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Enzymatic Properties of Uroporphyrinogen III Cosynthetase*

Ephraim Yale Levin

ABSTRACT: Uroporphyrinogen III cosynthetase is inactivated during the formation of uroporphyrinogen III catalyzed by uroporphyrinogen I synthetase. However, cosynthetase has the physical and chemical properties of a protein, and is active in catalytic concentrations. This indicates that cosynthetase is an enzyme which is inactivated during the course of the reaction which it catalyzes. Although under standard conditions the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase inactivated, the proportionality can be altered by changing the amount of synthetase present. Hence, the inactivation of cosynthetase is probably not an essential part of its catalytic activity. Cosynthetase from mouse spleen can be purified 18-fold with a 5% yield by ammonium sulfate fractionation and chromatography on Bio-Rex 70. It is inhibited about 80% by 2×10^{-5} M concentrations of uroporphyrins and coproporphyrins, or their corresponding porphyrinogens. Its pH optimum is about 7.7–7.9, the same as that for mouse spleen uroporphyrinogen I synthetase. At higher pH values, around 9.0, cosynthetase stimulates the activity of synthetase, and this stimulation can be used as a rapid assay for purified cosynthetase.

Two separate fractions are required for the formation of uroporphyrinogen III by extracts of plant or animal tissues (Bogorad, 1962; Levin and Coleman, 1967). One of these fractions is the enzyme uroporphyrinogen I synthetase, which catalyzes the formation of 1 mole of uroporphyrinogen I from 4 moles of porphobilinogen. Uroporphyrinogen III is formed instead of uroporphyrinogen I when synthetase acts upon porphobilinogen in the presence of the second fraction, called uroporphyrinogen III cosynthetase. Cosynthetase alone does not catalyze the disappearance of porphobilinogen, and it is not an isomerase which catalyzes interconversion of the uroporphyrinogen isomers.

Studies on uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase separated from erythropoietic mouse spleen revealed that cosynthetase activity is destroyed during the formation of uroporphyrinogen III. Under certain reaction conditions, the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase disappearing (Levin, 1968a). This relationship raised the possibility that cosynthetase functions as a nonrecycling cofactor rather than as a catalyst. However, a number of enzymes are known to be inactivated during the course of the reactions which they catalyze (McLemore and Metzler, 1968; Powers and Dawson, 1944; Hager *et al.*, 1957; Zervos *et al.*, 1971), and the question of the enzymatic nature of uroporphyrinogen III cosynthetase has remained unsettled.

The present report shows that uroporphyrinogen III cosynthetase is a proteinaceous material which is active in such low concentrations that it must be assumed to be participating in the formation of a large number of equivalents of uroporphyrinogen III before being inactivated—that is, to be functioning catalytically, as an enzyme. The proportionality between cosynthetase inactivation and uroporphyrinogen III formation can be altered by changing the incubation conditions, which suggests that the reaction which inactivates cosynthetase is not an essential part of the formation of uroporphyrinogen III. A modified assay for cosynthetase, which gives absolute values for the amount of uroporphyrinogen III formed rather than the relative values obtained previously,

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has been used to show that *in vitro* it would be possible to obtain 100% uroporphyrinogen III in the reaction product only with an infinite concentration of cosynthetase. In addition, it has been found that cosynthetase is inhibited by uro- and coproporphyrins and porphyrinogens.

Finally, an observation that uroporphyrinogen III cosynthetase stimulates uroporphyrinogen I synthetase activity at pH 9, which is a pH value far above the optimum one for either enzyme, has been used as the basis of a rapid assay for partially purified cosynthetase. A procedure is described which purifies cosynthetase 18-fold from crude extracts, providing a preparation suitable for use with the rapid assay.

Experimental Section

Uroporphyrinogen I synthetase was purified by heat treatment, ammonium sulfate fractionation, and Sephadex chromatography, starting either with crude mouse spleen extracts (Levin and Coleman, 1967), or with the discarded ammonium sulfate fraction of cosynthetase preparations (see Results). Synthetase activity was measured in terms of the rate of disappearance of porphobilinogen, and uroporphyrinogen formation was calculated from the stoichiometry (Levin, 1968a). One unit of synthetase is defined as the amount which catalyzes the consumption of 1 nmole of porphobilinogen/hr, at the temperature and pH specified.

