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Uroporphyrinogen III Cosynthetase from Mouse Spleen*

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ABSTRACT: Uroporphyrinogen III cosynthetase activity from hematopoietic mouse spleen does not affect the rate or the stoichiometry of the conversion of porphobilinogen into total uroporphyrinogen (I + III), catalyzed by the enzyme uroporphyrinogen I synthetase obtained from the same tissue. The percentage of the product uroporphyrinogen as isomer III is proportional to the amount of cosynthetase added. Cosynthetase activity disappears from the reaction mixture during the synthetase-catalyzed reaction. This inactivation of cosynthetase is dependent upon the presence of native synthetase and porphobilinogen, and occurs under conditions where cosynthetase is not measurably thermolabile. The inactivation occurs with synthetase obtained from spinach leaves as well as synthetase obtained from mouse spleen; it is not dependent upon visible light, and it is not caused by ammonium ion generated during the reaction. Under certain reaction conditions the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase activity disappearing. This means that uroporphyrinogen III cosynthetase either participates stoichiometrically rather than catalytically in the formation of uroporphyrinogen III, or else that it is inactivated stoichiometrically by some intermediate or product of the reaction. The cosynthetase levels in crude homogenates from various organs can be measured by assay with spleen synthetase. Cosynthetase appears to be widely distributed in the tissues of the mouse, even where concentrations of uroporphyrinogen I synthetase are very low.

In extracts of plant tissue, the conversion of porphobilinogen into uroporphyrinogen I is enzymatically catalyzed by uroporphyrinogen I synthetase (Bogorad, 1962). In the presence of a second fraction, uroporphyrinogen III cosynthetase, the product formed by the synthetase is uroporphyrinogen III, the physiological intermediate in the biosynthesis of protoporphyrin IX. The cosynthetase does not catalyze an isomerization of the uroporphyrinogen isomers, and its mode of action is not known.

Synthetase and cosynthetase fractions have recently been identified in extracts of hematopoietic spleens from mice made anemic with phenylhydrazine (Levin and Coleman, 1967). Under standard reaction conditions, the percentage of isomer III in the reaction product was proportional to the amount of cosynthetase added. It has now been found that synthetase catalyzes a PBG[1]-dependent disappearance of the cosynthetase activity, and that the amount of uroporphyrinogen III formed is proportional to the cosynthetase consumed. Therefore, cosynthetase appears either to participate stoichiometrically rather than catalytically in synthetase-catalyzed uroporphyrinogen III formation or else to be stoichiometrically inactivated by some intermediate or product of the reaction.

Experimental Procedure

All methods and materials not specified in the following were those previously described or cited (Levin and Coleman, 1967). Mouse spleen cosynthetase from phenylhydrazine-treated animals was prepared from the 25,000*g* supernatant of homogenates by acidification to pH 5, followed by ammonium sulfate fractionation. This procedure does not purify cosynthetase in terms of activity per milligram of protein, but separates it from synthetase. The amounts of cosynthetase used

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[1] The abbreviation used is: PBG, porphobilinogen.

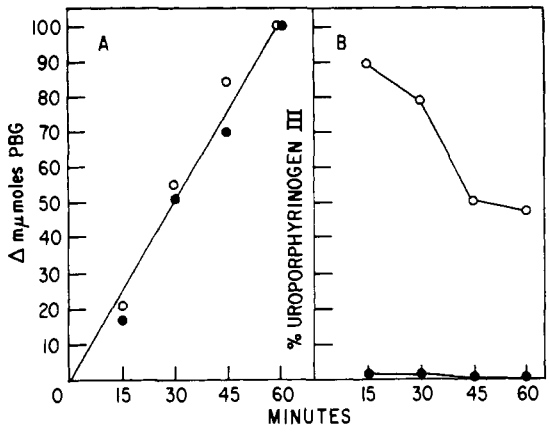

FIGURE 1: Time course of synthetase and cosynthetase activities. (A) Standard conditions for synthetase assay using 0.1 ml of purified enzyme. (○—○) With 0.15 ml of a one-tenth dilution of cosynthetase (in 0.01 M buffer) added; (●—●) control without cosynthetase. (B) Isomer composition of the product under the same conditions.

did not contain measurable synthetase activity under the experimental conditions. Mouse spleen synthetase was purified by heat treatment, ammonium sulfate fractionation, and Sephadex G-100 chromatography. The Sephadex step was scaled up so that 9 ml of enzyme solution was chromatographed on a 2 × 90 cm column (outer volume measured with blue dextran, 78 ml), at a flow rate of 15 ml/hr. A unit of synthetase activity is defined as the amount which catalyzes the consumption of 1 mμmole of PBG/hr. The buffers used were dilutions of 1 M potassium phosphate (pH 7.65).

