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Pteridine Regulation of Inhibition of Hepatic Uroporphyrinogen I Synthetase Activity by Lead Chloride

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SUMMARY

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A dialyzable factor has been purified from rat hepatic cytosol that protects against $PbCl_2$-mediated inhibition of uroporphyrinogen I synthetase (EC 4.3.1.8), an enzyme of the heme biosynthetic pathway. This factor also stimulated enzymatic activity which was antagonized by folic acid. The factor also produced growth of the folate-requiring microorganisms *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043), and antagonized binding of [^{3}H]folic acid to a specific folate-binding protein. These results suggested that the factor was a pteridine derivative. The protective effect against inhibition of uroporphyrinogen I synthetase activity by $PbCl_2$ was abolished by prior treatment of factor with γ-glutamyl carboxypeptidase (EC 3.4.12.10), indicating that the factor is a polyglutamated pteridine derivative. The estimated concentrations of factor (as pteroylglutamate) protecting against inhibition of enzyme activity by $PbCl_2$ (10 μM) were 3.3 pM (competitive protein binding radioassay) and 16 pM (microbiological assays), which indicates that the glutamate portion of the pteroylpolyglutamate factor does not merely chelate lead. Additional evidence ruling out chelation as a mechanism is the finding that concentrations of the factor in excess of those protecting against inhibition of uroporphyrinogen I synthetase activity by $PbCl_2$ failed to protect against inhibition of another lead-sensitive enzyme, δ-aminolevulinic acid dehydratase (EC 4.2.1.24). Pteroylhexaglutamate was found to protect against inhibition of uroporphyrinogen I synthetase activity by $PbCl_2$ at 1-10 μM concentrations. Pteroyltriglutamate and pteroylmonoglutamate were unable to protect against inhibition of enzymatic activity by $PbCl_2$. It is suggested that pteroylpolyglutamate derivatives may be regulators of uroporphyrinogen I synthetase activity, and may serve as coenzymes for this enzyme.

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INTRODUCTION

Uroporphyrinogen I synthetase (EC 4.3.1.8), an enzyme of heme biosynthesis, catalyzes the conversion of porphobilinogen to uroporphyrinogen I. Under certain circumstances (acute intermittent porphyria) this step can become rate-limiting for the synthesis of heme. It has been demon-

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strated by Strand *et al.* (1) and Meyer *et al.* (2) that the erythrocyte levels of activity for this enzyme are significantly lower in individuals afflicted with acute intermittent porphyria, an observation that has led to a rapid, simple test for carriers of this disease. It is also known that patients with lead intoxication may excrete abnormally high amounts of porphobilinogen into the urine (3, 4), which prompted us to study the effect of lead chloride on uroporphyrinogen I synthetase activity.

It has previously been shown that $PbCl_2$ *in vitro* produces inhibition of uro I[2] synthetase activity present in hemolysates of the rat, but has no effect on enzyme activity present in the cytoplasmic fraction of rat liver (5) unless a low molecular weight, heat-stable factor is removed by dialysis or purification of the enzyme (6). In this report results are presented which identify pteridine derivatives as protectors against inhibition of uro I synthetase by PbCla62, and also as activators of the enzyme.

MATERIALS AND METHODS

Materials. Ecteola cellulose, folic acid, 5-methyltetrahydrofolic acid, and *o*-phthalaldehybe were purchased from Sigma, and 5-formyltetrahydrofolic acid, from Lederle. 10-Formyltetrahydrofolic acid was prepared from 5-formyltetrahydrofolic acid by the method of Rabinowitz (7). Dihydrofolic acid was either purchased from Sigma or prepared by reduction of folic acid with sodium dithionite (8). Pteroyltriglutamate and pteroylhexaglutamate were generous gifts from Dr. E. L. R. Stokstad. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann; porphobilinogen, from Porphyrin Products, Salt Lake City; Bio-Gel P-30 (100–200 mesh) and DEAE-cellulose, from Bio-Rad; and crystalline bovine serum albumin, from Calbiochem. Cultures of *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043) were obtained from the American Type Culture Collection, and folate culture media, from Difco. Thin-layer chromatography plates (MN300F- cellulose) were purchased from Analtech. [3′,5′,9(*n*)-^{3}H]Folic acid (63 Ci/mmole) was purchased from Amersham/Searle.