Unless otherwise specified, the mouse spleen uroporphyrinogen III cosynthetase used in these experiments was purified through the first ammonium sulfate step reported in Results, or was further purified twofold by adsorption on alumina Cγ gel and elution with 0.075 M potassium phosphate buffer (pH 7.7) (E. Y. Levin, unpublished). Cosynthetase activity was routinely assayed by determining the per cent III in the reaction product formed from porphobilinogen by 40 units of synthetase in 30 min at 31°. This percentage is proportional to the cosynthetase added between 10 and 70% III (Levin and Coleman, 1967). The uroporphyrinogens were oxidized to the porphyrins, isolated on talc, esterified, and separated chromatographically, the separated isomers being determined fluorometrically when unlabeled porphobilinogen was used as substrate (Levin and Coleman, 1967), or radiometrically when tritiated porphobilinogen was used as substrate (Romeo and Levin, 1969). One unit of cosynthetase is defined as the amount needed to give 50% isomer III (2.5 nmoles) under these conditions. When cosynthetase was assayed by the alternative method described under Results, in which the uroporphyrinogens were chemically converted to the coproporphyrins which were then separated chromatographically, the reaction mixture was the same as that in the routine assay described above, using tritium-labeled porphobilinogen as substrate. Other details of this procedure have been described previously (Romeo and Levin, 1969).

Trypsin was assayed by the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (Hummel, 1959), using the modified procedure described in the Worthington catalog. One unit of activity is defined as the amount which hydrolyzes 1 μmole of ester/min at 25° and pH 8.1 in the presence of 0.01 M Ca^{2+}. For the study of the effect of trypsin on cosynthetase, 0.05–2.0 unit of trypsin was incubated with 1.6 mg of cosynthetase (950 units) and 12 μmoles of Tris buffer (pH 8.2) for 60 min at 22°, in a volume of 0.72 ml. The reaction was stopped with 0.9 mg of trypsin inhibitor, and aliquots of this mixture were assayed for the residual cosynthetase activity. Control tubes contained the same components, except that the trypsin inhibitor was added before the incubation with trypsin. Trypsin and its ester substrate were obtained from Worthington Biochemicals, and soybean trypsin inhibitor from Calbiochem.

Uroporphyrin I methyl ester and coproporphyrin I and III methyl esters were obtained from Calbiochem. Uroporphyrin III methyl ester was prepared from touraco feathers (obtained from the Baltimore zoo) as described by Nicholas and Rimington (1950), carrying out the reduction step with sodium borohydride (Sigma) instead of with sodium amalgam. To obtain the porphyrins and porphyrinogens used as inhibitors of cosynthetase, the esters were hydrolyzed in 6 M HCl, and the porphyrins were precipitated at pH 3–4, washed with water, and dissolved in dilute alkali. Reduction to the porphyrinogens was carried out with sodium amalgam (Mauzerall and Granick, 1958), or with sodium borohydride. For the latter, about 3 mg of porphyrin in 0.5–1.0 ml of 0.1 M NaOH was added to 55 mg of $NaBH_4$ and stirred in dim light, while 1 ml of 1 M HCl was added dropwise. Then 0.2 ml of deaerated 1 M potassium phosphate buffer (pH 7.9) was added to stabilize the pH at 7.5–8.1. The absorption at the Soret maximum was determined before and after treatment with iodine and thiosulfate, and the concentration of porphyrinogen was calculated from the published extinction coefficients (Bogorad, 1962; Mauzerall and Granick, 1958). When the different porphyrinogens were tested as inhibitors of cosynthetase, the control tubes contained all the components of the reduction mixture except the porphyrin, and the porphyrinogen was added to these tubes at the end of the incubation.

Protein concentration was determined by biuret, Folin, or optical density methods, depending on concentration (Layne, 1957). Bio-Rex 70, 200–400 mesh, sodium form (Bio-Rad), was washed seven times with water before use, decanting the fines. All materials and methods not specified in the above were those previously described or cited (Levin, 1968a; Romeo and Levin, 1969).

Results

Protein Nature of Cosynthetase. The inactivation of cosynthetase is synthetase-catalyzed, porphobilinogen-dependent, and proportional to the amount of product formed. However, cosynthetase is a highly thermolabile material, with size and solubility properties like those of a protein (Levin, 1968a; Bogorad, 1962; Stevens and Frydman, 1967). If cosynthetase itself is a protein, rather than a small molecule nonspecifically adhering to protein, it must be functioning catalytically, because the amount of protein added to a reaction mixture is very small compared to the amount of product formed. Crude preparations of cosynthetase from mouse spleen lead to the formation of 30–60 nmoles of uroporphyrinogen III/mg of protein, in the usual assay system. This indicates a yield of 1 mole of product/33,000–66,000 g of protein in the crude extract. Since cosynthetase probably constitutes 1% or less of the protein in the crude extract, the molecular weight of the active protein unit would have to be 330–660, if it participated only once in uroporphyrinogen formation. This is unreasonable. Hence, cosynthetase activity must be enzymatic if it resides in a protein.

