metallothionein was unable to protect against inhibition of uro I synthetase activity by $PbCl_2$. Factor was also found to be devoid of reduced or oxidized sulfhydryl content. However, approximately one-third of the amino acids present in metallothionein are known to be cysteine (Kagi et al., 1974; Pulido et al., 1966). Metallothionein exhibited a characteristic band pattern by disc-gel electrophoresis when stained with Coomassie blue (Shaikh and Lucis, 1971). When factor was subjected to gel electrophoresis, no bands were observed. The inability of metallothionein to protect against inhibition of uro I synthetase activity by $PbCl_2$, the absence of sulfhydryl content in factor preparations, and the inability to observe gel electrophoretic patterns characteristic of proteins indicated that the factor was a substance distinctly different from metallothionein.

C. Purification and properties of uroporphyrinogen I synthetase:

It became apparent that a better understanding of the properties of the factor could be achieved only by utilizing purified uro I synthetase preparations. Uro I synthetase has been purified from several non-mammalian sources such as wheat germ, spinach leaves and Rhodopseudomonas spheroides (Lambais and Batlle, 1971; Davis and Neuberger, 1973; Jordan and Shemin, 1973; Stevens and Frydman, 1968; Frydman and Frydman, 1970; Higuchi and Bogorad, 1975; Sancovich et al., 1969). However, the mammalian enzyme has been primarily isolated from erythrocytes (Stevens et al., 1968; Frydman and Feinstein, 1974). Only Sancovich et al (1969 a,b) have reported the study of a hepatic uro I synthetase (bovine liver). Extensive purification of the rat hepatic enzyme has not been reported. Most of the published purification procedures use ammonium sulfate precipitation, heat treatment, and ion exchange or gel filtration chromatography. However, the procedure reported herein utilizes all of these techniques for the purification of the enzyme from rat liver. It is therefore uniquely different from the previously published procedures. The elution of rat hepatic uro I synthetase from DEAE cellulose is shown in figure 5 and from Sephadex G-100 in figure 6. A typical example of the purification of rat hepatic uro I synthetase with yields, specific activities and purifications is presented in table 3.

1. Other heavy metal inhibitors.

In addition to lead, other divalent cations were tested for their ability to inhibit uro I synthetase activity. Several divalent heavy metal cations such as mercury, cadmium, and zinc were also found to be inhibitors of uro I synthetase activity (table 4). Factor was tested for its ability to protect against inhibition of enzymatic activity by these other divalent metals. Factor exhibited no protection against enzymatic inhibition produced by Hg^{+2}. A slight but significant protection against inhibition of enzymatic activity by Cd^{+2} and Zn^{+2} was observed. In addition to these metal ions, divalent magnesium and iron failed to produce significant ihiibition at 10^{-4} M concentrations. These studies with divalent metal cations other than Pb^{+2} indicate that the interaction between uro I synthetase, Pb^{+2}, and the protective factor must be somewhat specific.

D. Properties of the purified factor.

Factor preparations that had been purified by column chromatography were concentrated by lyophilization, dissolved in small volumes of water and subjected to ultraviolet, visible and fluorescent spectral analysis. The ultraviolet spectra of the factor showed only one characteristic absorption peak at 262 nm (Figure 7). No absorption peaks were observed in the visible spectrum (340-600 nm). Fluorescence spectra of the factor produced an

excitation maximum of 368 nm and an emission maximum of 420 nm. Spectral analysis were performed at pH 6.5. The excitation and emission wavelengths of the factor resembled the values reported for various folates. Thus, folic acid was immediately evaluated for its ability to protect against inhibition of uro I synthetase activity by $PbCl_2$. Folic acid (10^{-4} M) was unable to protect enzymatic activity from inhibition by $PbCl_2$ (figure 9). However, such high concentrations of folic acid produced a small but significant stimulation of purified rat hepatic uro I synthetase activity. An exciting observation was that freshly prepared factor preparations also produced a dramatic stimulation of purified rat hepatic uro I synthetase activity in addition to providing protection against inhibition of enzymatic activity by $PbCl_2$. Furthermore, the factor stimulation of uro I synthetase activity was antagonized by folic acid, which indicated that the factor might be a pteridine derivative.

In order to further test whether the factor was a folate, use was made of specific folate-dependent microbiological and competitive protein binding radioassays. Factor was added to cultures of two folate dependent bacterial strains, L. casei (ATCC 7469) and S. faecalis (ATCC 8043). Factor (152 units) was able to support the growth of both organisms, which supports the hypothesis that the factor is a folic acid derivative (table 5).

Competitive protein binding radioassays are highly specific for measurement of folate derivatives (Rothenberg et al., 1974; Mortensen, 1976). Therefore an assay was developed using a purified milk binding protein and ^{3}H-folate (Mortensen, 1976). Aliquots of factor were added to the assay system to determine whether the factor could antagonize ^{3}H-folate binding. Factor preparations (greater than 19 units) were found to antagonize ^{3}H-folate binding to the purified folate binding protein (table 6), which provides additional evidence that the factor is a folate derivative.

E. Evaluation of various pteridine derivatives for protection against inhibition of uro I synthetase activity by $PbCl_2$:

A number of folic acid derivatives were tested at $10^{-5}M$ concentration for their ability to affect uro I synthetase activity or to protect enzymatic activity from inhibition by $PbCl_2$ (table 7). None of the particular derivatives tested were able to protect against inhibition of uro I synthetase activity by $10^{-5}M$ $PbCl_2$. However, dihydrofolic acid produced a very marked stimulation of enzymatic activity. In contrast, tetrahydrofolate was an inhibitor of enzymatic activity at concentrations greater than $10^{-7}M$. Since none of the folates tested was capable of protecting against inhibition of uro I synthetase activity by $PbCl_2$, various

unconjugated pteridine derivatives were tested. Unconjugated pteridines refer to those compounds which contain the pteridine ring structure but do not have a p-amino-benzoylglutamyl moiety. Several of these compounds have been reported to be coenzymes for the hydroxylation of phenylalanine and other aromatic amino acids (Kaufman, 1963; Fukushima and Shiota, 1972). Several different unconjugated pteridines failed to protect against inhibition of uro I synthetase activity by $PbCl_2$, (table 8). An interesting finding was that dihydrobiopterin produced a significant activation of uro I synthetase activity.

F. Studies with the factor and pteroylpolyglutamates.

Folates isolated from animal tissue contain more than one glutamic acid residue and are referred to as pteroylpolyglutamates. Rat liver has been shown to contain over 90% of its folate in pteroylpolyglutamate forms with the penta- and hexa- glutamate forms being predominant (Shin et al., 1972; Leslie and Baugh, 1974). γ-glutamyl carboxypeptidases are present in tissues which can cleave successive glutamate residues from pteroylpolyglutamates. It was possible that the factor was a pteroylpolyglutamate. To test the hypothesis, γ-glutamyl carboxypeptidase was prepared from chick pancreas (Bird et al., 1965) and incubated with a sample of factor. Factor treated with boiled γ-glutamyl carboxypeptidase served as the control.

As shown in figure 10, pretreatment of factor greatly reduced the ability of it to protect against inhibition of uro I synthetase activity by $PbCl_2$, indicating that the factor is a pteroylpolyglutamate. Pretreatment of the factor with boiled γ-glutamyl carboxypeptidase failed to destroy the ability of the factor to protect, which indicates that the destruction of the factor was an enzymatic process.

Pteroylpolyglutamates were obtained and tested for their ability to protect against inhibition of uro I synthetase activity by $PbCl_2$. Pteroylhexaglutamate (10^{-5} M to 10^{-6}M) was able to protect enzymatic activity from inhibition by $PbCl_2$ (table 9). Pteroyltriglutamate or pteroylmonoglutamate were unable to protect enzymatic activity from inhibition by $PbCl_2$. Thus it is apparent that the glutamate side chain has an important role in enabling pteroylglutamates to protect against inhibition of uro I synthetase activity by $PbCl_2$.

Although the previous experiments have demonstrated a relationship between the factor and uro I synthetase, the relationship need not be a specific one. Naturally occurring amino acids such as aspartate or glutamate, which are present in liver, can in sufficient quantities protect against inhibition of uro I synthetase activity by $PbCl_2$. However, these amino acids were effective only at 10^{-3} M or

higher concentrations, which far exceeds the concentration of $PbCl_2$ (10^{-5} M).

Hence, to test the specificity of the protective factor, its effects were studied on another enzyme. ALA dehydratase is known to be very sensitive to inhibition by $PbCl_2$. The data in table 10 demonstrates that the factor was unable to protect ALA dehydratase activity from inhibition by $PbCl_2$. The amount of factor used was much higher than that required to protect uro I synthetase activity and indicates that the factor must be specific for uro I synthetase.

G. Stimulation of uro I synthetase activity by dihydrofolic acid:

In order to more fully describe the stimulation of uro I synthetase activity by dihydrofolate, enzymatic activity was measured at various dihydrofolate concentrations. Peak activation occurred at 5×10^{-5} M dihydrofolate. However, a significant stimulation of enzymatic activity was observed at concentrations of dihydrofolate as low as 5×10^{-7} M (figure 11). Concentrations greater than 10^{-4} M interfered with the fluorescence analysis of the product. Activation with dihydrofolate was not seen in the presence of boiled enzyme. Thus the activation must be enzyme dependent.

The time course of dihydrofolate activation of uro I synthetase activity is shown in figure 12. It is evident that product formation is linear with time in the presence of dihydrofolate. The concentration of dihydrofolate was measured by fluorometry at each point during the time course for activation of enzymatic activity. The emission and excitation wavelengths of dihydrofolate (λ ex 317nm ;λ em 425nm) are quite different from other folate derivatives (Uyeda and Rabinowitz, 1963). Thus, if dihydrofolate was being consumed during the activation of enzyme activity a decrease in fluorescence would be easily detectable. However, no decrease in dihydrofolate concentration was detected during the activation of uro I synthetase. Thus, this data indicates that dihydrofolate is not being transformed during the activation of uro I synthetase, but is catalytically enhancing the product formation. Such behavior is typical for coenzymatic regulation of certain enzymatic reactions.

Table 1

Purification of Protective Factor from Rat Liver

Purification Step	Volume	OPT Reactive Material[a]	Factor Content	Specific Activity	Yield	Fold Purification
	ml	mg/ml	units	units/mg	%	
Hepatic cytosol	30.0	23.2	1745	3.0	100	1
Bio-Gel P-30	60.5	3.97	1745	7.2	100	2.4
Ecteola cellulose	1.0	0.31	1666	5376	95	1792

[a] The standard used was bovine serum albumin.

[b] Factor content of hepatic cytosol was calculated from the Bio-Gel P-30 data assuming 100% recovery.

Table 2

Effect of Metallothionein on Lead Inhibition of
Uro I Synthetase Activity in Dialyzed Hepatic Cytosol

	uro I synthetase activity[c] pmoles/mg protein/hr	% inhibition
Control	131.0 ± 3.0	
$PbCl_2$[a]	103.0 ± 1.9	21.4
Metallothionein[b]	137.7 ± 2.2	
$PbCl_2$	103.0 ± 1.9	25.2

[a] The lead concentration was 10^{-4} M.

[b] The Metallothionein final concentration was 66.7 ug/ml.

[c] Values represent mean ± S.E.M. for 3 determinations.