Uroporphyrinogen I synthetase activity was measured by the rate of disappearance of PBG. The initial concentration of PBG in the reaction mixture was usually 3.6×10^{-4} M. Changes in the concentration of PBG between 7.2×10^{-4} and 1×10^{-4} M did not alter the reaction rate, but at lower concentrations the substrate became rate limiting. Therefore, assays in which less than 10^{-4} M porphobilinogen remained at the end of the incubation were repeated with less enzyme or at shorter incubation times. The standard reaction mixture (0.5 ml), containing 50 μmoles of buffer, 150–180 mμmoles of PBG, and the enzyme to be assayed, was incubated for 30 to 60 min at 31°. The reaction was stopped with 4.0 ml of 5% trichloroacetic acid (w/v). An aliquot of the trichloroacetic acid supernatant was appropriately diluted, and the amount of PBG present was determined with modified Ehrlich's reagent (Mauzerall and Granick, 1956). Control tubes were incubated without enzyme. It has previously been shown that the tetrapyrrole formed by the partially purified enzyme is uroporphyrinogen (Levin and Coleman, 1967). Experiments are described under Results which demonstrate that the stoichiometry between the PBG disappearing and the uroporphyrinogen appearing is approximately 4:1 under the conditions used in the current work. It is therefore possible to describe the activity of the synthetase in terms of the millimicromoles of uroporphyrinogen synthesized, calculated as one-fourth the millimicromoles of PBG disappearing.

Uroporphyrinogen III cosynthetase activity can be assayed by the per cent III in the reaction product (Levin and Coleman, 1967). Between 10 and 75% III, the proportion of isomer III in the reaction product is proportional to the amount of cosynthetase added, for a specified concentration of synthetase (Levin and Coleman, 1967). The rates of disappearance of PBG and formation of total uroporphyrinogen, as catalyzed by synthetase, are not altered by the presence of cosynthetase in the reaction mixture (see Results). It is therefore possible to calculate the amount of uroporphyrinogen III formed from the measured amount of synthetase added and the measured percentage composition of the reaction product.

$$\frac{\text{units of synthetase added}}{4} \times \text{time of incubation (hr)} \times \%\,\text{III} = \text{mμmoles of uroporphyrinogen III formed} \quad (1)$$

All the experiments on cosynthetase were carried out under conditions where the rate of PBG disappearance was constant with time and proportional to the amount of synthetase added.

The standard reaction mixture for the cosynthetase assay was the same as that for the synthetase assay, with the addition of the cosynthetase to be assayed, except that the amount of synthetase added was known from a previous assay. The amount of synthetase and the time of incubation are specified for each experiment described. The incubations were carried out at 31°, at which temperature cosynthetase is not measurably thermolabile in 100 min at pH 7.65.[2] The reaction was stopped with 0.035 ml of acetic acid, and the uroporphyrinogen oxidized to uroporphyrin with 0.01 ml of 0.2 M iodine in 0.3 M KI. The uroporphyrin was isolated, esterified, and extracted into chloroform by the method of Bogorad (1962), scaled down fivefold. The uroporphyrin methyl ester concentrations in the chloroform extracts were measured on a Turner fluorometer, using an F4T4B1 frosted lamp, a Corning 5113 primary filter, and a Corning 3486 secondary filter. A standard curve for the fluorometer was constructed from samples of uroporphyrin methyl ester assayed by optical density in chloroform at 406 mμ (Bogorad, 1962). From each extract four aliquots, each containing 0.25 μg of ester, were evaporated to dryness under a jet of nitrogen, redissolved in a small volume of chloroform, and applied to Whatman No. 1 paper in adjacent but noncontiguous spots. The samples were chromatographed in a modification (Cornford and Benson, 1963) of the double-development system of Falk and Benson (1953). Segments containing uroporphyrin I and III methyl esters, located by fluorescence or from appropriate guide strips, were cut out and eluted with chloroform (5 ml), combining the four replicates for each sample. The fluorescence was read as before, and the results were expressed as the percentage of the total uroporphyrin ester eluted as isomer III. Since erroneous readings

[2] Cosynthetase is thermolabile at 37° (Stevens and Frydman, 1967), the temperature used in previous experiments.