Animals. Sprague-Dawley rats (180–220 g) were obtained from Simonson Labs, Gilroy, Cal.

Uroporphyrinogen I synthetase purification. Rats were killed by decapitation, livers were perfused *in situ* with ice-cold 0.9% NaCl, and 33⅓% (w/v) homogenates were prepared in 50 mM Tris-HCl buffer, pH 8.0, using a hand-operated glass (Dounce) homogenizer. This buffer was used throughout the enzyme purification studies. Homogenates were centrifuged at 25,000 × *g* for 20 min at 5°, and the supernatants were removed and centrifuged at 105,000 × *g* for 1 hr to obtain the hepatic cytosol fraction. The cytosol fraction was incubated in a water bath at 60° for 5 min, followed by resuspension, and centrifugation. The supernatant was brought to 40% ammonium sulfate saturation and centrifuged, the pellet was discarded, and the remaining supernatant was brought to 60% ammomium sulfate saturation followed by centgifugation. The pellet was suspended in and dialyzed against buffer. This enzyme preparation was subjected to chromatography on DEAE-cellulose (1.7 × 24 cm). Columns were equilibrated with buffer, and the enzyme was eluted with a linear gradient of 0–0.4 M KCl in buffer. The eluted fractions containing the enzyme were combined and concentrated with an Amicon model 52 ultrafiltgation unit, using a PM-10 filter. The concentrated enzyme preparation was then subjected to gel chromatography with Sephadex G-100 (2.5 × 66 cm) which had been equilibrated with buffer. Unless designated otherwise, preparations at the Sephadex G-100 purification step were used as the enzyme source for experiments.

Isolation of factor that protects against inhibition of hepatic uroporphyrinogen I synthetase activity by lead chloride. Rat hepatic cytosol preparations were added to Bio-Gel P-30 columns (2.5 × 60 cm) that had been equilibrated with water. The protective factor was located by test-

[2] The abbreviations used are: uro I, uroporphyrinogen I; ALA, δ-aminolevulinic acid.

ing aliquots of fractions for their ability to protect against inhibition of purified enzyme by $PbCl_2$ (6). Fractions containing the factor were pooled, concentrated by lyophilization, and subjected to chromatography on Ecteola cellulose (0.9 × 40 cm) which had been conditioned by suspension in 5 mM NaCl and adjustment of the pH to 7.0 with 1 N HCl (9), followed by equilibration with water. Columns were equilibrated and eluted with a freshly prepared linear gradient of 0–0.1 M ammonium formate at pH 7.0. Fractions (4.5 ml) containing the protective factor were pooled, concentrated by lyophilization, dissolved in small volumes of water, and stored frozen at −20°.

The quantity of purified factor was expressed in terms of units, with 1 unit being defined as that amount of factor necessary to cause a 10-fold increase in the $PbCl_2$ concentration required to maintain 50% inhibition of uro I synthetase activity (Fig. 1).

Microbiological assay for folates. The microbiological assays of folates using *L. casei* and *S. faecalis* were modifications of the methods of Herbert (10) and Waters *et al.* (11). Growth of the organisms was

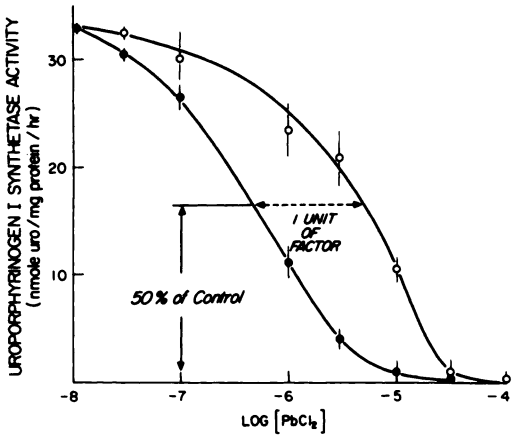

FIG. 1. *Inhibition of purified rat hepatic uroporphyrinogen I synthetase activity by lead chloride*

Each point represents the mean ± standard error of three determinations. ●——●, uro I synthetase activity in the absence of factor; ○——○, uro I synthetase activity in the presence of 1 unit of factor.

determined by measurement of turbidity at 660 nm after incubation in a water bath at 37° for 18 hr.

