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PORPHYRIN BIOSYNTHESIS

III. PORPHYRIN METABOLISM IN EXPERIMENTAL LEAD POISONING

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SUMMARY

Porphyrinogen and heme synthesis in whole blood, hemolysates, and supernatant of hemolysates from lead-poisoned rabbits and control rabbits were studied *in vitro*. [2-^{14}C]Glycine, δ-aminolaevulic acid, δ-amino[4-^{14}C]laevulic acid, porphobilinogen, [^{14}C]uroporphyrinogen III, [^{14}C]coproporphyrinogen III and Fe^{2+} ions were the basic substrates employed.

With [2-^{14}C]glycine as the substrate, free porphyrinogen synthesis by the lead-poisoned system was depressed as was incorporation of the substrate into heme. When starting with δ-aminolaevulic acid, there was a general decrease in all porphyrinogens synthesized, but most particularly for uro- and phyriaporphyrinogens. Incorporation of δ-amino[4-^{14}C]laevulic acid into heme was also diminished. In contrast, when porphobilinogen and [^{14}C]uroporphyrinogen III were the substrates, there were no significant differences in porphyrin synthesis between the lead-poisoned and control systems and comparable amounts of uroporphyrinogen III and phyriaporphyrinogen III were metabolized. Nevertheless, an accumulation of phyriaporphyrinogen III, relative to the amounts of coproporphyrinogen III, occurred. These results demonstrate that one of the principal blocks in the biosynthetic pathway of protoporphyrin 9 in lead poisoning is at the δ-aminolaevulic acid dehydrase (EC 4.2.1.24) level, and that a second block, quantitatively less important, occurs between phyriaporphyrinogen III and coproporphyrinogen III.

[^{14}C]Coproporphyrinogen III incorporation into free protoporphyrin 9 and heme was decreased in the lead-poisoned systems. The well-documented accumulation of both these intermediates could be satisfactorily explained on the basis of lead impairing the transport of iron or other metabolites through the mitochondrial membrane.

INTRODUCTION

The enzymic systems controlling porphyrin and heme synthesis have been located in two distinct fractions of the immature red blood cell. The glycine to δ-amino-

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laevulic acid steps take place in particulate preparations. Those in which δ-aminolaevulic acid is the basic substrate leading to the formation of coproporphyrinogen III are found in the soluble fraction and remain active after the erythrocytes mature. The conversion of coproporphyrinogen III to protoporphyrin 9 and heme appears to be linked to the mitochondria of erythrocyte precursors including reticulocytes[1–8].

There have been numerous reports of abnormal levels of porphyrins and porphyrin precursors in urine, serum, circulating erythrocytes, and bone marrow in lead poisoning (for review see ref. 9). Included among these are elevated urinary levels of δ-aminolaevulic acid, porphobilinogen, uroporphyrin I (refs. 9–12), and coproporphyrin III (refs. 11–15). In addition, there are reports of elevated serum δ-aminolaevulic acid[9,16], increased glycine[11], uroporphyrin I (refs. 17, 18), coproporphyrin III (refs. 17, 19), and protoporphyrin 9 (ref. 17) in bone marrow. Increased levels of the latter two porphyrins have been found in circulating erythrocytes[11,17].

Further evidence of disturbed heme metabolism in lead poisoning can be inferred from the following findings: elevated levels of serum iron[20], abnormally increased number of siderocytes[21], and an accumulation of iron granules in marrow reticulum cells[22]. Lead, in addition to acting on the biosynthesis of hemoglobin, also has been shown to shorten erythrocyte life span[23].

Previous studies have demonstrated that the coproporphyrinuria, which is secondary to elevated coproporphyrinemia[11], is not the result of hemoglobin catabolism, but rather from an anabolic origin[14].

In an effort to clarify some of the mechanism responsible for these changes in porphyrin and heme synthesis, studies of the metabolic steps between glycine and heme were undertaken. Measurement of the newly synthesized porphyrinogens and heme in both soluble and particulate fractions of the blood of lead-poisoned rabbits incubated *in vitro* with different substrates, was taken as an index of the enzymic activity.