Table 3

Purification of Uroporphyrinogen I Synthetase from Rat Liver

	Volume	Protein Concentration	Total Activity	Specific Activity	Yield	Fold Purification
	ml	mg/ml	nmoles/hr	nmoles/mg/hr	%	
Homogenate	250	43.7	365	0.034	100	1
25,000 xg super	155	43.7	353	0.080	97	2.4
Cytosol	119	23.2	259	0.094	71	2.8
Heat treated cytosol	91	4.59	318	0.761	87	22.8
Ammonium sulfate 40-60%	14.4	14.11	164	0.810	45	24.2
DEAE cellulose	56	0.492	114	4.12	31	123
Sephadex G-100	33	0.047	57.4	37.0	16	1107

Table 4

Inhibition of Uroporphyrinogen I Synthetase Activity by Several Divalent Metals

Metal added	without factor	with factor[a]
	nmoles uro/mg protein/hr[b]	
Control	26.2 ± 0.33	24.6 ± 1.06
Pb 10^{-5} M	1.33 ± 0.07	20.2[c] ± 0.40
Hg 10^{-5} M	0.38 ± 0.04	0 ± 0.14
Cd 10^{-4} M	4.06 ± 0.11	7.62[c] ± 0.27
Zn 10^{-4} M	6.13 ± 0.13	9.36[c] ± 0.23

[a] 16.7 units.
[b] mean ± S.E.M. for 3 determinations.
[c] $P < 0.01$.

Table 5

Assay of Factor with the Pteroylglutamate-dependent Microorganisms *Lactobacillus casei* and *Streptococcus faecalis*.

Factor Addition	Pteroylglutamate Content[a]	
	L. casei	*S. faecalis*
Units	pmoles	
16	0	0
32	0	0
152	3.6 (0.024[b])	3.2 (0.021)

[a] The minimum detectable PteGlu contents by microbiological assay were 0.4 and 1.5 pmoles for *L. casei* and *S. faecalis*, respectively.

[b] The numbers in parentheses denote pmoles of factor (as PteGlu) per unit.

Table 6

Assay of Factor by Competitive Protein Binding Radioassay

Factor Addition units	Pteroylglutamate Content[a] pmoles
13	0
19	0.10 (0.005)[b]
38	0.19 (0.005)
76	0.46 (0.006)

[a] The minimum detectable PteGlu content by competitive protein binding assay employing ^{3}H-folate was 0.1 pmole.

[b] The numbers in parentheses denote pmoles of factor (as PteGlu) per unit.

Table 7

The Effects of Some Pteroylmonoglutamates on Inhibition of Uroporphyrinogen I Synthetase Activity by Lead Chloride

Pteroylmonoglutamate[a]	Uro I Synthetase Activity nmoles/mg/hr[b]	
	$-PbCl_2$	$+PbCl_2$[c]
none	32.9 ± 0.6	1.0 ± 0.2
PteGlu	31.2 ± 0.5	0.9 ± 0.1
H_2 PteGlu	55.5 ± 0.5	2.9 ± 0.2
H_4 PteGlu[d]	10.5 ± 0.7	---
5-methyl H_4 PteGlu	27.3 ± 0.5	0.8 ± 0.1
5-formyl H_4 PteGlu	33.1 ± 0.8	0.9 ± 0.1
10-formyl H_4 PteGlu	26.5 ± 1.2	0.1 ± 0.1
5,10 methylene H_4 PteGlu	15.5 ± 0.8	0.1 ± 0.1

[a] All pteroylmonoglutamates were tested at 10^{-5} M.

[b] Values represent the mean ± S.E.M. for 3 determinations.

[c] The $PbCl_2$ concentration was 10^{-5} M.

[d] Assays containing H_4PteGlu were run anaerobically. This had no effect on control assays.

Table 8

The Effects of Some Unconjugated Pteridines on Inhibition of Uroporphyrinogen I Synthetase Activity by Lead Chloride.

Pteridine	Uro I synthetase activity
	nmoles uro/mg protein/hr.
None	23.4 ± 0.69[b]
+ $PbCl_2$[c]	0.27 ± 0.11
Pterine[a]	24.8 ± 0.46
+ $PbCl_2$	0.28 ± 0.25
6,7,dimethyl-5,6,7,8 tetrahydropterine	22.7 ± 0.35
+ $PbCl_2$	0.32 ± 0.17
D,L-6 methyl-5,6,7,8 tetrahydropterine	24.1 ± 0.46
+ $PbCl_2$	0.25 ± 0.19
Biopterin	22.9 ± 0.26
+ $PbCl_2$	0.22 ± 0.18
Dihydrobiopterin	31.5 ± 0.20
+ $PbCl_2$	0.39 ± 0.15

[a] All pteridines were tested at 10^{-5} M.

[b] Mean ± S.E.M. for 3 determinations.

[c] $[PbCl_2] = 10^{-5}$ M.

Table 9

The Effect of Pteroylglutamates of Variable Glutamate Chain Lengths on Inhibition of Uroporphyrinogen I Synthetase Activity by Lead Chloride

Addition to Reaction Mixture	Pteroylglutamate Concentration	Uro I Synthetase Activity
		nmoles uro/mg protein/hr[a]
None	None	31.1 ± 0.1
$PbCl_2$[b]	None	0.8 ± 0.1
PteGlu	10^{-5} M	29.4 ± 0.5
PteGlu + $PbCl_2$		0.8 ± 0.1
$PteGlu_3$	10^{-5} M	29.0 ± 0.4
$PteGlu_3$ + $PbCl_2$		0.9 ± 0.1
$PteGlu_6$	10^{-5} M	32.3 ± 0.3
$PteGlu_6$ + $PbCl_2$		30.2 ± 0.7[c]
$PteGlu_6$	10^{-6} M	32.5 ± 0.3
$PteGlu_6$ + $PbCl_2$		3.6 ± 0.5[c]

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

[b] The $PbCl_2$ concentration was 10^{-5} M.

[c] Denotes significant difference from $PbCl_2$ alone ($P<0.05$).

Table 10

Failure of Factor to Protect Against Inhibition of 5-Aminolevulinic Acid Dehydratase Activity by Lead Chloride

Addition to Reaction Mixture	ALA Dehydratase Activity
	nmoles/mg protein/hr[a]
None	6.59 ± 0.06
$PbCl_2$[b]	2.59 ± 0.08
Factor	6.57 ± 0.05
$PbCl_2$ + Factor	2.66 ± 0.07

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

[b] The $PbCl_2$ concentration was 2.5×10^{-5} M.

[c] Each reaction mixture contained 8.4 units of factor.

Figure 1

The Condensation Reaction of Uroporphyrinogen Synthetase.

Uro I is formed by uro I synthetase. Uro III formation requires the simultaneous action of uro I synthetase and uroporphyrinogen cosynthetase. A = acetate; P = n-propionate.

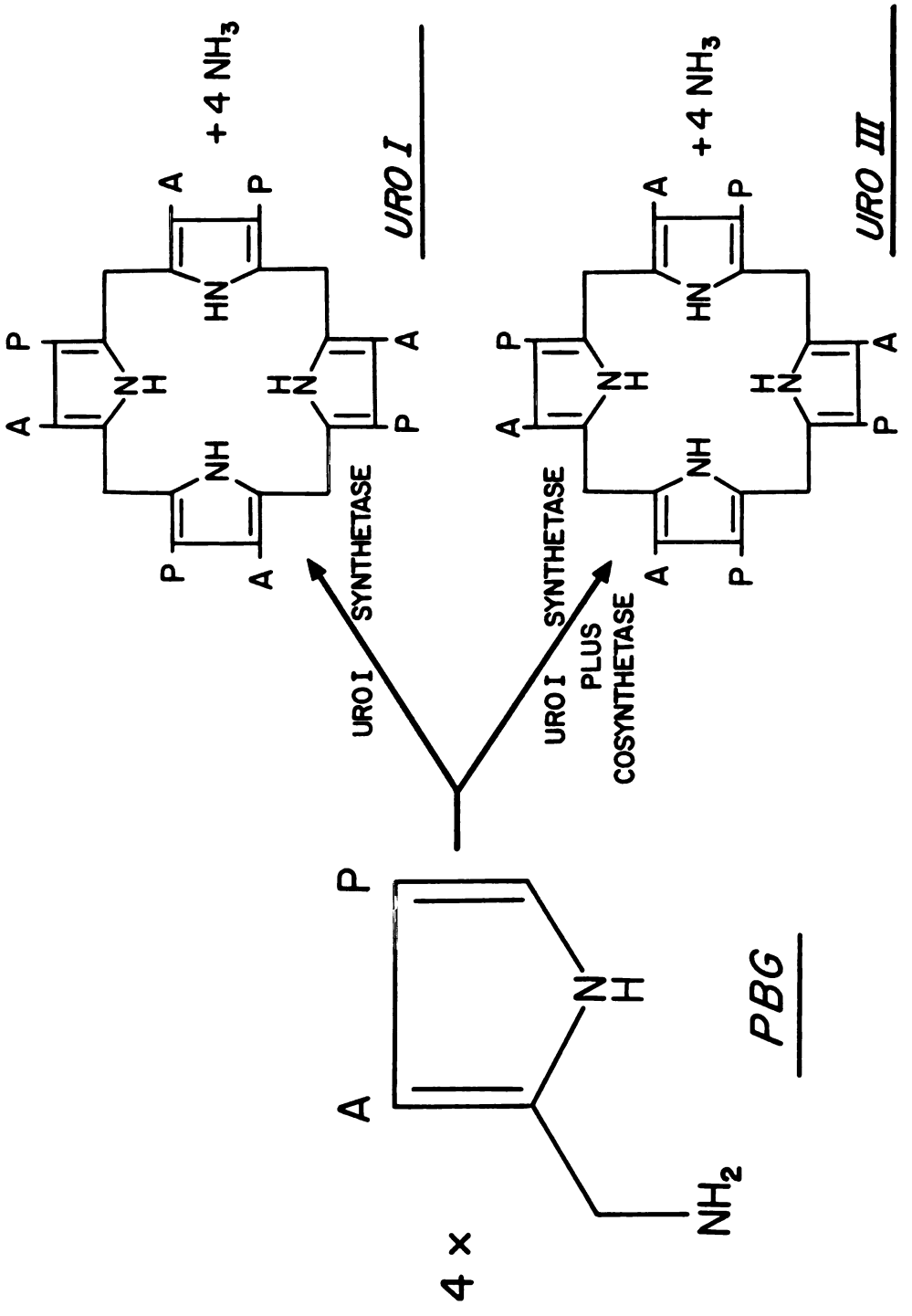

4 x
PBG
NH_2
URO I SYNTHETASE
URO I SYNTHETASE PLUS COSYNTHETASE
URO I
URO III
+ 4 NH_3
+ 4 NH_3

Figure 2

Isolation of a Factor from Rat Hepatic Cytosol which Protects Against Inhibition of Uroporphyrinogen I Synthetase Activity by $PbCl_2$.

Rat hepatic cytosol was added to a 2.5 x 60 cm column of Bio-Gel P-30 and 2.5 ml fractions were collected. The factor was located by adding 0.1 ml aliquots of every third fraction to dialyzed hepatic cytosol in the presence of substrate and 10^{-4} M $PbCl_2$, (O---O). Control uro I synthetase activity was 102 picomoles/mg/hr. $PbCl_2$ reduced enzymatic activity to 22 picomoles/mg/hr. Absorbance at 280 nm, (●---●).