Figure 1 demonstrates that cosynthetase is inactivated by incubation with trypsin, but it is not inactivated by incubation with trypsin plus trypsin inhibitor. The rate of the inactivation of cosynthetase is about two-thirds of the rate for the inactivation of uroporphyrinogen I synthetase in the presence of the tested amount of cosynthetase (E. Y. Levin, unpublished data). When added to evidence

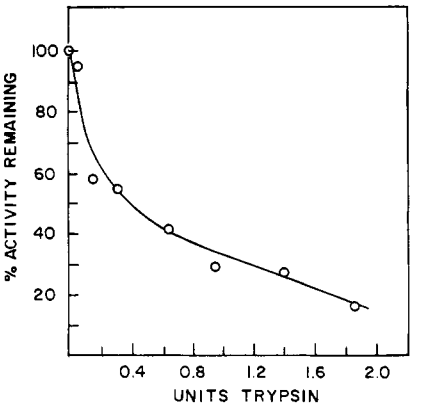

FIGURE 1: Inactivation of cosynthetase by trypsin (60 min, 22°).

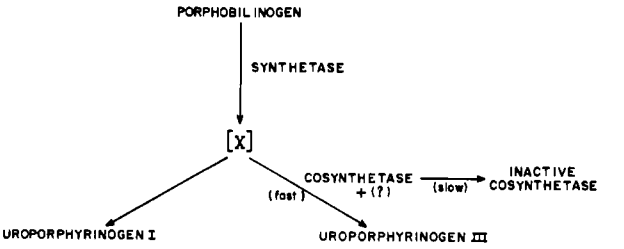

FIGURE 2: Proposed relationship between cosynthetase-catalyzed and cosynthetase-inactivating reactions.

that cosynthetase is nondialyzable and heat labile (Levin, 1968a) and is purified by ammonium sulfate fractionation (see below), these data indicate that the cosynthetase activity is an enzymatic one.

Alteration of the Stoichiometry between Cosynthetase Inactivation and Uroporphyrinogen III Formation by Change in the Rate of the Synthetase-Catalyzed Reaction. Identification of cosynthetase activity as enzymatic does not conflict with the previous finding that the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase inactivated. It means that although the disappearance of cosynthetase and the appearance of uroporphyrinogen III are proportional, the stoichiometric relationship is not 1:1. Since cosynthetase must cycle a large average number of times through the reaction leading to uroporphyrinogen III before being inactivated, the inactivation process is apparently not an essential part of uroporphyrinogen III formation. Probably cosynthetase inactivation is a separate reaction, competing for cosynthetase with the cosynthetase-catalyzed reaction, as diagrammed in Figure 2. In this figure, the symbol [X] represents the polypyrrole intermediate postulated by Bogorad (1963) to be the substrate for cosynthetase activity. According to this formulation, the relative rates of the cosynthetase-catalyzed and cosynthetase-inactivating reactions determine the stoichiometry between uroporphyrinogen III formed and the cosynthetase disappearing.

In support of the idea that uroporphyrinogen III formation and cosynthetase inactivation are separate reactions, it has been found that it is possible to vary the stoichiometry between them by a factor of 2, thus indicating that the rates of the two reactions involving cosynthetase have been affected independently. This experiment was carried out by assaying cosynthetase in the presence of different amounts of synthetase, simultaneously varying the incubation time inversely to the synthetase concentration, so that the total amount of uroporphyrinogen (I plus III) formed would be the same in all reaction vessels. As the amount of synthetase present was lessened and the incubation time lengthened, more uroporphyrinogen III was formed for a given amount of cosynthetase inactivated (Figure 3). That is, when the synthetase-catalyzed reaction proceeded more slowly, the cosynthetase functioned more effectively: the cosynthetase-catalyzed reaction was favored relative to the cosynthetase-inactivating reaction. This greater stability of cosynthetase on longer incubation is of course in a direction opposite to that expected for thermal destruction of cosynthetase. Cosynthetase is in any case not measurably thermolabile in 100 min at the temperature of these incubations (31°), in the presence of either porphobilinogen or synthetase alone (Levin, 1968a).