Competitive protein binding assay for folate. The assay of folate by competitive protein binding was a modification of the method of Mortensen (12), in which the extraction procedure was eliminated and 0.1 M Tris-HCl, pH 9.3, was used as the reaction buffer. Folic acid was used as the standard. The binding protein was purified from milk by the method of Rothenberg *et al.* (13).

Preparation of γ-glutamyl carboxypeptidase. Chick pancreas acetone powder (14) was used as the source of γ-glutamyl carboxypeptidase, which was isolated by the method of Bird *et al.* (15).

Uroporphyrinogen I synthetase assay. The procedure of Strand *et al.* (1) was used for the measurement of uroporphyrinogen I synthetase activity. Enzyme was incubated at 37° in the presence of 50 μmoles of Tris-HCl, pH 8.0, and 0.15 μmoles of porphobilinogen in a volume of 1.5 ml. Reaction mixtures were stopped and the uroporphyrinogen oxidized to uroporphyrin by addition of 1.5 ml of 2 N $HClO_4$–95% ethanol (1:1, v/v). The tubes were centrifuged at 2000 × *g* to remove any precipitated protein. Uroporphyrin in the supernatants was 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.

δ-Aminolevulinic acid dehydratase assay. The activity of ALA dehydratase was assayed by measurement of the rate of porphobilinogen formation, using a modification of the method of Gibson *et al.* (16).

Protein. The concentration of protein was determined by measurement of the absorbance at 280 nm, the procedure of Lowry *et al.* (17), or the *o*-phthalaldehyde method (18, 19), using crystalline bovine serum albumin as the standard.

Statistics. For determining the significance of differences between means, data were subjected to analysis by Student's *t*-test.

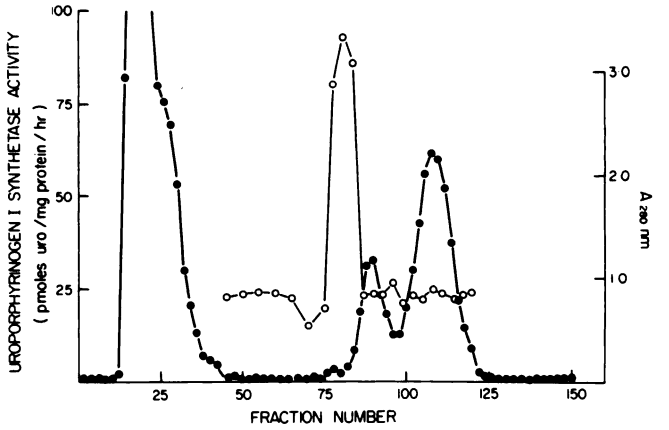

FIG. 2. *Bio-Gel P-30 purification of factor protecting against inhibition of hepatic uroporphyrinogen I synthetase activity by lead chloride*

Aliquots of hepatic cytosol were applied to a Bio-Gel P-30 column (2.5 × 60 cm), and the protective factor was located in the column fractions by analyzing their ability to reverse inhibition of uro I synthetase activity by lead chloride (100 μM) in dialyzed hepatic cytosol. ●——●, A_{280}; ○——○, uro I synthetase activity in the presence of eluate (0.1 ml) and lead chloride (100 μM). The control activity of uro I synthetase was 102 pmoles of uro I per milligram of protein per hour. Lead chloride produced a decrease in activity to 22 pmoles/mg of protein per hour.

RESULTS

Purification of protective factor from hepatic cytosol. Chromatography on Bio-Gel P-30 was initially employed (Fig. 2) in order to isolate the protective factor from hepatic cytosol, and the elution position was followed by testing aliquots of each fraction for their ability to protect against inhibition of purified uro I synthetase by $PbCl_2$ (6). An additional purification step using Ecteola cellulose was added (Fig. 3).