TABLE I: Stoichiometry of the Reaction in the Presence of Cosynthetase.[a]

Expt	Synthetase Added (ml)	Cosynthetase Added (ml)	PBG Disappearing (mμmoles)	Uroporphyrinogen Formed mμmoles	Uroporphyrinogen Formed % III	Ratio of ΔPBG to ΔUroporphyrinogen
1	0.20	0.02	145	32	100	4.5
2	0.10	0.01	73	16	93	4.6
3	0.20	0.02	180	41	30	4.4
4	0.15	0.02	142	33	40	4.3
5	0.20		156	37	3	4.2
6	0.10		63	16	10	3.9

[a] Standard reaction mixtures for the synthetase assay contained 360 mμmoles of PBG and the indicated amounts of synthetase and cosynthetase, incubated at 31° for 60 min. Uroporphyrin determinations (Bogorad, 1962) were carried out on a Cary Model 14 spectrophotometer equipped with a double slide wire of range 0–0.1 and 0.1–0.2 absorbance. Samples were read against blanks containing all the components of the reaction mixture, but with synthetase added at the end of the incubation. The synthetase and cosynthetase preparations used in expt 3 and 4 were not the same as those used in the other experiments.

sometimes resulted from chloroform-soluble contaminants transferred to the paper from fingers, the chromatographic paper was washed in chloroform before use and handled thereafter with gloves.

Results

Synthetase Activity in the Presence of Cosynthetase. It was reported by Bogorad (1958) that uroporphyrinogen III cosynthetase from wheat germ did not alter the rate of PBG consumption catalyzed by uroporphyrinogen I synthetase from spinach leaves, if the PBG concentration was at saturation for the synthetase. This information is essential to any study of the mechanism of action of cosynthetase, and the experiment has therefore been repeated with the synthetase and cosynthetase fractions from mouse spleen. Figure 1A shows a standard assay for synthetase, demonstrating that the rate of disappearance of PBG is the same in the presence or absence of cosynthetase. The amount of cosynthetase activity added is indicated by the results shown in part B of Figure 1, where duplicates of the reaction mixture shown in A were analyzed for the isomer composition of the reaction product. After 15-min incubation the uroporphyrinogen formed was nearly 90% III, but the proportion of III in the product decreased to 47% after 60 min. This apparent loss of the cosynthetase effect is described further below.

Stoichiometry of the Synthetase-Catalyzed Reaction in the Presence of Cosynthetase. At 60°, partially purified uroporphyrinogen I synthetase from mouse spleen catalyzes the formation of 1 mole of uroporphyrinogen from 4 moles of PBG (Levin and Coleman, 1967)

$$4\text{PBG} \dashrightarrow \text{uroporphyrinogen} + 4\text{NH}_3 \qquad (2)$$

Table I demonstrates that this stoichiometry is the same at 31° with a large amount of cosynthetase present (expt 1 and 2). Similar results were obtained in the presence of smaller amounts of cosynthetase, when the product contained 30–40% uroporphyrinogen III (expt 3 and 4), and also in the absence of added cosynthetase at 31° (expt 5 and 6). Since the rate of PBG disappearance and the stoichiometry between PBG and uroporphyrinogen are independent of alterations in the isomer composition of the product, the amount of uroporphyrinogen III formed in a reaction mixture can be calculated from the synthetase activity and the measured isomer composition, as described under Experimental Procedure.

Time Course of Uroporphyrinogen III Formation. It has been suggested that synthetase catalyzes formation of a linear polypyrrole which cyclizes to uroporphyrinogen I, and that cosynthetase catalyzes the cyclization of this polypyrrole to uroporphyrinogen III (Bogorad, 1963). The production of a mixture of isomers I and III could be ascribed to a steady-state competition of the cosynthetase-dependent and -independent cyclization reactions for the intermediate generated by synthetase. It has now been found, however, that unless cosynthetase is present in great excess, the per cent III of the product decreases with time of incubation, under conditions where cosynthetase is thermostable (Figure 1B). The formation of uroporphyrinogen III, as calculated from eq 1, slows to a stop, while the activity of the synthetase, as measured by the disappearance of PBG, is proceeding at an undiminished rate. In the experiment shown in Figure 2A varying amounts of cosynthetase were added to a standard reaction mixture, and the isomer composition of the product was determined after different incubation times. The conversion of PBG into total uroporphyrinogen (I + III) is indicated by the curve marked "line for 100% III," which was calculated from the measured synthetase activity. At incubation times of less than 10 min, all the amounts of cosynthetase used gave nearly 100% III; the observed points fall near the calculated line. The amount of uroporphyrinogen III formed correlated with the amount of cosynthetase added only