The magnitude of this cosynthetase-sparing effect, a 2.5-fold change in activity over an 8-fold range of synthetase concentration, is too small to have been noted in earlier experiments in which the time course of uroporphyrinogen III formation was followed over a 2.5-fold range of synthetase concentration (Levin, 1968a).

Assay of Cosynthetase Activity by Chemical Decarboxylation of Enzymatically Formed Uroporphyrinogen and Chromatographic Separation of the Resulting Coproporphyrin Isomers. The assay for cosynthetase depends upon chromatographic separation of the isomers of the methyl esters of uroporphyrins derived from the synthetase-generated uroporphyrinogens. However, this chromatographic method is difficult to standardize. Uroporphyrin I and III methyl esters have very different R_F values, but when mixtures of the two are chromatographed, each isomer is contaminated with some of the other, apparently held as an intermolecular complex (Bogorad and Marks, 1960). Moreover, the magnitude of the cross contamination is affected by the amount of ester applied to the paper (Cornford and Benson, 1963). A more accurate method of separating the isomers of uroporphyrinogen is to oxidize and decarboxylate them to form the corresponding coproporphyrins (Edmondson and Schwartz, 1953), and then separate the coproporphyrin isomers (Mauzerall, 1960). With this method, it is possible to determine more precisely the percentage of isomer III in the reaction

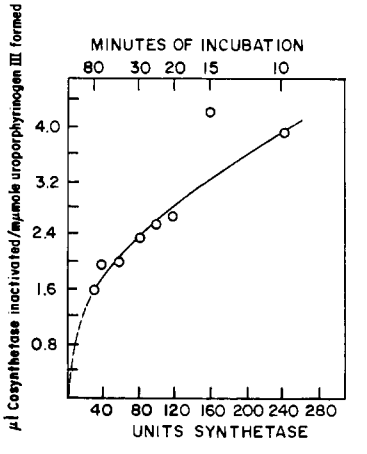

FIGURE 3: Relationship between cosynthetase inactivation and synthetase concentration, under conditions where 10 nmoles of uroporphyrinogen (I and III) are formed. The ratio of cosynthetase inactivated:uroporphyrinogen III formed was determined on the basis of the standard assay, as described previously (Levin, 1968a).

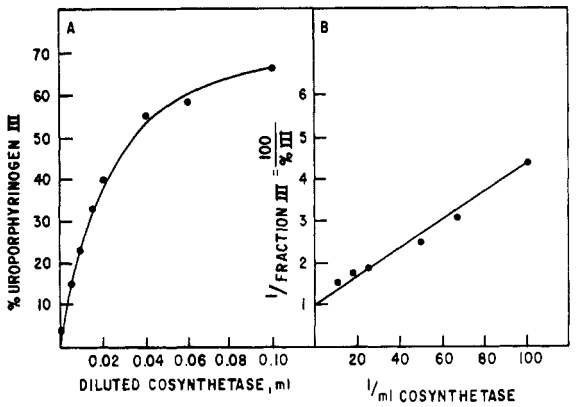

FIGURE 4: Modified assay for cosynthetase, in which the isomer composition of the enzymatically formed uroporphyrinogen was determined after chemical oxidation and decarboxylation to the corresponding coproporphyrins. (A) Plot of per cent isomer III against the amount of cosynthetase added. (B) Reciprocal plot of A.

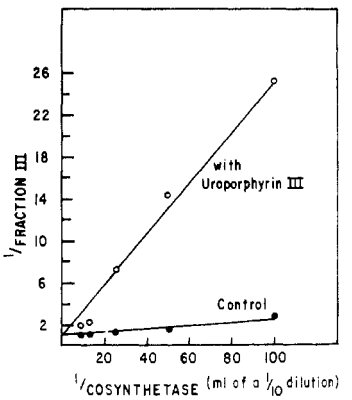

FIGURE 5: Inhibition of cosynthetase by 2×10^{-5} M uroporphyrin III, as determined with the assay shown in Figure 4B.

product, and calculate the amount of uroporphyrinogen III formed for a given amount of cosynthetase.

When cosynthetase preparations are assayed by this method, the shape of the curve formed by a plot of per cent isomer III *vs.* cosynthetase concentration is not a straight line (Figure 4A). Inspection of a number of such assays suggested that these curves have the shape of a rectangular hyperbola. Curves of this kind can be rectified to a straight line by replotting them as reciprocals. Figure 4B shows that such a plot of 1/fraction III *vs.* 1/cosynthetase concentration approximates a straight line which intersects the ordinate at the numeral 1.0, that is, 100% III. This indicates that, *in vitro*, 100% III in the reaction product can actually be obtained only with an infinite concentration of cosynthetase. This result is consistent with an hypothesis of the mode of action of cosynthetase which proposes the polypyrrole intermediate [X] (Bogorad, 1963), diagrammed in Figure 2. On the other hand, it may simply reflect the inactivation of cosynthetase during the formation of uroporphyrinogen III.