EXPERIMENTAL

Materials

δ-Aminolaevulic acid was purchased from the Sigma Chemical Co. (U.S.A.) and used without further purification. δ-Amino[4-^{14}C]laevulic acid (specific activity 3.1 mC/mmole) and [2-^{14}C]glycine (specific activity 6.1 mC/mmole) were purchased from New England Nuclear Corporation (U.S.A.). Porphobilinogen was isolated from the urine of a patient suffering from acute porphyria, according to the method described by COOKSON AND RIMINGTON[24]. [^{14}C]Uroporphyrin III (specific activity 780 counts/min per μg) and [^{14}C]coproporphyrin III (specific activity 980 counts/min per μg) were prepared from a biosynthetic system (chicken red blood cell supernatant) *in vitro*, using δ-amino[4-^{14}C]laevulic acid as the substrate. Unlabelled uroporphyrin III was isolated from turacine by a method to be published. Unlabelled coproporphyrin III was isolated from the urine of lead-poisoned rabbits (tetramethyl ester m.p. 142–144°, double 167–170°). $CaCO_3$ used for column chromatography was obtained from May and Baker, Ltd. (Great Britain); Al_2O_3 (chromatography grade) used for batch adsorption of porphyrins was obtained from Merck Co. All reagents were of A.R. grade. Glass-distilled water was used throughout.

Methods

New Zealand rabbits of both sexes, weighing 1.5–2.5 kg, were kept in metabolic cages and fed a standard laboratory diet (Forramez, Molinos Río de la Plata). Lead poisoning was induced by the daily subcutaneous injection of a solution of lead acetate (20 mg Pb^{2+} per ml; pH 5.5) with a dosage range of 20–40 mg as Pb^{2+} per kg body weight per day. Approx. 24 days after the end of a 7–14-day period of treatment, blood was drawn by cardiac puncture and collected in flasks containing heparin (0.5 mg/ml of blood). At this time, these animals showed a typical anemia of lead poisoning: hematocrit ranging from 23 to 35 %, mean 28.8 %; hemoglobin ranging from 6.8 to 10.4 g %, mean 8.3 g %, with punctate basophilia, and reticulocytosis ranging from 5 to 16.6 %, mean 8.6 %. Coproporphyrinuria was present, ranging from 127 to 710 μg %, mean 365 μg %.

Control studies were carried out on either normal rabbits or anemic rabbits in which reticulocytosis was induced by repeated cardiac puncture (15–30 ml removed on two to four occasions at intervals of 3–5 days, followed by iron therapy).

For incubation the tissue systems were prepared as follows: (a) Whole blood; used directly without further manipulations. (b) Washed cells; the whole blood was centrifuged, the plasma aspirated, and the cells were washed three times with 0.9 % NaCl at 0°. The buffy coat was not removed. The cells were then re-suspended in normal saline to restore the volume to that of the original aliquot of blood. (c) Hemolysate; the washed cells as in (b) were mixed with 1–2 vol. of cold, distilled water, kept in a refrigerator for 60 min and occasionally shaken. The supernatant of these hemolysates was obtained by centrifuging for 60 min at 24000 × *g* at 0°.

Red blood cell counts and hematocrits were done by standard methods. Reticulocytes were counted in dried blood smears stained with 1 % brilliant cresyl blue. From the red cell blood count, the per cent of reticulocytes, and the volume of the aliquot used, the total number of reticulocytes present in each flask was calculated. The number of red cells showing basophilic stippling was counted in peripheral blood using two different methods: Löffler's methylene blue and May–Grunwald–Giemsa. All determinations were performed in duplicate.

Urinary coproporphyrin was determined by the method of SCHWARTZ *et al.*[25]; δ-aminolaevulic acid and porphobilinogen by the method of MAUZERALL AND GRANICK[26]. Free erythrocyte protoporphyrin was measured by the micro-method of GRINSTEIN AND WINTROBE[27]. Porphyrinogens were prepared as described by BATLLE AND GRINSTEIN[28].

Incubations

Incubation was carried out in 100-ml conical flasks, either under continuous N_2 current or aerobically, at 37° with mechanical shaking at approx. 100 times per min. When porphyrinogens were the added substrate, the incubation was run in the dark. For each 10 ml of tissue preparation, hemolysate or supernatant of hemolysate, 0.5 ml of 0.12 M $MgCl_2$, 0.5 ml of 0.604 M KCl, and 0.1 ml of phosphate buffer (pH 7.0) were added. 0.5 mg of both streptomycin and penicillin, and 0.1–0.2 ml of octanol (anaerobic incubations) were placed in each flask along with the substrates: 5.0 mg of δ-aminolaevulic acid or 2.5 mg of porphobilinogen, both dissolved in 0.9 % NaCl (ref. 29), or the labelled porphyrinogens.