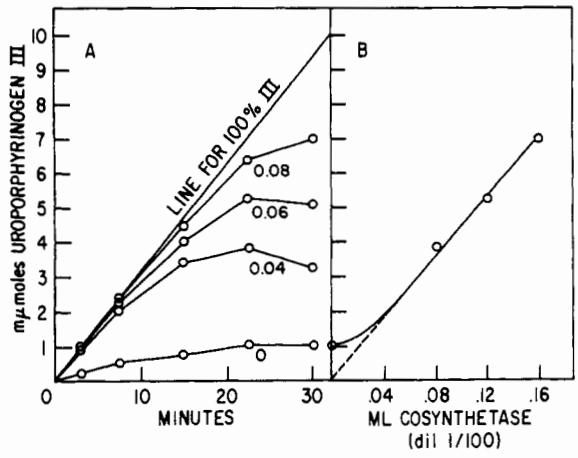

FIGURE 2: Uroporphyrinogen III formation in the presence of varying amounts of cosynthetase. (A) The numbers beside the curves indicate the volumes (in milliliters) of a 100-fold dilution of mouse spleen cosynthetase (in 0.01 M buffer) which were added to the standard reaction mixtures. The "line for 100% III" is the total uroporphyrinogen formed in the reaction, calculated from the measured synthetase activity. (B) A plot of the maximum height reached by each of the curves in A against the amount of cosynthetase added.

after 20 min, when the cosynthetase-dependent reaction had almost ceased, as indicated by the flattening out of the curves. Thus, there is a time-dependent loss of the activity of cosynthetase during the reaction between synthetase and its substrate.

The shapes of the curves shown in Figure 2A were not changed if the amounts of synthetase and cosynthetase were altered so as to increase the scale of the reaction threefold, or decrease it fourfold. This indicates that the loss of the cosynthetase effect does not result from an enzyme inhibition which is proportionate to the concentration of some product of the reaction, although it might be due to a stoichiometric inactivation of cosynthetase by some reaction intermediate or product. In all these experiments, the amount of fluorescence recovered after esterification of the uroporphyrin was linearly related to the time of incubation,

TABLE II: Stability of Cosynthetase at 31° in an Incomplete Reaction Mixture.[a]

Condn of First Incubn (30 min)	% III Formed in a 2nd Incubn (10 min)	
	Cosynthetase (5 μl)	Cosynthetase (10 μl)
Cosynthetase alone	45	94
Cosynthetase with PBG	53	95
Cosynthetase with synthetase (262 units)	48	96
First incubation omitted	43	96

[a] The omitted components of a standard reaction mixture were added at the end of the first incubation.

TABLE III: Failure of Ammonium Ion in Concentrations up to 4.8×10^{-4} M to Affect the Activity of Mouse Spleen Synthetase or Cosynthetase.[a]

Ammonium Ion Added (mμmoles/0.5 ml)	Act. of Cosynthetase (0.01 ml) (% of uroporphyrinogen as III)	Act. of Synthetase (Δmμmoles of PBG)
0	65	61
60	63	64
120	65	64
180	62	64
240	64	61

[a] Standard assay conditions as described under Experimental Procedure, except that the incubations were carried out for 40 min with 91 units of synthetase. Ammonium ion was added as $(NH_4)_2SO_4$.

regardless of the isomer composition. This is consistent with measurements of the rate of PBG disappearance and the experiments on stoichiometry, which together show that the rate of the synthetase-catalyzed conversion of PBG into total uroporphyrinogen (I + III) is not affected by the level of cosynthetase present.

Dependencies of Cosynthetase Inactivation. The time-dependent inactivation of cosynthetase does not occur if either PBG or synthetase is omitted from the reaction mixture. In the experiment shown in Table II, cosynthetase was incubated alone, and with each of the components of the reaction mixture separately, for 30 min at 31°. The omitted components were then added to each mixture and the per cent III was measured after a short second incubation. The results show that cosynthetase is stable in an incomplete system. Other experiments showed that cosynthetase is stable for 30 min in the presence of PBG and boiled synthetase, or in the complete system at 0°. The disappearance of cosynthetase at 31° may therefore be described as an enzyme-catalyzed, substrate-dependent reaction.

It is possible that cosynthetase is inactivated in some synthetase-catalyzed, PBG-dependent side reaction, although partially purified spinach synthetase (Bogorad, 1962) also catalyzes the disappearance of cosynthetase activity. Uroporphyrin sensitizes light-dependent oxidations which might inactivate cosynthetase, but very little uroporphyrinogen is oxidized to uroporphyrin under the reaction conditions (Levin and Coleman, 1967), and the inactivation of cosynthetase also occurs in the dark. It seems probable that cosynthetase is either inactivated stoichiometrically by some product of the reaction, or that cosynthetase participates noncatalytically in the formation of uroporphyrinogen III.