Inhibition of Cosynthetase by Porphyrinogens and Porphyrins. The availability of this modified assay for cosynthetase activity provided a means of testing this enzyme for inhibition by various porphyrins and porphyrinogens, because the separation method for coproporphyrin isomers is not affected by variations in the amount of material chromatographed, as is the separation method for uroporphyrin isomers (Cornford and Benson, 1963). To carry out these experiments, tritium-labeled porphobilinogen was incubated at 31° in the dark with synthetase and the cosynthetase to be assayed, in the presence and absence of unlabeled porphyrins and porphyrinogens. The isomer ratio of the radioactive coproporphyrin, derived from the enzymatically produced radioactive uroporphyrinogen by oxidation and chemical decarboxylation, was analyzed radiometrically (Romeo and Levin, 1969).

These experiments revealed that uro- and coproporphyrins I and III, and their corresponding porphyrinogens, all inhibit cosynthetase from 50 to 90% at a concentration of 2×10^{-5} M. Uroporphyrinogen I synthetase activity was not affected under these conditions. Figure 5 demonstrates the inhibition of cosynthetase by 2×10^{-5} M uroporphyrin III; at one-tenth of this concentration the inhibition is only about 10%. The relationship of the inhibition by porphyrinogens to the reaction-dependent inactivation of cosynthetase has not yet been determined. However, the reaction-dependent inactivation of cosynthetase is probably not simply due to binding of the product as a competitive inhibitor, because a second addition of cosynthetase to a reaction mixture containing inactivated cosynthetase causes uroporphyrinogen III formation to resume (Levin, 1968a). Moreover, product inhibition is not consistent with an effect of a change in rate of the synthetase-catalyzed reaction on the inactivation process as described above.

Purification of Cosynthetase from Erythropoietic Mouse Spleen. Evidence that cosynthetase activity resides in a protein moiety provided a stimulus to attempts to purify it by conventional enzyme fractionation methods. These attempts have been hampered by instability of the enzyme during purification procedures, and the cumbersome nature of the routine assay, but a procedure has been devised which purifies cosynthetase 18-fold from mouse spleen, with a recovery of 5%. Subfractions have been obtained with purifications as high as 25-fold. The yield of the procedure is low, but the starting material is rich in cosynthetase, containing about 3700 units/g of spleen, or 2700 units/animal.

Spleens were removed from about 200 phenylhydrazine-treated white ICR mice on the fourth day after cessation of the phenylhydrazine treatment (Levin and Coleman, 1967). All subsequent steps were carried out at 2–4°. The chilled spleens were homogenized for 40 sec in a Waring Blendor with thrice their weight of 0.05 M potassium phosphate buffer (pH 7.9). The homogenate was centrifuged for 15 min at 25,000*g* and the sediment was discarded. The crude extract was stirred while 280 mg of ammonium sulfate/ml was added slowly. The mixture was equilibrated for 30 min, and the precipitate was removed by centrifugation as before, and set aside for recovery of the uroporphyrinogen I synthetase, beginning with the heat step (Levin and Coleman, 1967). To the supernatant liquid, ammonium sulfate was added as before, 140 mg for each ml of the original crude extract. The precipitate was collected by centrifugation, redissolved in one-quarter of the original volume of the same buffer as used for the homogenization, and dialyzed in a rocker for 3 hr against at least a 25-fold excess of 0.01 M potassium phosphate buffer (pH 6.8), changed twice. The dialysate was frozen overnight at −20°, and the precipitate was discarded.

Aliquots of this preparation containing 220 mg of protein were chromatographed on a 12 × 0.9 cm column of Bio-Rex 70, 200–400 mesh, previously equilibrated with the pH 6.8 buffer by flushing it for 2–4 days (1500–2500 ml) before the enzyme was applied. Development was continued with this

TABLE I: Partial Purification of Uroporphyrinogen III Cosynthetase from Mouse Spleen (204 Mice).

Preparation	Sp Act.[a] (Units/mg of Protein)	Purification, fold	Yield (%)
Crude extract (25,000*g*)	31		
First ammonium sulfate fractionation	118	3.8	79
Bio-Rex chromatography	414	13.3	18
Second ammonium sulfate fractionation	548	17.7	5

[a] Assays were performed with pH 7.9 buffer.