Glycine carrier was added to give a concentration of 1 mg/ml in the incubation

mixture. [2-^{14}C]Glycine was added in solution and the chosen activity was approx. $1 \cdot 10^6$ counts/min per flask (1 μC = $3.11 \cdot 10^5$ counts/min by our methods). Fe^{2+} was added as a freshly prepared solution of $FeSO_4 \cdot 7\ H_2O$: 2–3 μg of Fe^{2+} per ml in the final incubation mixture.

Isolation and assay procedures

After incubation, the system was treated with a mixture of 3 vol. of ethyl acetate and 1 vol. of acetic acid; the precipitated proteins were filtered off and washed three to four times with an identical solution. The washings were pooled and added to the filtrate. The combined mixture was then washed with distilled water to eliminate the acetic acid. The water extracts were treated in order to recover any porphyrins that might have been lost into the water. The porphyrins in the ethyl acetate solution were then completely extracted with 10% HCl. During these procedures, porphyrinogens are oxidized to porphyrins.

The total porphyrin concentration of the HCl extract was measured at the peak of the Soret band in a Beckman DU spectrophotometer. For calculations, the extinction coefficients given by SALUM *et al.*[30] were used.

The free porphyrins were esterified in different ways with similar results: (a) The HCl extract was adjusted to pH 3.0–3.2 with sodium acetate crystals (gray to congo red paper), the porphyrins were re-extracted into ethyl acetate and washed several times with distilled water to remove the excess acid. Finally the extract was evaporated to dryness under reduced pressure. The residue was left in a desiccator with NaOH pellets. For esterification the dry residue was dissolved in H_2SO_4–CH_3OH (5%, v/v) and allowed to stand at room temperature in the dark for 24–48 h. (b) The neutralized solution as in (a) was adsorbed on talcum and the porphyrins eluted with the H_2SO_4–CH_3OH mixture. (c) The neutralized solution as in (a) was adsorbed on Al_2O_3 and the porphyrins eluted with the same esterification mixture.

After esterification was completed, the esters were transferred to chloroform. Further analyses were carried out on $CaCO_3$ columns[31] and by paper chromatography[32]. Quantitative determinations were made by the spectrophotometric micromethod of BATLLE AND GRINSTEIN[33].

The porphyrins that had been separated and identified by paper chromatography for radioactivity determinations were eluted with chloroform, transferred to aluminum planchettes, and evaporated to dryness. Their activity was measured in a thin-window gas-flow counter (Nuclear–Chicago Model D 47), as a very thin layer so that no correction for self-absorption was necessary. From the specific activity so determined, and the amount of each present in the mixture, the total radioactivity due to each porphyrin was calculated.

After the free porphyrins had been removed from the hematin solution, the heme protoporphyrin was isolated. The ethyl acetate layer containing the acid hematin was washed twice with a similar volume of distilled water. Further washings were carried out with a great excess of water until all the hematin precipitated. This precipitate was collected on filter paper and was allowed to stand overnight at room temperature. The dry hematin was dissolved in CH_3OH–oxalic acid solution and protoporphyrin 9 was liberated and crystallized as its dimethyl ester, by the method of GRINSTEIN[34].

The radioactivity of the labelled [^{14}C]protoporphyrin 9 dimethyl ester was

TABLE I

INCORPORATION OF [2-^{14}C]GLYCINE INTO HEME BY WHOLE-BLOOD PREPARATIONS

Whole blood (20.0 ml for the lead-poisoning preparation, 10.0 ml for the control) was incubated for 4 h at 37° with [2-^{14}C]glycine (11.6 mM, specific activity 3324 counts/min per μmole). Fe^{2+} was added at a concentration of 2 μg/ml at time zero. Protoporphyrin dimethyl ester from heme was isolated as described in the text.