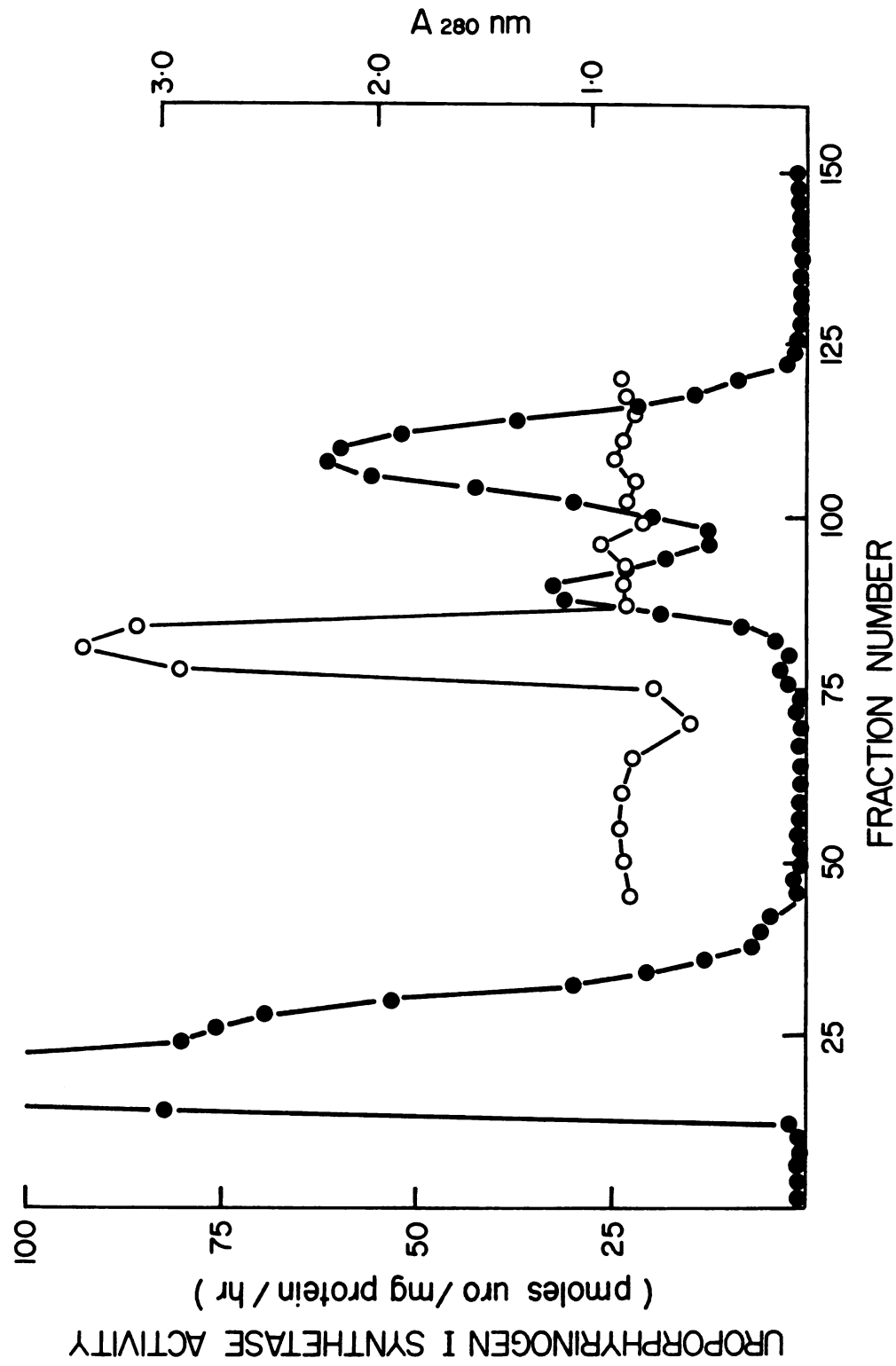

A 280 nm
3·0
2·0
1·0
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY
(pmoles uro /mg protein / hr)
100
75
50
25
FRACTION NUMBER
25
50
75
100
125
150

Figure 3

Purification of a Factor Protecting Against $PbCl_2$ Inhibition of Rat Hepatic uroporphyrinogen I Synthetase Activity by Ecteola Cellulose Chromatography.

Factor isolated by chromatography on Bio-Gel P-30 was lyophilized, dissolved in water (1 ml) and applied to an Ecteola cellulose column (0.9 x 24 cm). The factor was eluted with a linear gradient of 0-0.1 M NH_4COOH, pH 7.0. Fractions (5 ml) were collected at a flow rate of 15 ml/hr. Aliquots (0.5 ml) of every third fraction were assayed with purified rat hepatic uro I synthetase in the presence of 10^{-5} M $PbCl_2$, (○---○). Absorbance at 280 nm, (●---●).

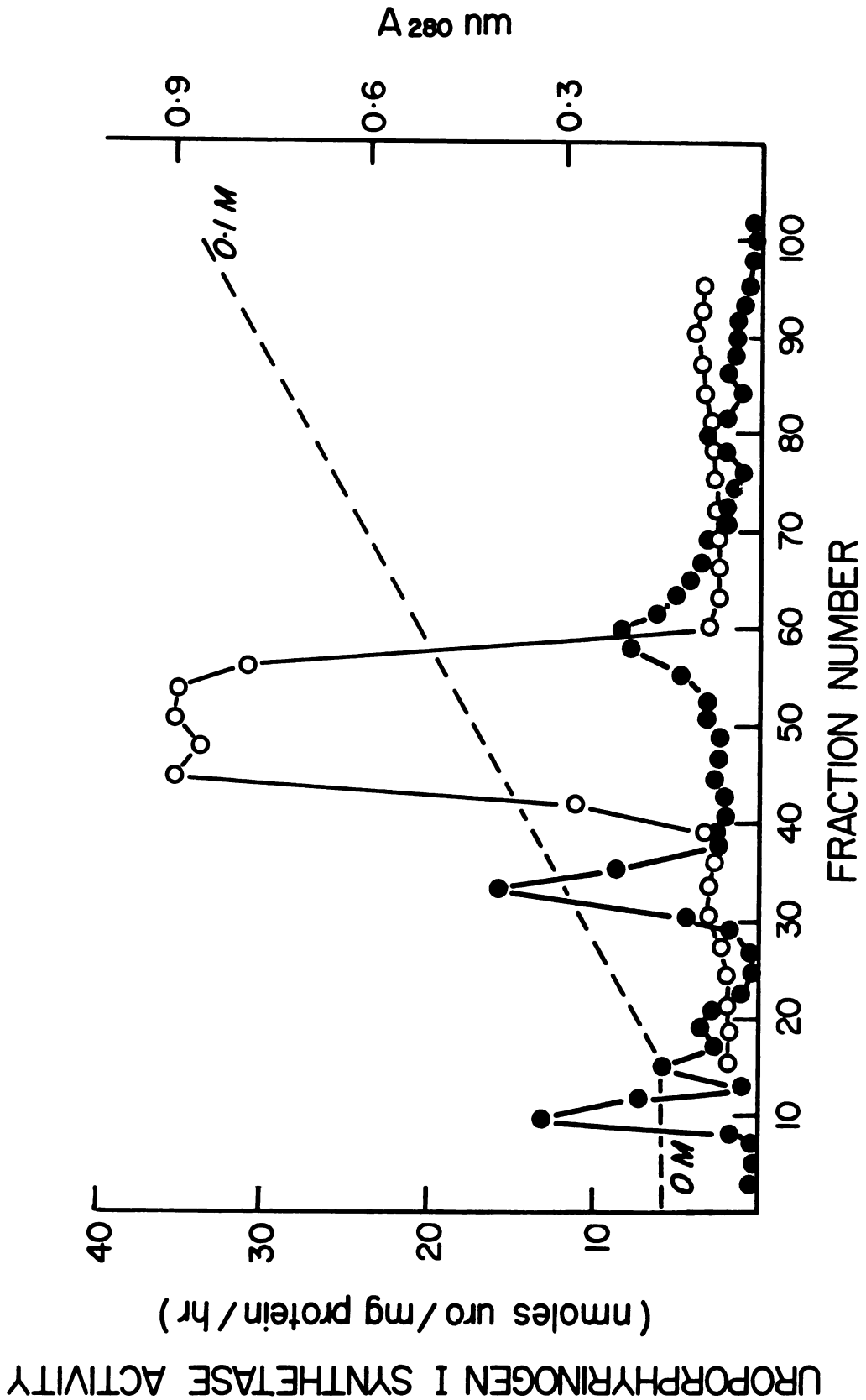

A280 nm
0·9
0·6
0·3
0·1 M
0 M
40
30
20
10
(nmoles uro / mg protein / hr)
UROPORPHYRINOGEN I SYNTHETASE ACTIVITY
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 4

Inhibition of Purified Rat Hepatic Uroporphyrinogen I Synthetase Activity by Lead Chloride.

Each point represents the mean of 3 determinations ± S.E.M. (●---●), uro I synthetase activity in the absence of factor. (O---O), uro I synthetase activity in the presence of 1 unit of factor.

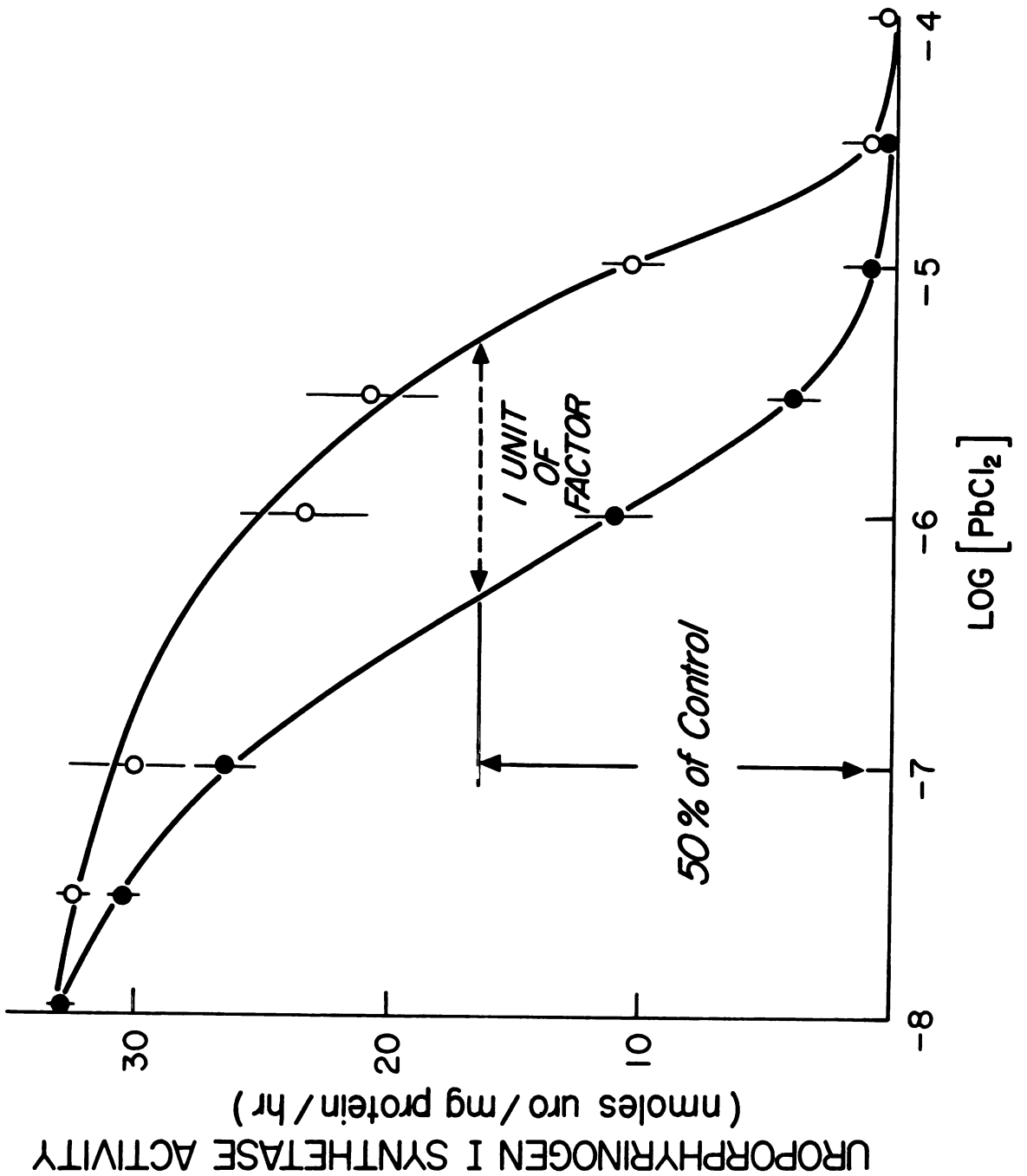

UROPORPHYRINOGEN I SYNTHETASE ACTIVITY
(nmoles uro / mg protein / hr)
30
20
10
-8
-7
-6
-5
-4
LOG [PbCl2]
50% of Control
1 UNIT OF FACTOR

Figure 5

Purification of Uroporphyrinogen I Synthetase on DEAE Cellulose.