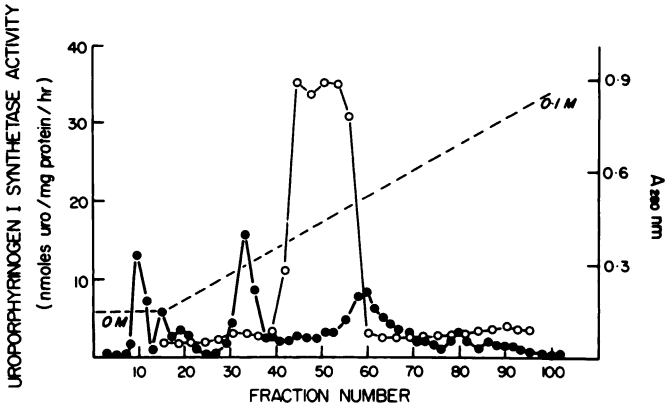

FIG. 3. *Ecteola cellulose purification of factor protecting against inhibition of hepatic uroporphyrinogen I synthetase activity by lead chloride*

Pooled fractions of factor from chromatography of hepatic cytosol on Bio-Gel P-30 were concentrated by lyophilization and chromatographed on Ecteola cellulose (0.9 × 40 cm) as described in MATERIALS AND METHODS, and the protective factor was located as described in Fig. 2. Purified uro I synthetase (Sephadex G-100 step) was used as the enzyme source. ●——●, A_{280}; ○——○, uro I synthetase activity in the presence of eluate (0.5 ml) and lead chloride (10 μM). The control activity of uro I synthetase was 36.7 nmoles of uro I per milligram of protein per hour. Lead chloride (10 μM) produced a decrease in activity to 2.1 nmoles/mg of protein per hour. The linear gradient of ammonium formate (0–0.1 M) is superimposed on the elution pattern.

Factor preparations purified by Ecteola cellulose were subjected to thin-layer chromatography on MN-cellulose in three different solvent systems: 5% NH_4HCO_3, pH 7.8; 5% ammonium carbamate, pH 9.0; and 95% ethanol–3% NH_4Cl (70:30), pH 6.0. The cellulose was scraped from the plate at 0.5-cm intervals and eluted with water, and aliquots were tested for their ability to protect against inhibition of uro I synthetase activity by $PbCl_2$. In each case a single spot corresponded to the protective factor, with R_F values of 0.74, 0.77, and 0.46 in the three solvent systems, respectively.

In order to estimate the degree of purification and recovery of factor from liver, *o*-phthalaldehyde-reactive (18, 19) material and units of factor were measured in aliquots at each purification step (Table 1). The *o*-phthalaldehyde reacts with primary amines and has been used for microanalysis of proteins at 0.1 μg/ml concentrations. As shown in Table 1, an estimated 1792-fold purification of the factor from hepatic cytosol was achieved. Factor preparations at the Ecteola cellulose purification step were judged to have no appreciable protein content, since factor was completely recoverable following ultrafiltration (Amicon UM2 membrane; molecular weight cutoff of 1000). Therefore the small amount of *o*-phthalaldehyde-reactive material at the Ecteola cellulose purification step is probably not protein in nature. The factor might contain a primary amine group, which would be capable of reacting with *o*-phthalaldehyde.

Purification of uroporphyrinogen I synthetase. The enzyme was purified from rat hepatic cytosol as described in MATERIALS AND METHODS. The purification of uro I synthetase is shown in Table 2. Purified enzyme (Sephadex G-100 step) was stable for several months when stored at −20°.

Properties of protective factor. Studies reported previously (6) demonstrated that the dialyzable factor was heat-stable (100° for 30 min), but that the protective effect was 80% destroyed by ashing and 50% destroyed by acid or base treatment. In addition, the factor was devoid of sulfhy-

TABLE 1

Purification of protective factor from rat liver

Step	Volume	*o*-Phthalaldehyde-reactive material[a]	Factor content	Specific activity	Yield	Purification
	ml	*mg/ml*	*units*	*units/mg*	*%*	*-fold*
Hepatic cytosol	25.0	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. *o*-Phthalaldehyde is known to react with primary amines, such as proteins and amino acids (18, 19).