	Reticulocytes (%)	*Reticulocytes per flask × 10⁻⁹*	*^{14}C specific activity* (counts/min per mg)*	*Total counts/min incorporated*	*Incorporation (%)*	*[2-^{14}C]Glycine incorporated (mμmoles)*	*[^{14}C]Protoporphyrin 9 formed (mμmoles)*	*[^{14}C]Protoporphyrin 9 formed per 10^9 reticulocytes (mμmoles)*
Lead poisoning	10.6	9.3	10	574	0.065	173	22	2.3
Control	20.2	7.8	341	8700	0.98	1310	164	21.0

* Corrected for self-absorption.

Fig. 1. Paper chromatogram of the methyl esters of the porphyrins isolated from the incubation systems when δ-aminolaevulic acid was the substrate. Other experimental conditions are identical to those described in Table II. Broken lines, first development; solid lines, second development.

TABLE II

CONVERSION OF δ-AMINOLAEVULIC ACID INTO PORPHYRINOGENS BY SOLUBLE SYSTEMS

Hemolysate supernatant (12.0 ml) was incubated as described in the text for 90 min at 37° with 30 μmoles of δ-aminolaevulic acid (3.7 μmoles of porphyrin equivalent)[29] in a final volume of 17.0 ml. Porphyrinogens were determined as described in *Methods*, and are expressed as average results of duplicate flasks in a typical example of a series of similar experiments.

	Porphyrins recovered (mμmoles)	
	Lead-poisoned system	*Control*
Uroporphyrin III	7	110
Phyriaporphyrin III	10	71
Coproporphyrin III	18	22
Total	35	203

measured as described by BANNERMAN *et al.*[35] in a thin-window gas-flow counter. Samples ranged from 10.2 to 20.5 mg. A minimum of 4000 counts was always recorded.

RESULTS

Incorporation of [2-^{14}C]glycine into porphyrinogens and heme

Whole blood from lead-poisoned rabbits and anemic rabbits was incubated aerobically with [2-^{14}C]glycine in the presence of Fe^{2+} for 4 h. At the end of the incubation protoporphyrin 9 from heme and free porphyrinogens were isolated. The amount of porphyrinogens present in the system was small, so further analyses were not performed.

As can be seen from Table I the reticulocytes from lead-poisoned rabbits synthesized heme from glycine and Fe^{2+} ions to a lesser extent than did the controls. Although the rate of heme synthesis was diminished, the activity of the final sample was sufficient to undertake further studies to clarify the metabolic steps from glycine to heme.

Incorporation of δ-aminolaevulic acid into porphyrinogens

The supernatants from hemolysates of red blood cells of lead-poisoned rabbits and normal rabbits were incubated with δ-aminolaevulic acid under a current of N_2 for 90 min. After incubation the resulting porphyrins were isolated, identified, and measured as described above.

No qualitative differences could be found in the porphyrins biosynthesized by the lead-poisoned system (Fig. 1). Quantitatively, however, there was a marked discrepancy as can be seen in Table II. Uroporphyrinogen III and phyriaporphyrinogen III were greatly diminished and there was only a relatively small accumulation of coproporphyrinogen III. It must be emphasized that these systems were particle-free and under anaerobic conditions. In such circumstances, neither free protoporphyrin 9 nor heme is formed. This artificial block in the biosynthetic pathway may be the cause of the similar accumulations of coproporphyrin III in both systems, the lead-poisoned and the control.

Incorporation of δ-amino[4-^{14}C]laevulic acid into heme

Washed cells from lead-poisoned rabbits and anemic rabbits were incubated aerobically with δ-amino[4-^{14}C]laevulic acid and Fe^{2+} ions for 4 h. After incubation porphyrins were isolated, identified, quantitated, and their radioactivity measured as described in *Methods*. The results were comparable with those when only the soluble fractions of the hemolysates were used. Substrate incorporation into heme was found to be markedly decreased as can be seen in Table III.