Concentrated enzyme from ammonium sulfate fractionation was applied to a 1.7 x 24 cm column of DEAE cellulose and eluted with a 0-0.4 M KCl gradient in 0.05 M tris-HCl pH 8.0 buffer. Uroporphyrinogen I synthetase activity, (O---O), absorbance at 280 nm, (●---●). Fractions of 3.0 ml were eluted at 15 ml/hr.

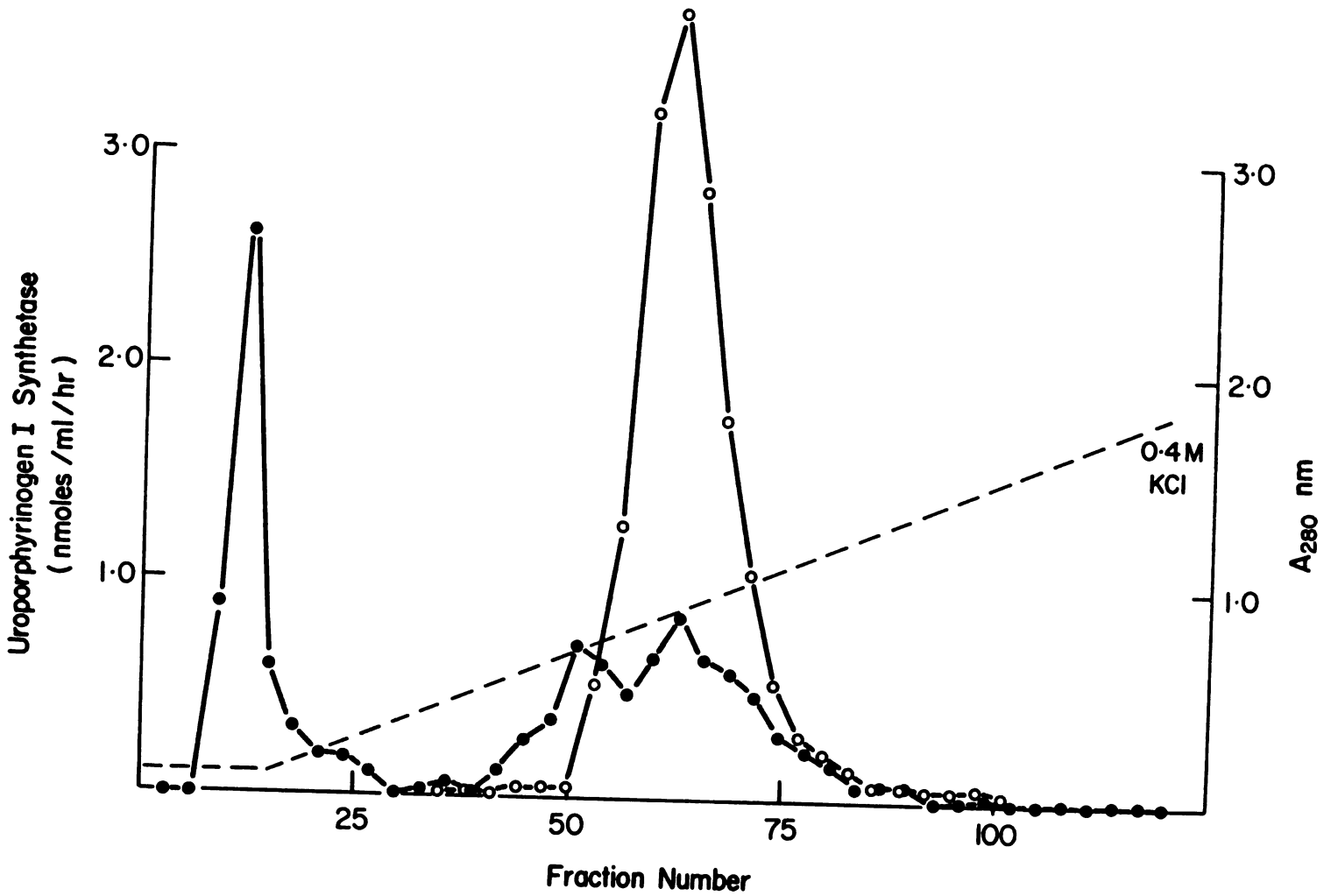

Uroporphyrinogen I Synthetase
(nmoles /ml/hr)
3·0
2·0
1·0
25
50
75
100
Fraction Number
0·4 M
KCl
3·0
2·0
1·0
A280 nm

Figure 6

Purification of Uroporphyrinogen I Synthetase on Sephadex G-100.

A concentrated solution of enzyme from DEAE cellulose chromatography was applied to a 2.5 x 66 cm column of Sephadex G-100. 150 two ml fraction were eluted with 50 mM tris-HCl, pH 8.0 at 12 ml/hr. Uroporphyrinogen I synthetase activity, (O---O); absorbance at 280 nm, (●---●).

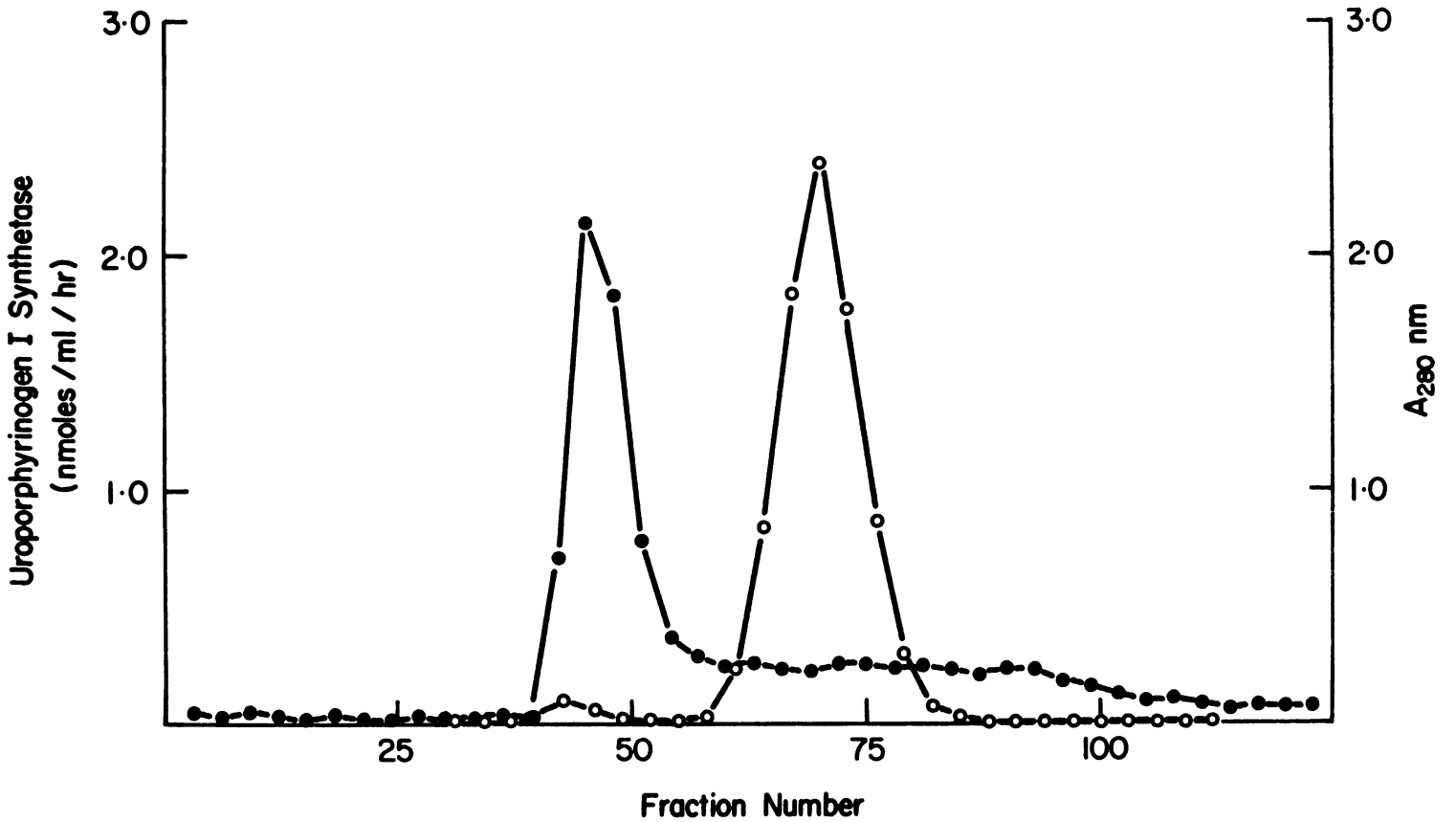

3·0
2·0
1·0
Uroporphyrinogen I Synthetase
(nmoles / ml / hr)
3·0
2·0
1·0
A280 nm
25
50
75
100
Fraction Number

Figure 7

The Ultraviolet Absorption Spectra of a Factor Protecting Against Inhibition of Uroporphyrinogen I Synthetase Activity by $PbCl_2$.

Concentrated factor (2160 units/ml) from Ecteola cellulose chromatography was used at pH 6.5. An absorption greater than 2.0 OD occurred below 225 nm which fell rapidly to a minimum at 238 nm. A peak occurred at 262 nm which fell to baseline at 292 nm. No further absorption peaks occurred in either the ultraviolet or visible spectrum.