The apparent disappearance of cosynthetase activity is not due to an inhibition of cosynthetase by ammonia formed during the reaction. This is indicated by the data shown in Table III. The amount of ammonia generated in those experiments (calculated from the stoichiometry

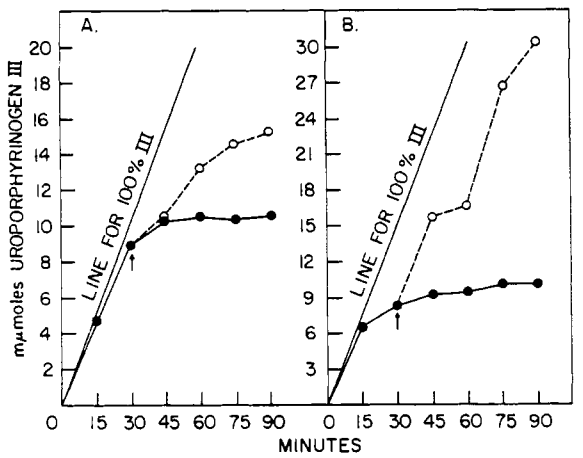

FIGURE 3: Reactivation of uroporphyrinogen III formation by a second addition of cosynthetase. In each experiment, the standard reaction mixture initially contained the equivalent of 1.6 μl of mouse spleen cosynthetase. (○—○) Tubes to which a second addition of cosynthetase was made at 30 min (arrow), 2 μl in A and 5 μl in B. (●—●) Control tubes. The amount of synthetase added is indicated by the calculated line for 100% III. The incubations were made in duplicate and the values plotted are the means.

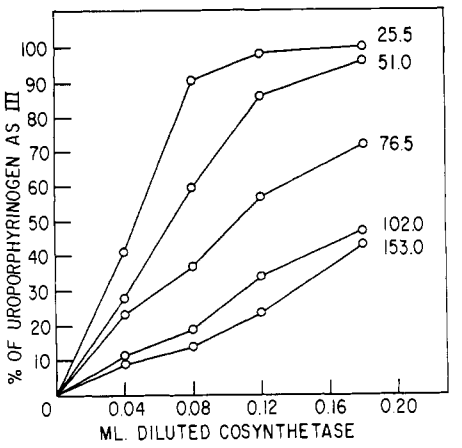

FIGURE 4: Assay of a cosynthetase preparation (diluted 1:50) in the presence of varying amounts of synthetase, incubated for 30 min. The units of synthetase added are indicated by the numbers beside the curves. The cosynthetase required for 50% III was read from the lines connecting the points, except for the lowest two curves, where it was read from an extention of the best straight line through all the points.

shown in eq 2) was 61–64 mμmoles. Since the isomer composition of the product was unaffected even by nearly four times this amount of NH_4^+, it seems unlikely that ammonia is the agent which causes the formation of III to cease. NH_4^+ also does not affect the per cent III obtained when concentrations of 240 mμmoles are preincubated with synthetase and cosynthetase for 30 min before the addition of PBG. In addition, Table III also shows that these amounts of NH_4^+ do not affect synthetase activity.

Reactivation of Uroporphyrinogen III Synthesis by a Second Addition of Cosynthetase. If the formation of uroporphyrinogen III stops because the cosynthetase has been inactivated, a second addition of cosynthetase should cause it to begin again. Figure 3 shows two experiments in which this occurred. When the amount of cosynthetase in the second addition was small, the formation of uroporphyrinogen III resumed, but again fell off with time (A); when the amount was large, the renewed formation of uroporphyrinogen III proceeded at a rate almost parallel to the line expected for 100% III (B). Hence the reaction system had not lost its sensitivity to cosynthetase. This again means that uroporphyrinogen itself does not inhibit cosynthetase by a mechanism that is dependent upon uroporphyrinogen concentration, although it is still possible that there is some kind of stoichiometric interaction between the uroporphyrinogen produced and the cosynthetase present.

Relationship between the Amount of Cosynthetase Disappearing and the Amount of Uroporphyrinogen III Formed. In the experiment described in Figure 2A, the maximum amount of uroporphyrinogen III formed, as judged by the highest point reached on each of the curves, was proportional to the amount of cosynthetase added. This is shown in Figure 2B. The three points fall on a line which passes through the origin, although not through the point for the amount of III formed in the absence of added cosynthetase. It is clear that the previously reported proportionality between the per cent III and the amount of cosynthetase added (Levin and Coleman, 1967) resulted from the different final amounts of III formed with different amounts of cosynthetase, rather than from a balanced steady state in which the reactions leading to uroporphyrinogens I and III were occurring at constant rate.