TABLE 2

Purification of uroporphyrinogen I synthetase from rat liver

Fraction	Volume	Protein concentration	Total activity[a]	Specific activity	Yield	Purification
	ml	*mg/ml*	*units* × 10^{-3}	*units/mg*	*%*	*-fold*
Homogenate	250	43.7	6.08	0.56	100	1
25,000 × *g* supernatant	155	28.4	5.89	1.34	97	2.4
Cytosol	119	23.2	4.31	1.56	71	2.8
Heat-treated cytosol	91	4.59	5.30	12.7	87	22.8
Ammonium sulfate, 40–60%	14.4	14.1	2.74	13.5	45	24.2
DEAE-cellulose	56	0.49	1.89	68.9	31	123.4
Sephadex G-100	33	0.047	0.96	619	16	1107

[a] One unit is defined as 1 pmole of uroporphyrinogen I formed per minute.

dryl content (6) and did not exhibit the definitive patterns of the metal-binding protein metallothionein (20) on polyacrylamide gels. Furthermore, metallothionein was unable to protect against inhibition of uro I synthetase activity by $PbCl_2$ (6). Thus it was readily apparent that the factor was a substance different from metallothionein, and might not be a macromolecule.

When purified factor preparations were analyzed by ultraviolet and fluorescence spectroscopy, they were found to have an absorption peak at 265 nm and fluorescence excitation and emission maxima at approximately 320 and 450 nm, respectively, which suggested that the factor might be a pteridine. Various folate (pteroylmonoglutamate) derivatives were tested and found to be incapable of protecting against inhibition of uro I synthetase activity by $PbCl_2$ (Table 3). Dihydrofolate (dihydropteroylglutamate) was observed to have a significant stimulatory effect on uro I synthetase activity. Dihydrofolate failed to produce significant increases in uro I formation in the presence of boiled enzyme, which indicates that the activation process is dependent upon enzymatic activity.

TABLE 3

Effects of some pteroylglutamates on inhibition of uroporphyrinogen I synthetase activity by lead chloride

Lead chloride and the pteroylglutamates [PteGlu, where H_2PteGlu denotes dehydropteroylglutamate (dihydrofolate), etc.] were present at 10 μM. Values are the means ± standard errors of three determinations.

Pteroylglutamate	Uro I synthetase activity
	nmoles urol/mg protein/hr
None	32.9 ± 0.6
+$PbCl_2$	1.0 ± 0.2
PteGlu	31.2 ± 0.5
+$PbCl_2$	0.9 ± 0.1
H_2PteGlu	55.5 ± 0.5
+$PbCl_2$	2.9 ± 0.2
5-Methyl-H_4PteGlu	27.3 ± 0.5
+$PbCl_2$	0.8 ± 0.1
5-Formyl-H_4PteGlu	33.1 ± 0.8
+$PbCl_2$	0.9 ± 0.1
10-Formyl-H_4PteGlu	26.5 ± 1.2
+$PbCl_2$	0.1 ± 0.1

It was also apparent that the factor was a stimulator of uro I synthetase activity and that folic acid (pteroylglutamate) produced a slight but significant stimulation of enzyme activity at a concentration of 100 μM (Fig. 4). Furthermore, the stimulatory effect of the factor on uro I synthetase activity could be antagonized by folic acid, which suggested that the factor might be a pteridine derivative. The factor failed to increase uro I formation in the presence of boiled enzyme, indicating that the activation process depends upon enzymatic activity.

In order to obtain additional evidence that the protective factor was a pteroylglutamate derivative, aliquots of factor preparations were added to the assay media for the folate-dependent organisms *L. casei* and *S. faecalis*.

As shown in Table 4, factor preparations at the lower quantities of 16 or 32 units were inactive in promoting growth of either organism. However, growth of both organisms was observed with higher amounts (152 units/assay) of factor.