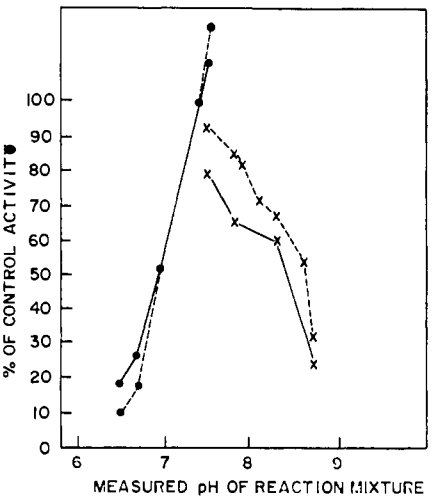

FIGURE 6: Plot of activity against pH for synthetase (solid line) and cosynthetase (dashed line). Control activity was that observed with pH 7.7 buffer (measured pH of reaction mixture 7.55). (●) Phosphate buffer and (×) Tris-HCl buffer.

buffer until a red band was eluted (50–60 ml), and the eluate became clear, after which the enzyme was eluted with 0.1 M potassium phosphate buffer (pH 7.7). The enzyme appeared in the effluent after about 50 ml. Fractions of 3 ml were collected, and those purified 2-fold or more were saved and pooled.

The combined active Bio-Rex fractions were fractionated again with ammonium sulfate. This time the fraction precipitated with the first 330 mg/ml of Bio-Rex eluate was discarded, and the precipitates obtained with four successive additions of 70 mg of ammonium sulfate/ml of Bio-Rex eluate were collected separately, dissolved in one-tenth the original volume of 0.05 M potassium phosphate buffer (pH 7.7), and dialyzed for 3 hr against the same buffer, changed once. The dialyzed fractions were kept frozen overnight, and any precipitate was discarded before they were assayed for protein and cosynthetase activity. Usually two of these fractions contained all the activity which was recoverd. A typical preparation is outlined in Table I.

pH–Activity Curves for Mouse Spleen Synthetase and Cosynthetase. Figure 6 shows that the optimum pH value for the activity of uroporphyrinogen I synthetase from mouse spleen is at 7.7–7.9, close to that for the same enzyme from other sources (Bogorad, 1958a; Sancovich *et al.*, 1969; Llambias and Batlle, 1971a,b). To obtain the corresponding pH optimum curve for cosynthetase, its activity was determined in reaction mixtures which contained 50 μmoles of buffer at the appropriate pH, 120 nmoles of porphobilinogen, and 40 units of synthetase, previously assayed at that pH value. In this way the variation of cosynthetase activity with pH was measured independently of the variations in synthetase activity with pH. The resulting curve is also shown in Figure 6. The pH–activity curve of cosynthetase is very similar to that for synthetase, as might be expected.

There are two flaws in the pH–activity curve for cosynthetase illustrated in this figure. A minor one is that control experiments showed that cosynthetase is slightly unstable on the alkaline side of the pH optimum, even in the absence of porphobilinogen and synthetase; about 8% of the activity is lost in 30 min at 31° at pH 8.3, and 18% at pH 8.7. More important, cosynthetase stimulates synthetase activity at pH values above 8.5. Previous work had shown that such a stimulation does not occur around the pH optimum for these enzymes (Levin, 1968a). Both the instability of cosynthetase to alkaline conditions, and the stimulation by cosynthetase of synthetase activity, make the apparent activity of cosynthetase lower than the true activity at pH values of 8.5 and above.

Assay of Cosynthetase by Measurement of Synthetase Stimulation. The stimulation of synthetase activity by cosynthetase at high pH values has been developed into a rapid assay for cosynthetase activity. Initial experiments indicated the maximum stimulation of porphobilinogen disappearance that could practicably be obtained ranged around 70%, using 2000 units of cosynthetase and 50 units of synthetase incubated at pH 9. However, under these conditions, the stimulation of synthetase was not proportional to the amount of cosynthetase added, since a 30–60% stimulation was observed with 500 units of cosynthetase. To obtain a more nearly linear response of synthetase activity to the amount of cosynthetase added, it was necessary to use much smaller amounts of cosynthetase and lower levels of stimulation. It was not feasible to measure these small stimulations by assaying the disappearance of porphobilinogen, as in the usual synthetase assay, so measurement of uroporphyrinogen formation was employed instead. This has so far limited practical use of this assay to preparations which do not have high optical density around 400 mμ in the concentrations to be assayed, *i.e.*, to preparations purified through the Bio-Rex step.