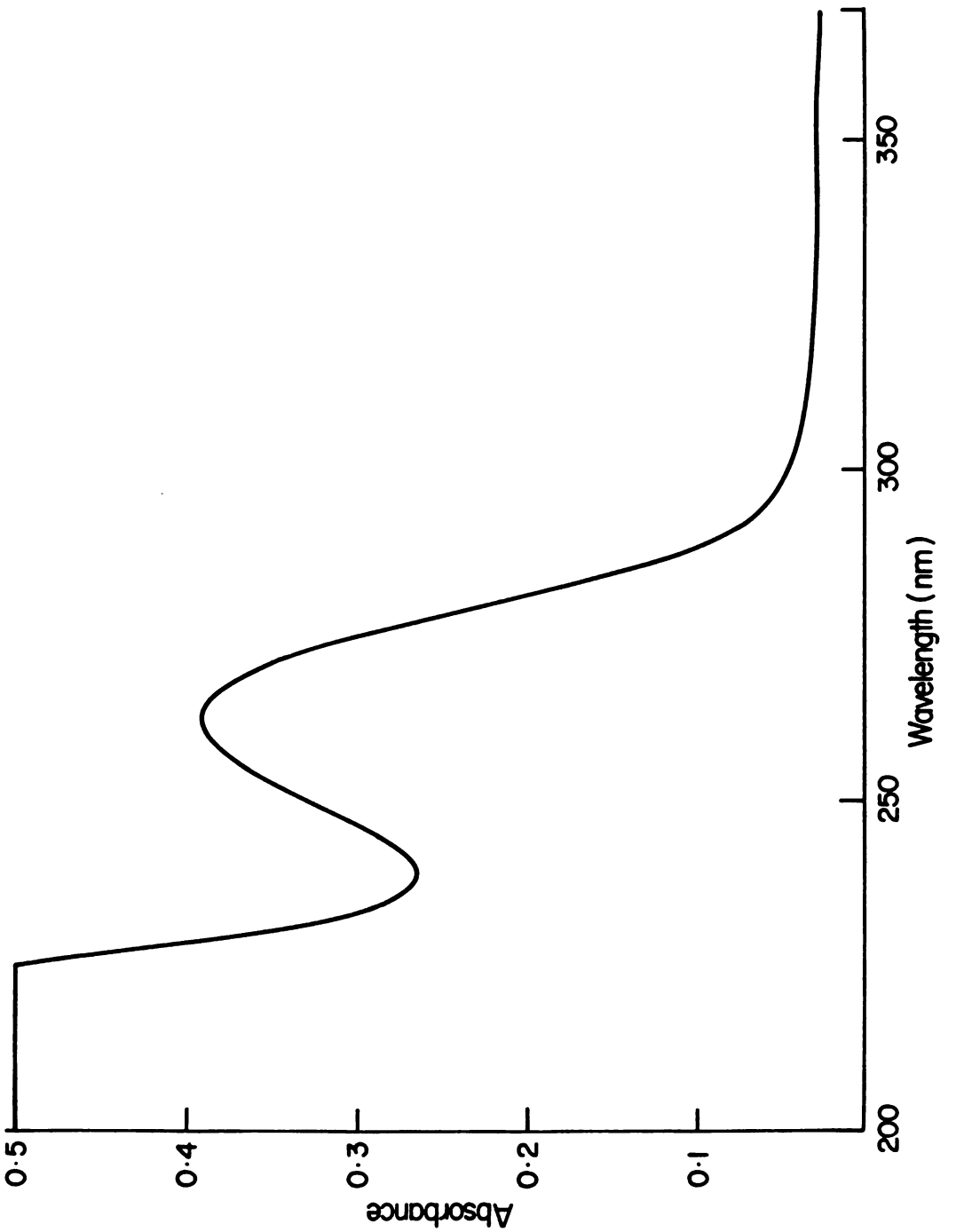

0·5
0·4
0·3
0·2
0·1
Absorbance
200
250
300
350
Wavelength (nm)

Figure 8

The Fluorescence Excitation and Emission Spectra of a Factor Protecting Against Inhibition of Uroporphyrinogen I Synthetase Activity by $PbCl_2$.

Concentrated factor (1080 units/ml) from Ecteola cellulose chromatography was used at pH 6.5. The excitation maximum occurred at 368 nm while measuring fluorescence at 450nm; the emission maximum occurred between 420 and 450 nm while exciting at 368 nm. The spectra have not been corrected for lamp intensity, and were recorded on an Aminco-Bowman spectrophotofluorometer.

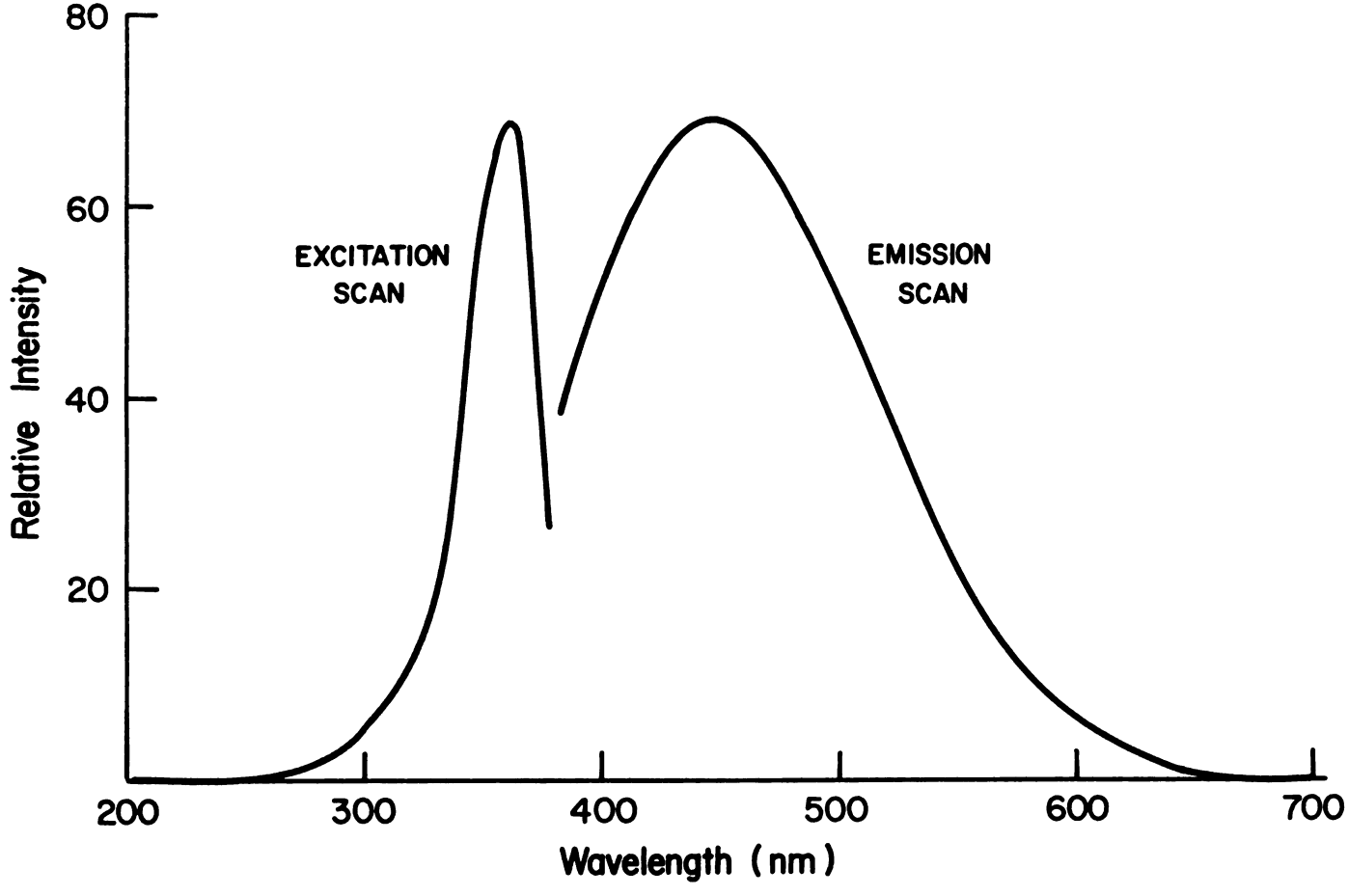

80
60
40
20
Relative Intensity
EXCITATION
SCAN
EMISSION
SCAN
200
300
400
500
600
700
Wavelength (nm)

Figure 9

Antogonism of Factor Stimulation of Hepatic Uroporphyrinogen I Synthetase Activity by Folic Acid.

Each value represents the mean ± S.E.M. for 3 determinations. Reaction mixtures with factor contained 8.4 units; 10^{-5}M $PbCl_2$ was used where noted. * Denotes significant difference from control ($P<0.05$). ** Denotes significant difference between factor plus folic acid and factor alone, ($P<0.05$).

1. Control
2. Factor, 8.4 units
3. 10^{-5} M PbCl2
4. Factor + $PbCl_2$
5. 10^{-4} M folic acid + $PbCl_2$
6. 10^{-4} M folic acid
7. 10^{-5} M folic acid
8. 10^{-6} M folic acid + factor
9. 10^{-4} M folic acid + factor
10. 10^{-5} M folic acid + factor
11. 10^{-6} M folic acid + factor

Uroporphyrinogen I Synthetase Activity (nmoles/mg/hr)
0
10
20
30
40
1
2
3
4
5
6
7
8
9
10
11
*
*
*
*
**
**

Figure 10

Effect of γ-Glutamyl Carboxypeptidase Pretreatment of Factor on Protection Against Inhibition of Uroporphyrinogen I Synthetase Activity by $PbCl_2$.

Factor (63.3 units) was preincubated with chick pancreas γ-glutamyl carboxypeptidase as described in materials and methods. Aliquots of treated factor preparations were then spotted on MN-300 cellulose thin-layer plates which were developed with 5% NH_4HCO_3, pH 7.8. Cellulose was scraped off the plates at 0.5 cm intervals, eluted with water and the eluates tested for their ability to protect against inhibition of uro I synthetase activity by 10^{-5} M $PbCl_2$. (O---O), uro I synthetase activity of reaction mixtures containing factor treated with γ-glutamyl carboxypeptidase, (●---●), uro I synthetase activity of control reaction mixtures containing factor treated with boiled γ-glutamyl carboxypeptidase.