To test further whether the factor was a pteroylglutamate derivative, aliquots of factor preparations were incubated with purified milk binding protein in the presence of [^{3}H]folic acid. Factor (13 units) was found to be incapable of antagonizing [^{3}H]folate binding to the binding protein (Table 5). However, antagonism of [^{3}H]folate binding was observed with higher amounts (19, 38, or 76 units/assay) of factor.

Growth of the folate dependent organisms and antagonism of [^{3}H]folate binding to folate-binding protein by the factor indicated that it is a pteroylglutamate derivative. Estimated values of picomoles of factor (as pteroylglutamate) per unit by competitive protein radioassay were approximately one-fifth those obtained from microbiological assays employing *L. casei* and *S. faecalis*. This variation in the magnitude of the pteroylglutamate content might possibly be explained by the differential responses of pteroyl*poly*glutamates in these assay systems. It is known that most of the pteroylglutamate isolated from tissues exists in pteroylpolyglutamate

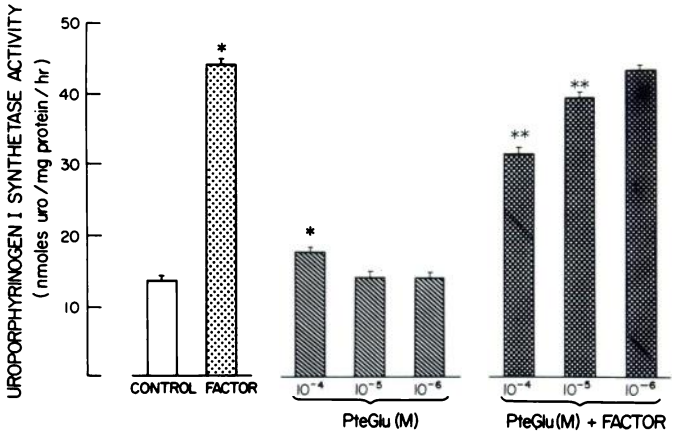

FIG. 4. *Antagonism of factor stimulation of hepatic uroporphyrinogen I synthetase activity by folic acid*
Each value represents the mean ± standard error of three determinations. Reaction mixtures with factor contained 8.4 units.
* Significant difference between result with pteroylglutamate (PteGlu) or factor and control ($p < 0.05$).
**Significant difference between result with factor plus pteroylglutamate and factor alone ($p < 0.05$).

TABLE 4

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 pteroylglutamate contents by microbiological assay were 0.4 and 1.5 pmoles for *L. casei* and *S. faecalis*, respectively.
[b] The numbers in parentheses denote picomoles of factor (as pteroylglutamate) per unit.

TABLE 5

Assay of factor by competitive protein binding radioassay

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

[a] The minimum detectable pteroylglutamate content by competitive protein binding assay employing [^{3}H]folate was 0.1 pmole.
[b] The numbers in parentheses denote picomoles of factor (as pteroylglutamate) per unit.

forms (21), with reduced pteroylpenta- and -hexaglutamates being the predominant forms isolated from rat liver (22-25). In addition, γ-glutamyl carboxypeptidases (conjugates) have been isolated and purified from liver and other tissues that hydrolyze these pteroylpolyglutamates (15, 26) to the various folate derivatives and glutamic acid. Various pteroylpolyglutamates do not differ appreciably from pteroylmonoglutamates when determined by competitive protein binding radioassays (27, 28). However, the growth response of *L. casei* and *S. faecalis* is known to be markedly depressed by increasing the glutamate chain length (21, 29). Therefore it is probable that the factor exists as a pteroylpolyglutamate derivative.