The standard assay for the measurement of cosynthetase activity in terms of synthetase stimulation contained 50 μmoles of Tris-HCl buffer (pH 9.0), 120 nmoles of porphobilinogen, 40 units of synthetase (previously assayed at pH 9.0), and 2–12 units of partially purified cosynthetase, in a volume of 0.5 ml, incubated for 60 min at 31°. For each assay, a control tube contained the same components, except that the cosynthetase was added at the end of the incubation. After the incubation, the tubes were cooled in ice, and 5 μl of iodine solution (25 g/l. of 0.3 M KI) was added, followed immediately by enough of a freshly prepared solution of sodium thiosulfate (three to four crystals in 50 ml of water) to destroy the iodine color. The volume was brought to 1 ml with water, and the optical density of the assay mixture was determined at the Soret maximum by scanning from 420 to 380 mμ in a Cary 14 recording spectrophotometer, using the cor-

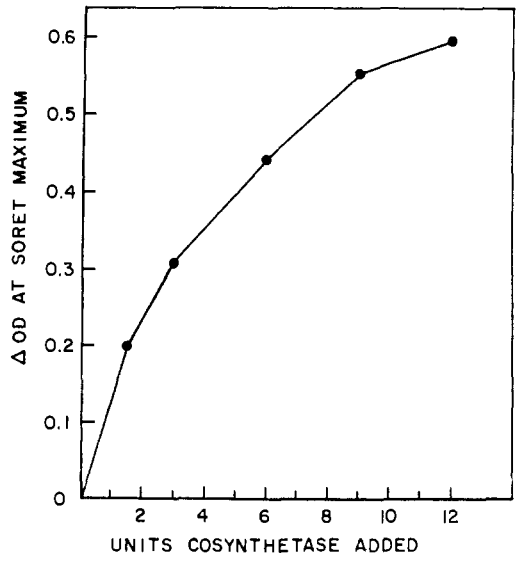

FIGURE 7: Stimulation by cosynthetase of synthetase-catalyzed uroporphyrinogen formation at pH 9.

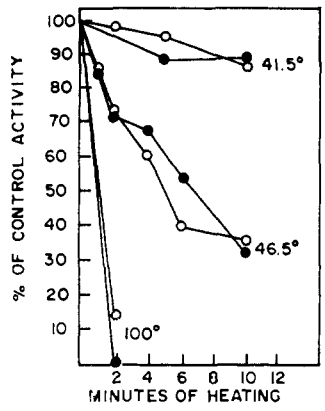

FIGURE 8: Similarity between the rates of heat inactivation of cosynthetase (●) and the synthetase-stimulating factor (○).

responding control tube as the blank. In this way, the optical density of the Soret band indicated directly the increase in uroporphyrinogen formation resulting from the stimulation of synthetase by cosynthetase. Maximum usable optical density readings in this assay were about 0.65, representing a stimulation of 2.9 nmoles of uroporphyrinogen, or 29%. At higher levels of stimulation, the readings deviated too far from linearity to be useful.

A standard curve for cosynthetase activity is shown in Figure 7, with the optical density reading plotted against the units of cosynthetase added (as determined by the standard assay at pH 7.9). This curve was reproducible and correlated well between different cosynthetase preparations. With the best preparations (second ammonium sulfate fractions), which were nearly colorless, a single control tube without cosynthetase could be used for all the samples being assayed, and the addition of cosynthetase to this control tube could be omitted.

Control Experiments Validating the Synthetase-Stimulation Assay for Cosynthetase. Use of the synthetase-stimulation assay for the measurement of cosynthetase activity requires assurance that the stimulation is due to a real increase in synthetase activity, and not to some artifact; that the stimulation is not produced by the presence of incidental synthetase activity in the cosynthetase preparations; and that it is due to cosynthetase itself, not to some third factor contaminating cosynthetase preparations. Since the stimulation is detectable as an increase in both porphobilinogen disappearance and uroporphyrinogen formation, it seems not to be due to some effect of cosynthetase on the analytical method when the assay is run at pH 9. The stimulation of synthetase is not a spurious effect caused by contamination of the cosynthetase preparations with synthetase, because it could not be observed at pH 7.9, the optimum for synthetase, but only above pH 8.5. Moreover, the amounts of cosynthetase being assayed did not contain measureable amounts of synthetase activity when incubated with porphobilinogen for 30 min at pH 9.0; in reaction mixtures from which synthetase had been omitted, no Soret peak could be observed, and not more than 3% of the amount of synthetase needed to give the observed stimulation could have been present in the cosynthetase. Finally, synthetase is stable for 30 min at 60°, while the synthetase-stimulating factor is destroyed under these conditions. Evidence that the stimulation of synthetase is due to cosynthetase itself, and not to some third factor, comes from the following observations: the purification of the synthetase-stimulating factor follows that of cosynthetase, as determined by the standard assay, through the Bio-Rex fractions and the second ammonium sulfate fractionation; and the heat lability of the synthetase-stimulating factor and cosynthetase are virtually identical at 41.5, 46.5, and 100° (Figure 8).