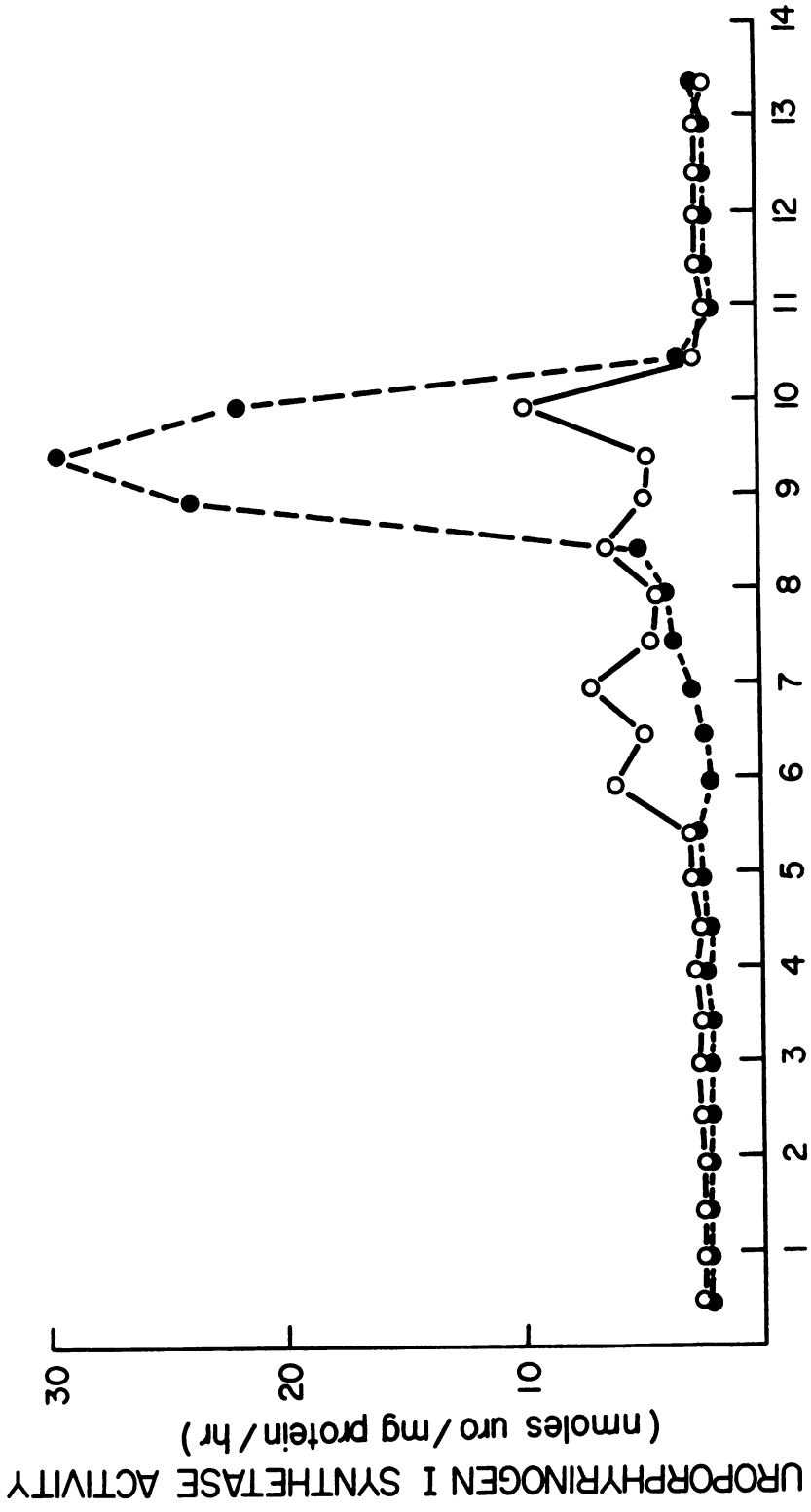

UROPORPHYRINOGEN I SYNTHETASE ACTIVITY
(nmoles uro / mg protein / hr)
30
20
10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
DISTANCE FROM ORIGIN (cm)

Figure 11

The Effect of Dihydrofolate Concentration on the Activation of Rat Hepatic Uroporphyrinogen I Synthetase Activity.

Each value represents the mean $\pm$ S.E.M. for 3 determinations. Uroporphyrinogen I synthetase activity without dihydrofolate was 26.0 nmoles/mg/hr. Significant activation ($P<.05$) occurred at all concentrations greater than or equal to 5×10^{-7} M dihydrofolate.

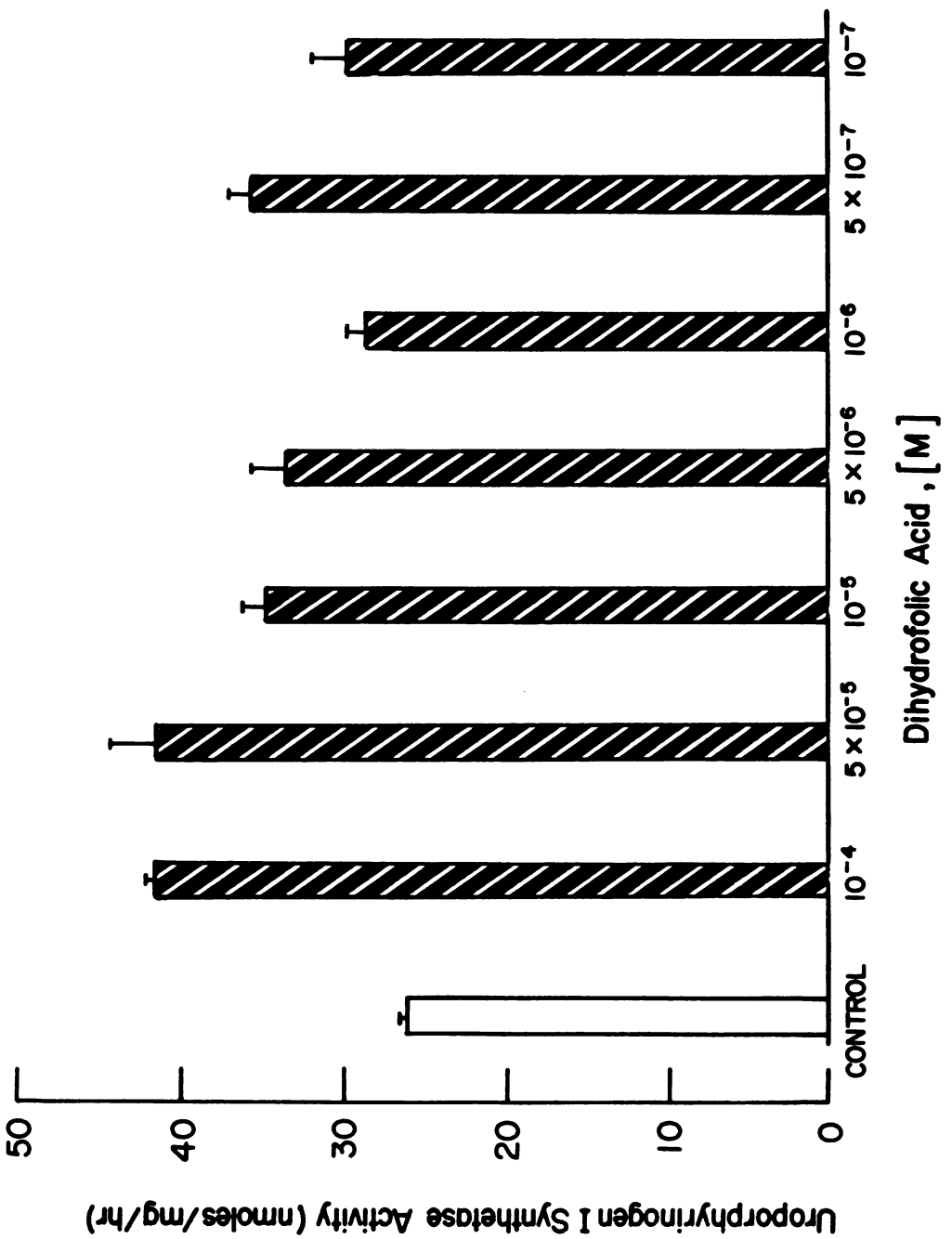
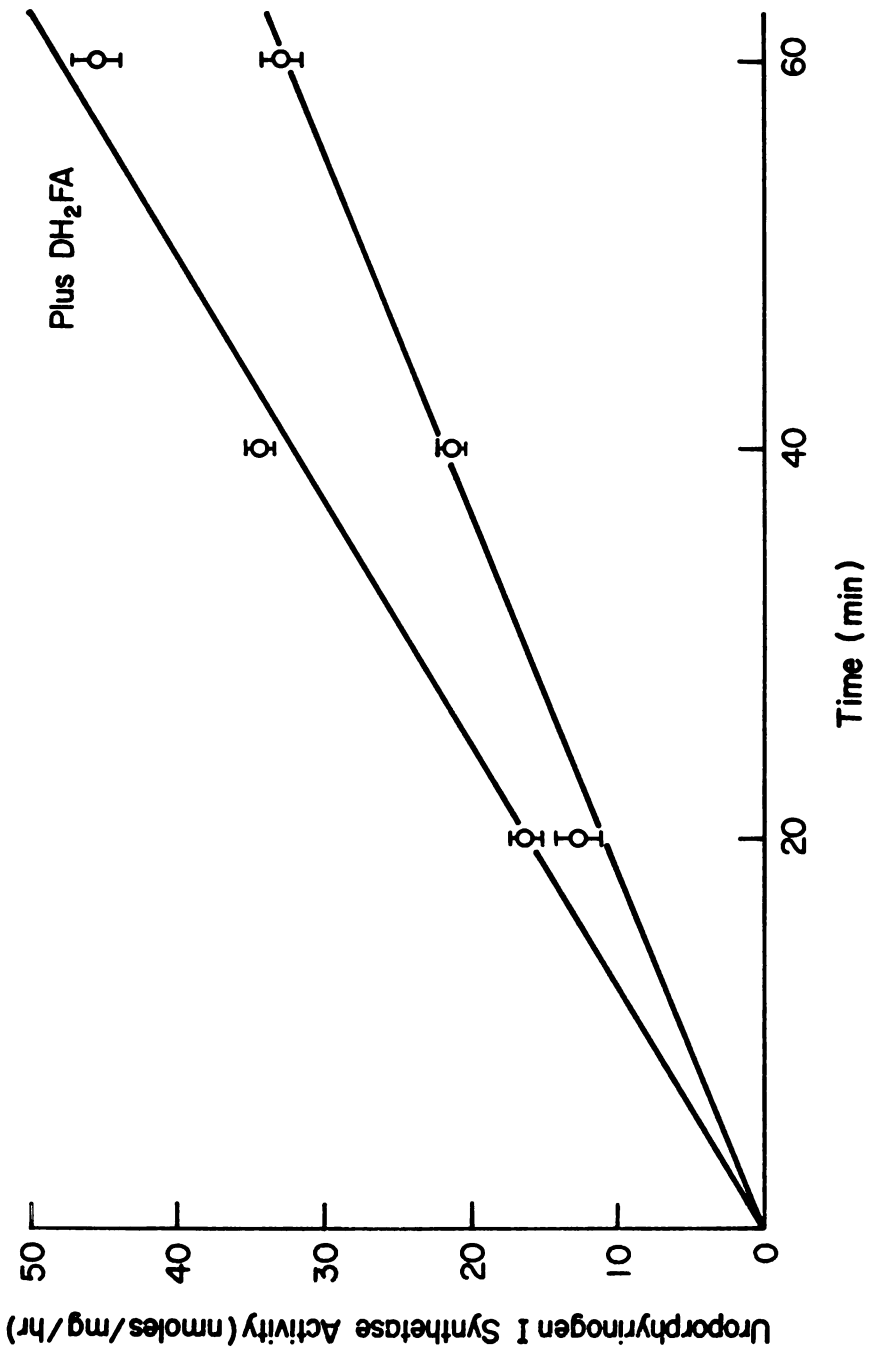

Uroporphyrinogen I Synthetase Activity (nmoles/mg/hr)
0
10
20
30
40
50
CONTROL
10⁻⁴
5 x 10⁻⁵
10⁻⁵
5 x 10⁻⁶
10⁻⁶
5 x 10⁻⁷
10⁻⁷
Dihydrofolic Acid, [M]

Figure 12

Time course for Activation of Uroporphyrinogen I Synthetase by Dihydrofolic Acid.

Each value represents the mean $\pm$ S.E.M. for 3 determinations. Control uro I synthetase, (O---O); Uro I synthetase activity in the presence of 10^{-5} M dihydrofolate, (●---●).