Incorporation of porphobilinogen into porphyrinogens

To determine whether or not the inhibition noted above was at the δ-aminolaevulic acid to porphobilinogen step, incubations and porphyrin measurement identical to those described in the first paragraph of this section were carried out using porphobilinogen as the substrate. In contrast to the findings with δ-aminolaevulic acid, there was no quantitative difference in the total amount of porphyrins produced compared with the control (Table IV). These results demonstrated that the main

TABLE III

INCORPORATION OF δ-AMINO[4-^{14}C]LAEVULIC ACID INTO HEME

Washed cells were resuspended in normal saline (17.0 ml) and incubated as described in the text for 4 h at 37° with 60.5 μmoles of δ-amino-[4-^{14}C]laevulic acid (7.65 μmoles of porphyrin equivalent)[29] in a final volume of 20.0 ml. Fe^{2+} ions in a concentration of 2 μg/ml were also added at time zero. Porphyrinogens and protoporphyrin 9 dimethyl ester derived from heme were isolated as described in the text. Results are the average of duplicate flasks in a typical example of a series of similar experiments.

	Reticulocytes (%)	*Reticulocytes per flask* × 10^{-9}	^{14}C *specific activity of heme protoporphyrin (counts/min per mg)*	*Total counts/min incorporated*	*Incorporation* (%)	*δ-Amino[4-^{14}C]-laevulic acid incorporated (mμmoles)*	*[^{14}C]Proto-porphyrin 9 formed (mμmoles)*	*[^{14}C]Proto-porphyrin 9 formed per 10^9 reticulocytes (mμmoles)*
Lead poisoning	14.5	5.4	9	316	0.034	20.6	2.5	0.46
Control	8.6	6.0	137	2490	0.026	162.5	20.3	3.38

TABLE IV

CONVERSION OF PORPHOBILINOGEN INTO PORPHYRINOGENS BY SOLUBLE SYSTEMS

Hemolysate supernatant (10.0 ml) was incubated as described in the text for 90 min at 37° with 9.5 μmoles of porphobilinogen (2.4 μmoles of porphyrin equivalent)[29] in a final volume of 15.0 ml. Porphyrinogens were determined as described in *Methods* and are expressed as average results of duplicate flasks in a typical example of a series of similar experiments.

	Porphyrins recovered (mμmoles)	
	Lead-poisoned system	*Control*
Uroporphyrin III	110	134
Phyriaporphyrin III	56	40
Coproporphyrin III	14	12
Total	180	186

Biochim. Biophys. Acta, 111 (1965) 110–123

inhibition was occurring at the δ-aminolaevulic acid to porphobilinogen step. In addition, however, there was a moderate difference in the relative proportion of the porphyrins such that there appeared to be a slight but consistent excess of phyriaporphyrinogen III in the lead-poisoned system. This indicated that the step from phyriaporphyrinogen III to coproporphyrinogen III might also be lead sensitive.

Enzymic conversion of [^{14}C]uroporphyrinogen III into other intermediate porphyrinogens

The supernatant of hemolysed red blood cells from lead-poisoned and anemic rabbits was incubated with [^{14}C]uroporphyrinogen III for 60 min under a continuous current of N_2.

Quantitatively, the amount of uroporphyrinogen III recovered at the end of incubation was of the same order in both systems; similarily, the amounts of newly synthesized phyriaporphyrinogen III were approximately equal. However, coproporphyrinogen III was decreased in the lead-poisoned preparations (Table V), thus showing a relative accumulation of phyriaporphyrinogen III and suggesting that the same accumulation found when porphobilinogen was the substrate was due to another block, which was quantitatively less important than the δ-aminolaevulic acid to porphobilinogen block.

Enzymic conversion of [^{14}C]coproporphyrinogen III into free protoporphyrin 9 and heme

The biosynthetic studies of heme synthesis in lead poisoning were further investigated as follows: hemolysates of red blood cells from lead-poisoned and control rabbits were incubated aerobically for 4 h at 37°, using Fe^{2+} ions and [^{14}C]coproporphyrinogen III as the substrates.

No qualitative differences were detected in the porphyrins isolated from the lead-poisoned systems compared to the control. Table VI summarizes the results of incorporation of [^{14}C]coproporphyrinogen III into [^{14}C]protoporphyrin 9 and heme. It can be seen that when the amount of newly synthesized [^{14}C]protoporphyrin 9 is correlated with the number of reticulocytes present per flask, the lead-poisoned preparations showed a markedly decreased incorporation of coproporphyrinogen III into the total [^{14}C]protoporphyrin 9. The decreased protoporphyrin 9 incorporation into heme also can be seen in this table. Since the specific activities of the [^{14}C]heme were identical in both the lead-poisoned and the control systems, the possibility of pool dilution playing any significant role in the decreased amounts formed is ruled out.