In the experiment described in Figure 2A, the amount of cosynthetase which has been consumed when uroporphyrinogen III formation ceases can be assumed to be the same as the amount initially added, since no cosynthetase activity is lost in an incomplete system (Table II). It can be seen from Figure 2A that under those particular conditions, uroporphyrinogen III formation had ended by the time the proportion of III had fallen to 50%. For example, the curve obtained with 0.04 ml of cosynthetase has become flat at 22 min, at which time the amount of uroporphyrinogen III which has been formed is about 50% of the value reached by the line calculated for total uroporphyrinogen, labeled "line for 100% III." Similarly, the curve indicating uroporphyrinogen III formation with 0.06 ml of cosynthetase has become flat at 22–30 min, at a level about half of the total uroporphyrinogen formed ("line for 100% III"). The occurrence of 50% III in the product provides a more objectively measured end point for III formation than does inspection of the time course, which approaches an asymptote. The plot of per cent III against the amount of cosynthetase, on the other hand, is a straight line which crosses 50% III at some easily measured point. Moreover, the method for chromatographic separation of uroporphyrin I and III is most reliable when there are approximately equal amounts of the methyl esters of the two isomers present (Chu and Chu, 1957). This end point for uroporphyrinogen III formation was used to test again the idea that

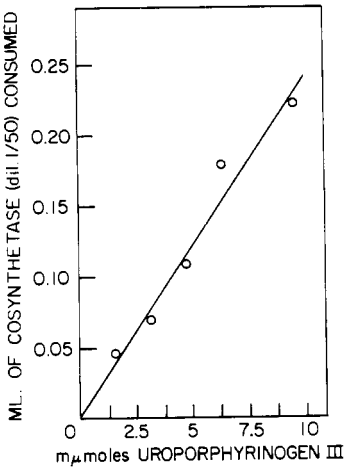

FIGURE 5: Relationship between the amount of uroporphyrinogen III formed and the amount of cosynthetase consumed, obtained from the curves shown in Figure 4.

the amount of III formed with a given amount of cosynthetase is independent of synthetase concentration, which is what would be predicted if cosynthetase activity is consumed in some stoichiometric way during the formation of uroporphyrinogen III. The amount of cosynthetase required to produce 50% uroporphyrinogen III was determined for five concentrations of synthetase. The results are shown in Figure 4. As expected from previous work (Bogorad, 1958) the per cent III in the product decreased as the amount of synthetase was increased. The amount of cosynthetase needed to give 50% III (the amount of cosynthetase consumed) was read from each curve and plotted against the amount of uroporphyrinogen III formed (50% of the total uroporphyrinogen formed). As shown in Figure 5, there was a proportionality between cosynthetase consumption and uroporphyrinogen III formation, measured by this method, over a sixfold range in the amount of synthetase added.

Time Course of Uroporphyrinogen III Formation in the Presence of Varying Amounts of Synthetase. From these data it would seem that the total amount of uroporphyrinogen III formed depends upon the amount of uroporphyrinogen III cosynthetase added, and is independent of the amount of synthetase present. If the amount of synthetase were varied while the cosynthetase added was constant, one would expect a family of curves which converged on the same final point. An experiment of this kind is demonstrated in Figure 6, where the curves all appear to converge at about 16 mμmoles of uroporphyrinogen III formed.

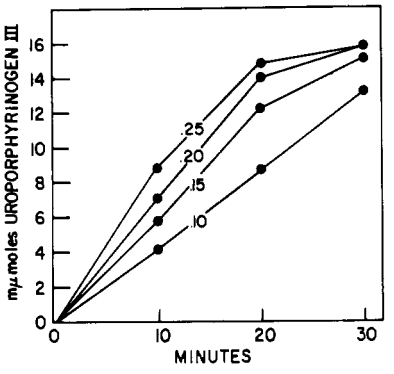

FIGURE 6: Time course for the formation of uroporphyrinogen III in the presence of varying amounts of synthetase. The standard reaction mixtures for cosynthetase assay contained the equivalent of 16 μl of a cosynthetase preparation. The synthetase preparation used had an activity of 1050 units/ml at 31°. The amounts of synthetase added (in milliliters) are indicated by the numbers beside the curves.

TABLE IV: Specific Activities of Synthetase and Cosynthetase in Homogenates of Organs of Male White Mice, Measured at 31°.[a]

Organ	Cosynthetase (units/mg of protein)	Synthetase (units/mg of protein)
Brain	5.5	0
Muscle	5.0	0
Testis	3.5	0
Heart	3.2	0
Kidney	2.9	0
Liver	2.4	0
Lung	1.6	0
Spleen (normal)	16.1	11.2
Spleen (phenylhydrazine-treated animal)	32.2	15.7

[a] Protein was determined by the biuret method (Layne, 1957). The phenylhydrazine treatment schedule was that previously described for synthetase (Levin and Coleman, 1967).