Uroporphyrinogen I Synthetase Activity (nmoles/mg/hr)
0
10
20
30
40
50
20
40
60
Time (min)
Plus DH_2FA

IV. DISCUSSION

The results of previous studies (Piper et al., 1976.; van Lier and Piper 1976) have demonstrated the existence of a small, heat-stable, dialyzable factor in rat hepatic cytosol which can protect uroporphyrinogen I synthetase activity form inhibition by lead chloride. Initial experiments reported herein showed that the factor contains neither sulfhydryl nor disulfide groups. Thus, the factor was not the sulfhydryl rich protein, metallothionein. Furthermore, purified rat hepatic metallothionein could not protect uro I synthetase activity from inhibition by $PbCl_2$ nor did the factor and metallothionein display similar staining patterns on disc-gel electrophoresis. Folate antagonism of factor activation of uro I synthetase activity, support of the growth of the folate-dependent bacteria, L. casei and S. faecalis, and antagonism of a competitive protein binding radioassay for folate indicated that the factor was a pteroylglutamate. Degradation of its protective capacity by pretreatment with γ-glutamyl carboxypeptidase indicated that the factor was a pteroylpolyglutamate. Protection of lead mediated inhibition of uro I synthetase activity by pteroylhexaglutamate also suggests that the factor is a pteroylpolyglutamate.

An estimation of the amount of factor required to protect against inhibition of uro I synthetase activity by $PbCl_2$ can be achieved from the microbiological and competitive protein binding assays. Approximately two units of factor as previously defined are required to protect uro I synthetase activity from the inhibitory effect of 10^{-5} M $PbCl_2$. This corresponds to 0.01 to 0.05 picomoles/assay expressed as folate which corresponds to 6.7 to 33 picomolar concentrations. This is considerably less than the concentrations of $PbCl_2$ (10 umolar) or uroporphyrinogen (ca. 0.2 umolar) which suggests that the factor may be a coenzyme for this enzymatic reaction. Much higher concentrations of pteroylhexaglutamate (2-20 umolar) than the factor (6.7 - 33 pmolar) were required to protect against inhibition of uro I synthetase activity by $PbCl_2$. The factor may contain substituent groups or may be in an oxidation state quite different from pteroylhexaglutamate. Such structural differences may enable it to more effectively interact with the enzyme to protect against inhibition by $PbCl_2$.

Although chelation of lead might be suggested as a possible mechanism for the factor's protective capability. this was unlikely since picomolar amounts of the factor protected against inhibition of uro I synthetase by 10^{-5} M $PbCl_2$. Furthermore. the factor was unable to protect against inhibition of ALA dehydratase activity by $PbCl_2$.

During the elution of uro I synthetase from DEAE cellulose with the KCl gradient a loss of activity was sometimes noted. When the effluent after concentration was tested, it was shown to support growth of the folate dependent microorganisms and to antagonize ^{3}H-folate binding in the competitive protein binding radioassay. These findings suggest that a folate is associated with uro I synthetase. This folate was also capable of protecting uro I synthetase activity from inhibition by $PbCl_2$.

The possible association of a folate derivative with uro I synthetase as well as its ability at very low concentrations to protect enzymatic activity from inhibition by $PbCl_2$ suggests that it may be a coenzyme for this reaction. This hypothesis is further strengthened by noting that the factor was also able to stimulate enzymatic activity. Since dihydrofolate was the only other folate tested which could stimulate uro I synthetase activity, it is possible that the factor might be a dihydropteroylpolyglutamate. Nevertheless, all other enzymatic systems which use a folate coenzyme have required a tetrahydro- form. Studies with other folate requiring enzymes have shown lower Km's and higher Vmax's when tetrahydropteroylpolyglutamates were used thus indicating their superior ability to function as coenzymes (Coward et al., 1975; Whitfield and Weissback, 1968). The fact that tetrahydropteroylmonoglutamate was an inhibitor of the uro I

synthetase reaction does not eliminate the possibility that a tetrahydropteroylpolyglutamate may in fact be the true coenzyme. Further work will be necessary to clarify this point.

R E F E R E N C E S .

Albro, P., Determination of Protein in Preparations of Microsomes, Analytical Biochem. 64:485-493. (1975).

Barry, P., and Mossman, D., Lead Concentrations in Human Tissues, Br. J. Ind. Med. 27:339-351. (1970).

Bashour, F., Urinary Uroporphyrin, Porphobilinogen and Coproporphyrin Excretion in Lead-Exposed Workers, J. Lab. Clin. Med. 44:764, (1954).

Battersby, A., McDonald, E., Williams, D., Wurziger, H., Biosynthesis of Natural (Type III) Porphyrins: Proof that Rearrangement Occurs After Head-to-Tail Formation. J. Chem. Soc. (Chemical Communications) 4:113-115, (1977a).

Battersby, A., Buckley, D., McDonald, E., Williams, D., Enzymic Formation of the Type III Porphyrin Macrocycle from Unrearranged AP.AP Pyrromethane, J.Chem.Soc. (Chemical Communications) 4:115-117, (1977b).

Battersby, A., Johnson, D., McDonald, E., Williams, D., Mechanistic Study of the Enzymic Incorporation of Unrearranged AP.AP Pyrromethane into Urogen III, J.Chem.Soc. (Chemical Communications) 4:117-118, (1977c).

Benson, J., and Hare, P., o-Phthalaldehyde: Fluorogenic Detection of Primary Amines in the Picomole Range. Comparison with Fluorescamine and Ninhydrin, Proc. Nat. Acad. Sci. USA 72(2):619-622, (1975).

Berlin, A., and Schaller, K., European Standardization Method for the Determination of 5-Aminolevulinic Acid Dehydratase Activity in Blood, Z. Klin. Chem. Klin. Biochem. 12:389-390, (1974).

Bird, O., McGlohon, V., Vaitkus, J., Naturally Occurring Folates in the Blood and Liver of the Rat, Analytical Biochem. 12:18-35, (1965).

Blakley, R. The Interconversion of Serine and Glycine: Preparation and Catalytic Derivatives of Pteroylglutamic Acid. Biochem. J. 65:331-342, (1957).

Bogorad, L., The Enzymatic Synthesis of Porphyrins from Porphobilinogen I. Uroporphyrin I, J. Biol. Chem., 233:501-509, (1958a).

Bogorad, L., The Enzymatic Synthesis of Porphyrins from Porphobilinogen II. Uroporphyrin III, J. Biol. Chem. 233:510-515, (1958b).

Bogorad, L., The Enzymatic Synthesis of Porphyrins from Porphobilinogen III. Uroporphyrinogens as Intermediates, J. Biol. Chem., 233:516-519, (1958c).

Bogorad, L., and Marks, G., The Enzymatic Synthesis of Uroporphyrins from Porphobilinogen IV. Investigations on the Participation of Formaldehyde, J. Biol. Chem. 237(7):2127-2129, (1960a).

Bogorad, L., and Marks, G., Studies on the Biosynthesis of Uroporphyrin III from Porphobilinogen and the Behavior of Uroporphyrin Esters in Paper Chromatography, Biochimica et Biophysica Acta, 41:356-358, (1960b).

Browder, A., Joselow, M., and Louria, D., The Problem of Lead Poisoning, Medicine 52:121-139, (1973).

Butt, E., Nasbaum, R., Gilmour, T., Didio, S., and Mariano, S., Trace Metal Levels in Human Serum and Blood, Arch. Environ. Health 8:52-57, (1964).

Chisolm, J., The Use of Chelating Agents in the Treatment of Acute and Chronic Lead Intoxication in Childhood, J. Pediatrics 73:1-38, (1968).

Coward, J., Chello, P., Cashmore, A., Parameswaran, K., DeAngelis, L., and Bertino, J., 5-Methyl-5,6,7,8-tetrahydropterovl oligo-γ-L-glutamates: Synthesis and Kinetic Studies with Methionine Synthetase from Bovine Brain. Biochem. 14:1548-1552, (1975).

Clarkson, T., and Kench, J., Uptake of Lead by Human Erythrocytes In Vitro. Biochem. J. 69:432-439, (1958).

Davis, B., Disc Electrophoresis - II: Methods and Application to Human Serum Proteins, Ann. N.Y. Acad. of Sci. 121(2):404-427, (1964).

Davies, R., Neuberger, A., Polypyrroles Formed from Porphobilinogen and Amines of Uroporphyrinogen Synthetase of Rhodopseudomonas Spheroides, Biochem. J. 133:471-492, (1973).

De Matteis, F., The Effects of Drugs on 5-Aminolevulinate Synthetase and Other Enzymes in the Pathway of Liver Haem Biosynthetasis, In: Enzyme Induction (Chapter 7) ed. D.V. Parke Basic Life Sciences, Vol. 6. (1975). Plenum, N.Y. A.H. Hollaender, general ed.

de Treville, R., Natural Occurrence of Lead. Arch. Environ. Health 8:212-221, (1964).

Diem, K., and Lentner, C., Documenta Geigy: Scientific Tables Publ: Geigy Pharmaceuticals, (1970). 7th ed.

Difco, Media for Microbiological Assay of Vitamins and Amino Acids, Difco Technical Information #247, July (1971).

Doss, M., and Schermuly, E., Urinary Porphyrin Excretion Pattern and Isomer Distribution of I and III in Human Porphyrin Disorders. In: Porphyrins in Human Disease, 1st International Porphyrin Meeting, ed. M. Doss and M. Lahn, S. Karger Pub. Basel, p. 189, (1976).

Ellman, G., Tissue Sulfhydryl Groups, Arch. of Biochem. and Biophys. 82:70-77, (1959).

Emmerson, B., Chronic Lead Nephropathy. The Diagnostic Use of Ca EDTA and the Association with Gout. Australian Annals of Med. 3:219-224, (1963).

Frydman, B., Frydman, R., Valasinas, A., Levy, E., and Feinstein, G. Biosynthesis of Uroporphyrinogens from Porphobilinogen Mechanism and Nature of the Process. Phil. Trans. R. Soc. Lond. B. 273:137-160, (1976).

Frydman, B., and Frydman, R., Biosynthesis of Uroporphyrinogens from Porphobilinogen. Mechanism and Nature of the Process, Accounts of Chemical Research 8:201-208, (1975).

Frydman, B., Frydman, R., Valasines, A., Levy, S., and Feinstein, G., The Mechanism of Uroporphyrinogen Biosynthesis, Ann. N.Y. Acad. Sci. 244:371-395, (1975).

Frydman, R., Feinstein, G., Studies on Porphobilinogen Deaminase and Uroporphyrinogen III Cosynthetase from Human Erythrocytes, Biochimica et Biophysica Acta, 350:358-373, (1974).

Fukushima, T., and Akino, M., Nuclear Magnetic Resonance Studies of Some Biologically Active Dihydropterins, Arch. Biochem. and Biophys. 128:1-5, (1968).

Fukushima, T., and Tetsuo, S., Pterins in Human Urine, J. Biol. Chem. 247(14):4549-4556, 1972.

Futterman, S., Preparation and Properties of Dihydrofolic Acid, in Methods of Enzymology, Vol VI, p. 801, Art. 112, (1963).

Gibson, K., Neuberger, A., Scott, J., The Purification and Properties of 5-Aminolaevulic Acid Dehydrase, Biochem. J. 61:618-629, (1955).

Gibson, S., MacKenzie, J., Goldberg, A., The Diagnosis of Industrial Lead Poisoning, Br. J. Ind. Med. 25:40-51, (1968).

Gibson, S., and Goldberg, A., Defects in Haem Synthesis in Mammalian Tissues in Experimental Lead Poisoning and Experimental Porphyria, Clinical Science 38:68-72, (1970).

Gilsinn, J., Estimates of the Nature and Extent of Lead Paint Poisoning In the United States, (NBS TN - 746) Washington, D.C., Dept. of Commerce, National Bureau of Standards, December (1972).

Goldberg, A., Lead Poisoning as a Disorder of Heme Synthesis, Seminars in Hematology 5(4):424-433, (1968).

Goyer, R., Toxicology of Lead on Kidney and Other Organs. p.86-96, Health Effects of Occupational Lead and Arsenic Exposure. ed. B.W. Carnow, US Dept of HEW, Feb. (1976).