DISCUSSION

In 1936, WATSON[36] theorized that the porphyrinuria observed in lead poisoning was the result of disturbed heme synthesis, and 2 years later, RIMINGTON[37] suggested that the toxic hematologic effect of lead was primarily that of inhibition of Fe^{2+} incorporation into heme. In recent years there has been increasing evidence to support these hypotheses. The action of lead upon porphyrin and heme synthesis *in vitro* from the earliest precursors has been studied by several workers[38–41]. With [^{14}C]acetate as the labelled precursor, ERIKSEN[41] showed that lead inhibition of heme formation was primarily an interference in the synthesis of the porphyrin moiety of heme. DRESEL AND FALK[38], and GOLDBERG *et al.*[40] showed decreased synthesis of porphyrins and heme from [2-^{14}C]glycine and defective uptake of ^{59}Fe into heme using unlabelled

Biochim. Biophys. Acta, 111 (1965) 110–123

TABLE V

CONVERSION OF [^{14}C]UROPORPHYRINOGEN III INTO OTHER PORPHYRINOGENS BY SOLUBLE SYSTEMS

Hemolysate supernatant (15.0 ml) was incubated as described in the text for 60 min at 37° with [^{14}C]uroporphyrinogen III (112 mμmoles, specific activity 76.3 counts/min per mμmole) in a final volume of 20.0 ml. Porphyrinogens were determined as stated in *Methods* and are expressed as average results of duplicate flasks in a typical example of a series of similar experiments.

	[^{14}C]*Porphyrins recovered (mμmoles)*	
	Lead poisoning	*Control*
[^{14}C]Uroporphyrin III	15.2	12.6
[^{14}C]Phyriaporphyrin III	14.5	11.0
[^{14}C]Coproporphyrin III	3.1	6.8

TABLE VI

ENZYMIC CONVERSION OF [^{14}C]COPROPORPHYRINOGEN III INTO FREE PROTOPORPHYRIN 9 AND INTO HEME

Hemolysate (13.5 ml) was incubated as described in the text for 4 h at 37° with [^{14}C]coproporphyrinogen III (65.7 mμmoles, specific activity 80 counts/min per mμmole) and Fe^{2+} ions (3 μg/ml) in a final volume of 17.5 ml. Free coproporphyrin III and protoporphyrin 9 as well as protoporphyrin 9 from heme were isolated. Results are the average of duplicate flasks in a typical example of a series of similar experiments.

	Reticulocytes (%)	*Reticulocytes per flask* × 10^{-9}	*mμmoles of* [^{14}C]*porphyrins*					
			Free [^{14}C]*coproporphyrin III recovered*	*Free* [^{14}C]*protoporphyrin 9 recovered*	*Heme-derived* [^{14}C]*protoporphyrin 9*	*Total* [^{14}C]*protoporphyrin 9 formed*	*Total* [^{14}C]*protoporphyrin 9 formed per 10^9 reticulocytes*	[^{14}C]*Protoporphyrin 9 incorporated into heme per 10^9 reticulocytes*
		(*a*)		(*b*)	(*c*)	(*b* + *c*)	(*b* + *c*)/*a*	(*c*/*a*)
Lead poisoning	16.6	9.6	18.4	2.9	13.8*	16.7	1.7	1.4
Control	8.2	4.1	15.4	0.9	15.4**	16.3	4.0	3.7

* Specific activity 27.3 counts/min·mg.
** Specific activity 27.6 counts/min·mg.

glycine, in the presence of lead. Those authors concluded independently that the step leading to the formation of δ-aminolaevulic acid was much more sensitive to lead than the ones beyond. SANO[11], however, working with erythrocytes of lead-poisoned rabbits showed a marked increase in protoporphyrin 9 synthesis from glycine.

In the current studies, when whole blood from lead-poisoned rabbits was incubated aerobically with [2-^{14}C]glycine and Fe^{2+} ions as the substrate, heme synthesis was greatly decreased. No significant amounts of free porphyrinogens were detected in the lead-poisoned systems, nor in the controls.

The present results cannot be explained on the basis of impaired protoporphyrin 9–iron linkage. An accumulation of free porphyrinogens would normally occur if that were the only inhibition.