The total amount of uroporphyrinogen formed is proportional to the amount of synthetase added. Since the amount of III formed with a given amount of cosynthetase seems to be a synthetase-independent constant, the amount of I formed must vary with the synthetase. This means that the inactivation of cosynthetase cannot result from a stoichiometric interaction with uroporphyrinogen I. Whether or not the inactivation is due to a reaction of cosynthetase with uroporphyrinogen III or some precursor of uroporphyrinogen III remains to be determined.

Distribution of Cosynthetase in Mouse Tissues. For this study, organs were pooled from ten normal white male mice weighing 25–30 g each, homogenates were prepared in the usual way, and assays for synthetase and cosynthetase were carried out on the 25,000*g* supernatants (Levin and Coleman, 1967). One unit of cosynthetase activity was defined as the amount needed to give 50% III when the synthetase catalyzed formation of 10 mμmoles of uroporphyrinogen in 30 min at 31°, and the specific activity as the units per milligram of protein. The results are shown in Table IV. In this experiment, synthetase activity was not detected in

organs other than spleen. Cosynthetase activity was easily measured in the extracts, and its specific activity was between 1.6 and 5.5 units per mg of protein in all organs tested except spleen. In spleen from untreated mice, the endogenous synthetase activity catalyzed the disappearance of 11.2 mμmoles of PBG/hr per mg of protein at 31°, indicating the formation of 2.8 mμmoles of uroporphyrinogen/hr. The endogenous cosynthetase measured, 16.1 units/mg of protein, was adequate for the formation of 80.5 mμmoles of uroporphyrinogen III/mg of protein (16.1 units × 50% of 10 mμmoles). Thus if a spleen homogenate were incubated with PBG at 31°, without any added synthetase, a period of 57.5 hr (161 mμmoles of uroporphyrinogen formed) would have been required before the proportion of III in the reaction product fell to 50%. This relative excess of cosynthetase over what is required to saturate the synthetase present is even greater in mouse spleen made more hematopoietic by phenylhydrazine treatment of the animal, and greater still in other, nonhematopoietic tissues, where the synthetase activity is low so that it was not measurable in 30 min at 31°. This tissue excess of cosynthetase explains why studies of the isomer composition of the product formed by various crude systems from normal tissues always gave 100% III (Margoliash, 1961) when only the endogenous synthetase was used to catalyze uroporphyrinogen formation.

The increase in the total amount of cosynthetase per spleen was more than 15-fold after phenylhydrazine treatment. The spleens enlarged 5-fold, from an average of 0.1 to 0.5 g each; the protein concentrations of the extracts increased over 50%, from 20.7 to 35.2 mg/ml; and the specific activity of the cosynthetase doubled (Table IV).

Discussion

In the mouse spleen system, the rate of uroporphyrinogen I formation increases when the rate of uroporphyrinogen III formation declines, because the rate of formation of total uroporphyrinogen remains constant. A simple way to explain these kinetics is to consider that two separate reactions take place (eq 3 and 4).

$$\text{synthetase} + \text{cosynthetase} + 4\text{PBG} \longrightarrow \text{synthetase} + \text{inactive cosynthetase} + \text{uroporphyrinogen III} + 4\text{NH}_3 \quad (3)$$

$$\text{synthetase} + 4\text{PBG} \longrightarrow \text{synthetase} + \text{uroporphyrinogen I} + 4\text{NH}_3 \quad (4)$$

It is clear that if the turnover number of synthetase for PBG is the same for the two reactions, their relative rates depend upon the initial concentrations of synthetase and cosynthetase. As the cosynthetase becomes inactivated, the rate of reaction 3 decreases and that of reaction 4 increases. Nothing in the data presented in this paper or in the above equations conflicts with the idea that cosynthetase interacts with some free intermediate which is a product of the activity of synthetase.