Experiments were conducted to determine whether the factor might be a pteroylpolyglutamate. Aliquots of factor were incubated with chick pancreas γ-glutamyl carboxypeptidase preparations, boiled to inactivate the enzyme, and centrifuged. Controls (boiled enzyme) were also conducted. Aliquots of the supernatants from controls and γ-glutamyl carboxypeptidase-treated reaction mixtures were spotted on a thin-layer plate of MN-cellulose and developed in 5% NH_4HCO_3, pH 7.8. The cellulose was then scraped off the plates at 0.5-cm intervals and eluted with water, and the eluates were incubated with uro I

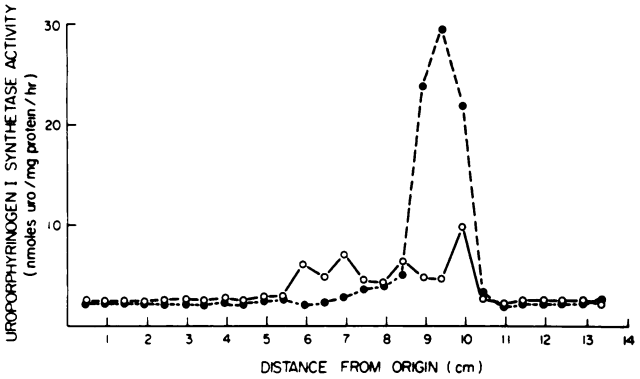

FIG. 5. *Abolition of factor protection against lead chloride-mediated inhibition of hepatic uroporphyrinogen I synthetase activity by prior treatment of factor with γ-glutamyl carboxypeptidase*

Factor (63.3 units) was incubated at 37° for 2 hr with chick pancreas γ-glutamyl carboxypeptidase and 50 mM ammonium formate, pH 7.0. Incubation mixtures were boiled for 10 min to inactivate the enzyme and centrifuged, and the supernatant was lyophilized. The material was dissolved in 25 μl of H_2O, and 20-μl aliquots were spotted on MN-cellulose thin-layer plates and developed in 5% NH_4HCO_3, pH 7.8. The cellulose was scraped off the plates at 0.5-cm intervals and eluted with 0.5 ml of water, and 0.4 ml of each eluate was incubated with uro I synthetase in the presence of 10 μM lead chloride. 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.

synthetase in the presence of lead chloride.

As shown in Fig. 5, the factor protection against inhibition of uro I synthetase activity by $PbCl_2$ was abolished by prior incubation with γ-glutamyl carboxypeptidase. Treatment of factor with boiled γ-glutamyl carboxypeptidase preparations failed to abolish the protective effect, which indicates that the destruction of the protective factor was an enzymatic process.

In order to substantiate further the role of pteroylpolyglutamates as regulators of inhibition of uro I synthetase activity by $PbCl_2$, synthetic derivatives of various chain lengths were obtained and tested. Pteroylmonoglutamate and pteroyltriglutamate were unable to protect against inhibition of uro I synthetase activity by $PbCl_2$ (Table 6). However, pteroylhexaglutamate was found to protect against inhibition of enzymatic activity by $PbCl_2$.

One possible mechanism for the ability of pteroylpolyglutamates to protect against inhibition of an enzyme by $PbCl_2$ would be chelation of the lead by the glutamate moieties. If pteroylpolyglutamates act by such a mechanism, they should protect against inhibition by $PbCl_2$ of other enzymes that are known to be sensitive to this metal. It order to test this concept, aliquots of factor were tested for their ability to protect against $PbCl_2$-mediated

TABLE 6

Effects of pteroylglutamates of variable glutamate chain lengths on inhibition of uroporphyrinogen I synthetase activity by lead chloride

The concentration of $PbCl_2$ was 10 μM. The pteroylglutamates (PteGlu, where $PteGlu_3$ denotes pteroyltriglutamate, etc.) were added at the concentrations shown. Values are the means ± standard errors of three determinations.

Addition	Pteroylglutamate concentration	Uro I synthetase activity
	μM	*nmoles uro I/mg protein/hr*
None	0	31.1 ± 0.1
+$PbCl_2$	0	0.8 ± 0.1
PteGlu	10	29.4 ± 0.5
+$PbCl_2$		0.8 ± 0.1
$PteGlu_3$	10	29.0 ± 0.4
+$PbCl_2$		0.9 ± 0.1
$PteGlu_6$	10	32.3 ± 0.3
+$PbCl_2$		30.2 ± 0.7[a]
$PteGlu_6$	1	32.5 ± 0.3
+$PbCl_2$		3.6 ± 0.5[a]

[a] Significant difference from result with $PbCl_2$ alone ($p < 0.05$).