There is evidence of a direct inhibitory effect of lead *in vitro* on δ-aminolaevulic acid dehydrase (EC 4.2.1.24), the first of the soluble enzymes (DRESEL AND FALK[39], GOLDBERG *et al.*[40]). There is also evidence of decreased activity of this enzyme both *in vivo* (SANO[11], TANABE[42]), and in the tissues of previously lead-poisoned rabbits tested *in vitro* (KOIKE[43]). LICHTMAN AND FELDMAN[44] studying δ-aminolaevulic acid dehydrase activity in erythrocytes from lead-poisoned patients found it to be decreased. They and VAVRA *et al.*[46] both found poor porphyrin production *in vitro* from δ-aminolaevulic acid. Contrary to these reports, SANO[11] found increased synthesis of porphyrins when incubating δ-aminolaevulic acid with blood from lead-poisoned rabbits.

In order to clarify the nature of these blocks more completely, intermediates in porphyrin synthesis were measured in the present studies. This was carried out with soluble and particulate systems obtained from the blood of lead-poisoned rabbits, and showed a marked decrease of δ-aminolaevulic acid dehydrase activity, measured by porphyrin and heme production. Confirmation that the block was located at the δ-aminolaevulic acid to porphobilinogen step was obtained by incubating the soluble system with porphobilinogen as the substrate. In this case, no quantitative differences were detected. While there was a general decreased synthesis of all porphyrinogens with δ-aminolaevulic acid as the substrate, the fraction of porphyrinogens with a high number of carboxyl groups, *e.g.* uroporphyrinogen III and phyriaporphyrinogen III was disproportionately reduced. The relative accumulation of coproporphyrinogen III (Table II) might well be due to the artificial block in the biosynthetic pathway resulting from the absence of mitochondria and O_2 from the test systems, both necessary for protoporphyrin 9 and heme synthesis[3,7].

GRANICK AND MAUZERALL[4] suggested that the direct effect of lead on δ-aminolaevulic acid dehydrase might be caused by the inactivation of sulfhydryl groups. Since there is an accumulation of protoporphyrin 9 in lead poisoning (for review see ref. 9) an alternative explanation might be proposed in terms of inhibition of the δ-aminolaevulic acid to porphobilinogen step by excess porphyrins through a feedback mechanism rather than by lead itself. In bacterial[40] as well as in human systems[47], it has been shown that protoporphyrin 9 inhibits this step and that this may be a part of the normal control mechanism of porphyrin synthesis.

The second step involving the soluble enzymes, porphobilinogen → uroporphyrinogen III has also been described as being directly affected by lead *in vitro* (DRESEL AND FALK[39]). GOLDBERG *et al.*[40] found that heme synthesis from porpho-

bilinogen, measured by ^{59}Fe incorporation was inhibited by lead but to a much lesser extent than the earlier steps. A higher excretion of porphobilinogen might be expected if a significant inhibition does occur at this point *in vivo*. On this question, divergent results have been reported concerning humans suffering from lead poisoning[9]. In experimental lead poisoning all the published data are in agreement showing that there is an increased excretion of porphobilinogen[16].

In the present studies, neither quantitative nor qualitative differences could be detected between lead-poisoned and control systems when porphobilinogen was the substrate and the soluble fraction was the enzymic source. The metabolic sequence between porphobilinogen and uroporphyrinogen III and phyriaporphyrinogen III showed no alterations; however, the step from phyriaporphyrinogen III to coproporphyrinogen III was slightly altered. A consistent moderate excess of phyriaporphyrinogen III appeared in the lead-poisoned systems in relation to the amount of coproporphyrinogen III present. No increase in the rate of synthesis of any of the porphyrinogens could be demonstrated in the lead-poisoned systems.

Our results fail to explain why porphobilinogen is excreted in increased amounts in the urine of lead-poisoned rabbits. Even though in the tissue under study (blood), no inhibition in the pathway beyond porphobilinogen could be found, there might well be another tissue, *e.g.* the kidney, where the effect of lead could be more injurious, therefore resulting in the increased urinary porphobilinogen observed in experimental lead poisoning.