Griggs, R., Lead Poisoning: Hematologic Aspects, pp. 117-137, In C.V. Moore and E.B. Brown, Eds. Progress in Hematology Vol III, New York: Grune and Stratton, Inc., (1964).

Haeger, B., Increased Content of 5-Aminolaevulic Acid-Like Substance in Urine from Workers in Lead Industry, Scandinav. J. Clin. and Lab. Invest. 9:211-212, (1957).

Hammond, P., Lead Poisoning. An Old Problem With a New Dimension. in Essays in Toxicology, F.R. Blood ed. Academic Press, (1969).

Heath, H., and Hoare, D., The Biosynthesis of Porphyrins from Porphobilinogen by Rhodopseudomones Spheroides, Biochem. J. 72:14-22, (1959).

Heath, H., and Hoare, D., The Partial Purification and Some Properties of Porphobilinogen Deaminase and Uroporphyrinogen Decarboxylase. Biochem. J. 73:679-690, (1959).

Herbert, V., Aseptic Addition Method for Lactobacillus Casei Assay of Folate Activity in Human Serum, J. Clin. Path. 19:12-16, (1966).

Itzhaki, R., and Gill, D., A.Micro-Biuret Method for Estimating Proteins, Analytical Biochem. 9:401-410, (1964).

Jordan, P., and Shemin, D., Purification and Properties of Uroporphyrinogen I Synthetase from Rhodopseudomona Spherodies, J. Biol. Chem. 248(3):1019-1924, (1973).

Kagi, J., Himmelhoch, S., Whanger, P., Bethane, J. and Vallee, B., Equine Hepatic and Renal Metallothioneins, J. Biol. Chem. 249(11):3537-3542. (1974).

Kappas, A., Levere, R., and Granick, R., The Regulation of Porphyrin and Heme Synthesis, Seminars Hematol. 5:323-334, (1968).

Kaufman, S., The Structure of the Phenylalanine-Hydroxylation Cofactor, Proc. Nat. Acad. Sci. USA 50:1085-1092, (1963).

Kehoe, R., Normal Metabolism of Lead, Arch. Environ. Health. 8:232-235, (1964a).

Kehoe, R., Metabolism of Lead Under Abnormal Conditions, Arch. of Environ. Health 8:235-243, (1964b).

Kehoe, R., Under What Circumstances is Ingestion of Lead Dangerous? pp. 51-58, Symposium on Environmental Lead Contamination, US Public Health Service Doc. #1440, Washington, D.C., (1966).

Kehoe, R., The Metabolism of Lead in Man in Health and Disease, The Harben Lectures, (1960), J. Roy. Inst. Public Health Hyg. 24:81-96, 101-120, 129-143, 177-203, (1961).

King, B., Schaplowsky, A., and McCabe E., Occupational Health and Child Lead Poisoning: Mutual Interests and Special Problems, Am. J. of Public Health, 62:1056-1059, (1972).

Lin-Fu, J. Undue Absorption of Lead Among Children -- A New Look at an Old Problem, N. Eng. J. of Med. 286:702-710, (1972).

Lin-Fu, J., Vulnerability of Children to Lead Exposure and Toxicity, N. Eng. J. of Med., Part I. 289(23):1229-1233, (1973), Part II. 289(24):1289-1293, (1973).

Llambias, E., and Batlle, A., Uroporphyrinogen III Cosynthetase, Evidence for the Existence of a Polypyrolic Substrate in Soybean Callus Tissue, FEBS Letters, 6(3):285-288, (1970).

Llambias, E., and Batlle, A., Porphyrin Biosynthesis in Soybean Callus. V. The Porphobilinogen Deaminase - Uroporphyrinogen Cosynthetase System. Kinetic Studies, Biochemica et Biophysica Acta. 220:552-559, (1970).

Llambias, E. and Battle, A., Porphyrin Biosynthetase VIII. Avian Erythrocyte Porphobilinogen Deaminase - Uroporphyrinogen Cosynthetase, Its Purification, Properties and the Separation of its Components. Biochemica et Biophysics Acta 227:180-191, (1971).

Llambias, E., Battle, A., Studies on the Porphobilinogen Deaminase - Uroporphyrinogen Cosynthetase System of Cultured Soyabean Cells, Biochem. J. 121:327-340, (1971).

Lockwood, W. and Benson, A., The Enzymic Condensation of Porphobilinogen to Porphyrins, Biochem. J. 75:372-381, (1960).

Lowry, O., Rosenbrough, N., Farr, A., and Randall, R., Protein Measurements with the Folin-Phenol Reagent, J. Biol. Chem. 193:265-275, (1951).

Mortensen, E., Determination of Erythrocyte Folate by Competitive Protein Binding Assay Preceeded by Extraction, Clinical Chemistry 22(7):982-992 (1976).

Nordmann, Y,, and Grandchamp, B., Conjugated effect of Light and Uroporphyrin on Porphobilinogen, Biochem. Med. 15:119-125 (1976).

National Academy of Sciences, Lead. Airborne Lead in Perspective. Biologic Effects of Atmospheric Pollutants. series. National Academy of Sciences, Wahington, D.C., (1972).

Piper, W., and Tephly, T. Differential Inhibition of Erythrocyte and Hepatic Uroporphyrinogen I Synthetase Activity by Lead. Life Sciences 14: 873-876, (1974).

Piper, W., van Lier, R., Rios, G., and Tephly, T., Isolation of a Factor that Protects Against Lead Inhibition of Hepatic and Erythrocytic Uroporphyrinogen I Synthetase Activity. Life Sciences 19:1225-1234 (1976).

Piper, W., and van Lier, R., Pteridine Regulation of Inhibition of Hepatic Uroporphyrinogen I Synthetase Activity by Lead Chloride. Molecular Pharmacology (to be published (1977)).

Plusec, J., and Bogorad, L., A Dipyrrylmethane Intermediate in the Enzymatic Synthesis of Uroporphyrinogen. Biochemistry 9:4736-4743 (1970).

Pulido, P., Kagi, J., and Vallee, B., Isolation and Some Properties of Human Metallothionein. Biochemistry 5(5):1768-1777 (1966).

Rabinowitz, M., Wetherill, G., and Kopple, J., Kinetic Analysis of Lead Metabolism in Healthy Humans. J. Clin. Invest. 58:260-270 (1976).

Radmer, R., Bogorad, L., A. Tetrapyrrolic Intermediate in the Enzymatic Synthesis of Uroporphyrinogen. Biochemistry 11(5):904-910 (1972).

Robinson, E., Symposium on Environmental Lead Contamination. U.S. Public Health Service Doc #1440 (1966).

Rothenberg, S., MaCosta, M., and Rosenberg, Z., A Radioassay for Serum Folate: Use of a Two-Phase Sequential-Incubation, Ligand-Binding System. N. Eng. J. of Med., 286(25):1335-1339, (1972).

Sancovich, H., Batlle, A., and Grinstein, M., The Porphobilinogen Deaminase-Uroporphyrinogen III Cosynthetase System (Porphobilinogenase) from Bovine Liver. Kinetic Studies. FEBS Letters, 3(3):223-226 (1969a).

Sancovich, H., Batlle, A., and Grinstein, M., Porphyrin Biosynthesis: VI Separation and Purification of Porphobilinogen Deaminase and Uroporphyrinogen Isomerase from Cow Liver. Porphobilinogenase an Allosteric Enzyme. Biochimica et Biophysica Acta. 191:130-143 (1969b).

Scott, A., Ho, K., Kajiuwa, M., Kakahashi, T., Biosynthesis of Uroporphyrinogen III from Porphobilinogen. Resolution of Enigmatic "Switch" Mechanism. J. Amer. Chem. Soc. 98:1589-1591, (1976).

Shaikh, Z., and Lucis, O., Isolation of Cadmium-Binding Proteins. Experentia 27(9):1024-1025 (1971).

Shaw, W., Slade, B., Harrison, J., Nino, H., Assay of Serum Folate: Difference in Serum Folate Values Obtained by L. Casei Bioassay and Competitive Protein-Binding Assay. Clinical Biochemistry 7:165-178 (1974).

Stevens, E., and Frydman, B., Isolation and Properties of Wheat Germ Uroporphyrinogen III Cosynthetase. Biochimica et Biophysica Acta. 151:429-437 (1968).

Stevens, E., Frydman, R., and Frydman, B., Separation of Porphobilinogen Peaminase and Uroporphyrinogen III Cosynthetase from Human Erythrocytes. Biochimica et Biophysica Acta. 158:496-498 (1968).

Strand,L., Meyer, U., Felsher, B., Redeker, A., Marver, H. Decreased Red Cell Uroporphyrinogen I Synthetase Activity in Intermittent Acute Porphyria. J. Clin. Invest. 51:2530-2536, (1972).

Tephly, T., Hasegawa, E., and Baron, J., Effects of Drugs on Heme Synthesis in the Liver. Metabolism 20:200-214 (1971).

Tepper, L., Renal Function Subsequent to Childhood Plumbismn. Arch. Environ. Health 7:76-85 (1963).

Torres, A., Alvarez, V., and Sandberg, L., The Use of o-Phthaldialdehyde in the Detection of Proteins. Biochimica et Biophysica. Acta. 434:209-214 (1976).

Uyeda, K., and Rabinowitz, J., Fluorescence Properties of Tetrahydrofolate and Related Compounds. Analytical Biochem. 6:100-108 (1963).

van Lier, R., and Piper, W., Some Characteristics of a Factor Protecting Uroporphyrinogen I Synthetase Activity from Inhibition by Lead. Fed. Proc. 35:503 (1976).

van Lier, R., and Piper, W., Regulation of Lead Inhibition of Hepatic Uroporphyrinogen I Synthetase Activity by Pteridine Derivatives. Fed. Proc. 36:355, (1977).

Vostal, J., and Heller J., Renal Excretory Mechanisms of Heavy Metals. Environ. Res. 2:1-10 (1968).

Waldron, H., The Anemia of Lead Poisoning: A Review. Br. J. Ind. Med. 23:83-100 (1966).

Waters, A., and Mollin, D., Studies on the Folic Acid Activity of Human Serum. J. Clin. Path. 14:335-344 (1961).

Watson, C., Hawkinson, V., and Bossenmair, V., Some Studies of the Nature and Clinical Significance of Porphobilinogen. Tr. Ass. Amer. Physicians 66:144 (1953).

Webb, M., Binding of Cadmium Ions by Rat Liver and Kidney. Biochem. Pharm. 21:2751-2765 (1972).

Weidekamm, E., Wallach, D., Fluckiger, R., A New Sensitive, Rapid Fluorescence Technique for Determination of Proteins in Gel Electrophoresis and in Solution. Analytical Biochem. 54:102-114 (1973).

Weissberg, J., and Voytek, P., Liver and Red-Cell Porphobilinogen Synthetase in the Adult and Fetal

Guinea Pig. Biochimica et Biophysica Acta. 364:304-319 (1974).

Whitfield, C., and Weissbach, H., Binding of Substrate to N5-Methyl-Tetrahydropteroyltriglutamate-Homocysteine Transmethylase. Biochem. Biophysis. Res. Comm. 33:996-1003 (1968).

Zahler, W., and Cleland, W., A Specific and Sensitive Assay for Disulfides J. Biol. Chem. 243(4):716-719 (1968).

Zamenhof, S., Preparation and Assay of DNA from Animal Tissue, P. 696 [103], Methods in Enzymology Vol. III p. 792, (1957), ed. S.P. Colowick and N.O. Kaplan.