The data indicate that the cosynthetase activity is lost during the synthetase-dependent formation of uroporphyrinogen, and that the amount of uroporphyrinogen III formed is dependent upon the amount of cosynthetase added. The most common explanation of the coupled disappearance of a substance required for a biosynthetic process, in a reaction which is substrate dependent, enzyme catalyzed, linked to the time of incubation, and proportionate to the amount of product formed, is the stoichiometric participation of the substance as a cofactor. However, mouse spleen cosynthetase is a thermolabile material with protein-like properties during ammonium sulfate precipitation and dialysis (Levin and Coleman, 1967). Cosynthetase also behaved like a protein when fractionated from wheat germ (Bogorad, 1962; Stevens and Frydman, 1967), although specific activities have not been reported, and it is not certain that the procedures truly represented purifications. Of course, if cosynthetase is a cofactor, it is possible that it is a substance of low molecular weight which is tightly protein bound. On the other hand, enzymes are known which are inactivated during the reactions which they catalyze, either by reaction with a substrate (McLemore and Metzler, 1968) or with a product (Powers and Dawson, 1944), and it is possible that the catalytic activity of cosynthetase is lost by an interaction with some reaction intermediate. In the inactivation of ascorbic acid oxidase by the peroxide which is a product of the enzyme activity, the amount of dehydroascorbate formed is proportional to the amount of ascorbic acid oxidase initially added (Powers and Dawson, 1944). Definition of the chemical nature of cosynthetase will require its further purification.

Humans and cattle with congenital erythropoietic porphyria excrete large amounts of uroporphyrin I in the urine. The formation of I has been ascribed to a deficiency of the enzymatic activity of cosynthetase (Bogorad, 1963; Granick, 1962). More recently, it has been reported that excretion of uroporphyrin III is also elevated in erythropoietic porphyria, which would be inconsistent with a deficiency of cosynthetase (Watson *et al.*, 1964). On this basis it has been suggested that an inherited defect in the regulation of uroporphyrinogen I synthetase leads to a markedly elevated level of this enzyme in marrow, so that the capacity of the cosynthetase enzyme to convert the synthetase-generated intermediate into uroporphyrinogen III is exceeded. However, if cosynthetase is inactivated during uroporphyrinogen synthesis *in vivo* as well as *in vitro*, an alternative possibility might be that elevated synthetase levels deplete the tissue stores of cosynthetase, permitting uroporphyrinogen I formation to proceed. Measurement of cosynthetase levels in the tissues of normal and porphyric cattle are in progress.

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Chick Microsomal Oxidases. Isolation, Properties, and Stimulation by Embryonic Exposure to 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane*

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ABSTRACT: The optimum conditions for the activity of the chick liver microsomal oxidative *N,N*-dimethylaniline demethylase have been established. Reduced nicotinamide–adenine dinucleotide phosphate was required and was partially replaced by reduced nicotinamide–adenine dinucleotide. The enzyme was competitively inhibited by β-diethylaminoethyl diphenylpropylacetate. Michaelis constants in the absence and in the presence of β-diethylaminoethyl diphenylpropylacetate were determined for *N,N*-dimethylaniline and *N,N*-dimethylaniline *N*-oxide. The data presented in this work demonstrate that the rate of *N,N*-dimethylaniline *N*-oxide demethylation by chick liver microsomal fraction is sufficiently high for the *N*-oxide to be considered as an intermediate in the oxidative demethylation of *N,N*-dimethylaniline. Ferrous ions activated the microsomal demethylase when incubated with nicotinamide–adenine dinucleotide phosphate and gave a greater oxidative reaction in the absence of nicotinamide–adenine dinucleotide phosphate. The microsomal fraction oxidized *N,N*-dimethylaniline when incubated with hydrogen peroxide generating system. The activity of this enzyme system in chicks hatched from eggs which had been injected with 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene prior to the incubation has been determined. 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane and its major metabolites in the chick stimulated microsomal oxidative activity. However, there were no changes in the enzyme activity requirements or in the Michaelis constants both in the presence and absence of β-diethylaminoethyl diphenylpropylacetate. The ability of chick microsomal enzymes to dealkylate some carbamate insecticides was found to be increased by the embryonic exposure to 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, and 2,2-bis(*p*-chlorophenyl)ethylene.

Numerous studies have been conducted on the oxidative demethylation of *N*-methyl compounds. In the presence of NADPH and oxygen the microsomal fraction catalyzed the oxidation of some of the *N*-alkyl compounds to the aldehydes and corresponding amines (Gillette *et al.*, 1957). This enzyme system appeared to be a mixed-function oxidase or mixed-function oxygenase (Ziegler and Pettit, 1966).

A number of studies have been done on the mechanism of the oxidative N dealkylation of lipid-soluble tertiary amines (Fish *et al.*, 1955; McMahon and Sullivan, 1964; Ziegler and Pettit, 1964, 1966).

The activity of microsomal oxidases may be stimulated by exposure of the animal to certain chemicals. Current data indicate that stimulation occurs by increased synthesis of the enzyme systems normally present rather than by modification or elaboration

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