Studies of the step starting with uroporphyrinogen III were also carried out with soluble fractions. Again, no qualitative differences in porphyrinogen synthesis were detected. The quantity of [^{14}C]uroporphyrinogen III which was recovered at the end of incubation, was of the same order in the lead-poisoned system as in the control; [^{14}C]phyriaporphyrinogen III appeared to be increased in proportion to the amount of coproporphyrinogen III present. It is suggested that the relative accumulation of phyriaporphyrinogen III found when porphobilinogen was the substrate, as well as when uroporphyrinogen III was the substrate, could be due to a second enzymic block by lead, though quantitatively of far less importance than the one between δ-aminolaevulic acid and porphobilinogen.

The present studies continued with the enzymic incorporation of [^{14}C]coproporphyrinogen III into protoporphyrin 9 and heme. Total protoporphyrin 9 synthesis, free and heme-linked, was diminished in the poisoned systems compared with the controls. Moreover, a decreased incorporation of [^{14}C]protoporphyrin 9 into heme (Table VI) was demonstrated. Thus the fact observed with lead added *in vitro* to incubation systems, *i.e.* diminished protoporphyrin 9 incorporation into heme, is the same as occurred in experimental preparations from lead-poisoned animals. This inhibition of the decarboxylase–oxidase system in the reticulocytes could explain the increased levels of coproporphyrin III found in the erythrocyte precursors and plasma of lead-poisoned subjects[11,48]. This correlates well with the findings of LASCELLES[49], YONEDA AND PAPPENHEIMER[50], and COOPER[51] who noted that in bacterial systems low Fe^{2+} concentration favored increased coproporphyrin III accumulation. In addition to the accumulation of coproporphyrin III, a concurrent increase of uroporphyrin I is sometimes observed[52–54], and this type of uroporphyrin is the one which has also been reported to be increased in lead poisoning[18,19,55]. An alternative explanation for accumulation of the latter of course would be direct inhibition of

the step uroporphyrinogen I → coproporphyrinogen I as suggested by WATSON[56] (Fig. 2).

Our results, when added to the observations cited above, allow us to postulate that the abnormalities of heme synthesis associated with lead poisoning could be explained, in part, on both the failure of an adequate amount of iron to enter the mitochondria (Fig. 2), as well as by a direct enzyme sensitivity to lead.

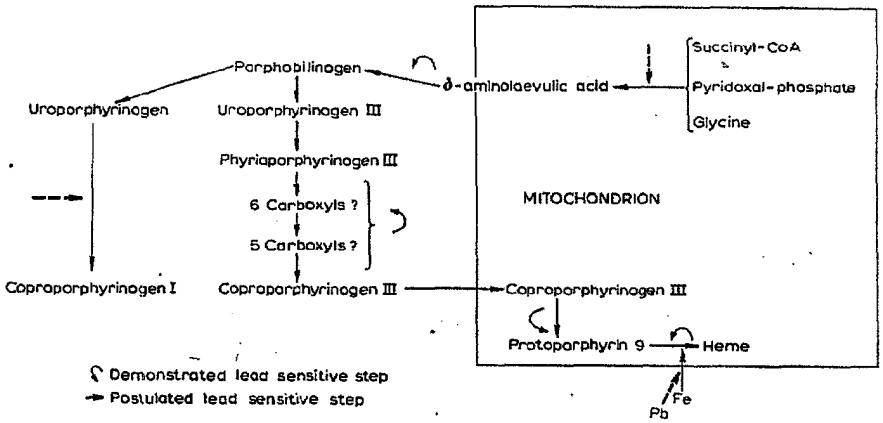

Fig. 2. Adapted from GRANICK[59]. Hypothesis on the control of porphyrin biosynthesis based on compartmentation of enzymes and transport of metabolites through membranes.

Thus we could explain:

(1) Impaired heme synthesis and accumulation of the other substrate of this reaction, protoporphyrin 9.

(2) Impaired coproporphyrinogen III to protoporphyrin 9 conversion being affected by either the absence of Fe^{2+}, that may be involved as a catalyst[51,57,58], and/or by the accumulated protoporphyrin 9 acting as a product inhibitor.

(3) The simultaneous appearance of uroporphyrin I and coproporphyrin III, since both these metabolites accumulate in bacteria grown in Fe^{2+}-deficient conditions[52-54].

(4) The depression of δ-aminolaevulic acid dehydrase activity might be due directly to inhibition caused by an accumulation of later products such as protoporphyrin 9 (refs. 46, 47) and hence indirectly also to unavailability of Fe^{2+}.

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