inhibition of δ-aminolevulinic acid dehydratase (EC 4.2.1.24), an enzyme of the heme biosynthetic pathway that is known to be extremely sensitive to lead (30). ALA dehydratase, like uro I synthetase, was assayed in 1.5-ml reaction mixtures. Two units of factor were sufficient to provide complete protection against inhibition of uro I synthetase activity by $PbCl_2$ (10 μM). However, concentrations of the factor approximately 4 times higher (8.4 units) than required to restore lead chloride (10 μM)-inhibited uro I synthetase activity to control values were unable to protect even partially against inhibition of ALA dehydratase activity by $PbCl_2$ (Table 7). Glutamic acid was found to protect against inhibition of uro I synthetase activity by $PbCl_2$, but only at 1 mM concentrations or greater.

DISCUSSION

Inhibition of hepatic uro I synthetase activity by lead chloride has been found to be regulated by a dialyzable, protective factor that was also found to be a stimulator of enzyme activity. Antagonism of factor stimulation of uro I synthetase activity by folic acid, growth of the folate-dependent microorganisms *L. casei* and *S. faecalis*, and antagonism of [^{3}H]folate binding to purified folate-binding protein are evidence that the factor is a pteroylglutamate derivative. Abolition of the ability of factor to protect against lead chloride-mediated inhibition of hepatic uro I synthetase activity by γ-glutamyl carboxypeptidase treatment indicates that the factor is a pteroyl*poly*glutamate derivative. Synthetic pteroylhexaglutamate was also found to protect against inhibition of uro I synthetase activity by lead chloride.

It is possible to estimate the amount of factor (as pteroylglutamate) that is required to protect uro I synthetase activity from inhibition by lead chloride, based upon data obtained from the microbiological and competitive protein binding assays. Approximately 2 units of factor are required for complete protection of uro I synthetase activity by 10 μM $PbCl_2$; this consists of only 0.01–0.05 pmol of factor (as pteroylglutamate), as shown in Tables 4 and 5. Since the volume of the reaction mixtures was 1.5 ml, factor (as pteroylglutamate) was estimated to be present at 6.7–33 pM concentrations. Thus the pteroylpolyglutamate factor does not appear to protect merely by chelating lead and preventing the metal from reaching the enzyme. Additional evidence to indicate that the pteroylpolyglutamate factor does not chelate lead are the findings that concentrations of factor in excess of those required to protect against inhibition of uro I synthetase activity by lead chloride were incapable of protecting against inhibition of another lead-sensitive enzyme, ALA dehydratase.

The pteroylpolyglutamate factor appears to be a better protector than pteroylhexaglutamate against inhibition of hepatic uro I synthetase activity by lead chloride. Only pteroylhexaglutamate concentrations in the range of 1–10 μM were found to protect uro I synthetase from inhibition by lead chloride; whereas, it is estimated that 6.7–33 pM concentrations of factor can protect. One plausible explanation is that the pteroylpolyglutamate factor has functional groups on the pteridine ring which enable it to interact more efficiently with the enzyme to prevent lead from reaching specific enzymatic sites and causing inhibition.

Factor preparations, as well as dihydrofolate, were consistently observed to produce activation of uro I synthetase activity. Upon storage, the activation produced by factor was found to diminish. These

TABLE 7

Failure of factor to protect against inhibition of δ-aminolevulinic acid dehydratase activity by lead chloride

The $PbCl_2$ concentration was 25 μM. Each reaction mixture contained 8.4 units of factor. Values are the means ± standard errors of three determinations.

Addition	ALA dehydratase activity
	nmoles/mg protein/hr
None	6.59 ± 0.06
$PbCl_2$	2.59 ± 0.08
Factor	6.57 ± 0.05
$PbCl_2$ + factor	2.66 ± 0.07

findings suggest that certain labile pteroylglutamate derivatives may be regulators of uro I synthetase activity, and that a pteridine coenzyme may exist for this enzyme. Studies are currently being conducted to define the role of various pteridine derivatives in the stimulation of uro I synthetase activity and the enzymatic conversion of porphobilinogen to uroporphyrinogen.

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