Discussion

The proposal by Bogorad (1963) that uroporphyrinogen I synthetase catalyzes the formation of a polypyrrole intermediate which can be cyclized to uroporphyrinogen III by uroporphyrinogen III cosynthetase remains the simplest hypothesis of the relationship between these two enzymes. Recently, evidence has been obtained that noncyclic polypyrroles are produced when synthetase acts on porphobilinogen in the presence of ammonia or hydroxylamine (Pluscec and Bogorad, 1970). The availability of these inhibitors of tetrapyrrole cyclization provides a new tool for the preparation of possible substrates for uroporphyrinogen III cosynthetase, which increases interest in suitable methods for the preparation and assay of the latter enzyme. Cosynthetase has been prepared from a number of plant and animal tissues (Bogorad, 1962; Stevens and Frydman, 1967; Stevens *et al.*, 1968; Llambias and Batlle, 1971a,b), but none of these reports describes quantitative assay of cosynthetase activity. Presumably this is because the only available assay (Levin, 1968a) is laborious and is difficult to standardize. The present report should contribute to a wider use of quantitative methods for studies of cosynthetase activity, because it describes a procedure for an initial 18-fold purification of cosynthetase, and a simple assay suitable for use in further purification attempts. In addition, it provides evidence that despite its inactivation during uroporphyrinogen III formation, cosynthetase is an enzyme active in catalytic concentrations, and not some protein-bound cofactor required in stoichiometric amounts.

Cosynthetase inactivation is only known to proceed when catalysis by synthetase is occurring, which implies that the inactivating reactant indicated by the question mark in Figure 2 is some intermediate or product of the process of tetrapyrrole formation. With a relationship like that shown in Figure 2,

the amount of uroporphyrinogen III formed will be proportional to the amount of cosynthetase inactivated, provided that the cosynthetase-catalyzed and cosynthetase-inactivating reactions are first order in cosynthetase.[1] When the reaction between synthetase and porphobilinogen is made to proceed more slowly, by decreasing the concentration of synthetase, cosynthetase functions more efficiently, catalyzing more uroporphyrinogen III formation before losing activity. Both this experiment, and the evidence that cosynthetase is a protein which is active in catalytic concentrations, suggest that cosynthetase inactivation is not a requirement for the enzymatic formation of uroporphyrinogen III. Nevertheless, the inactivation may have biological significance, because it could account for the low activity of cosynthetase which has been observed in human and animal subjects with congenital erythropoietic porphyria (Levin, 1968b; Romeo and Levin, 1969), a hereditary disease characterized by the overproduction of uroporphyrinogen I.

The stimulation of synthetase by cosynthetase at pH 9, which has been used as the basis of a rapid assay for cosynthetase, is another unusual feature of the relationship between these two enzymes. Like cosynthetase inactivation, it could provide an important clue for studies of the enzyme mechanism, despite the fact that it is not a phenomenon essential to uroporphyrinogen III production. Although previous studies have indicated that under optimal conditions cosynthetase does not affect the rate or stoichiometry of the synthetase-catalyzed reaction (Levin, 1968a), Bogorad (1958b) observed that cosynthetase stimulates the consumption of porphobilinogen at low substrate concentration. It may be that this stimulation of synthetase activity at suboptimal substrate concentration, as well as the stimulation at nonoptimal pH values, indicates that cosynthetase is removing some synthetase-generated intermediate which inhibits synthetase activity.

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[1] Let u = uroporphyrinogen III, c = cosynthetase. Then the first-order assumptions yield the system $dc/dt = -kc$, $du/dt = k'c$; k, k' constants, $du/dt = (-k'/k)(dc/dt)$, and $u = (k'/k)c +$ constant. The constant is fixed at $t = 0$, when $u = 0$ and $c = c_0$. Hence that constant is $(k'/k)\,c_0$. Thus $u = (k'/k)(c_0 - c) = k'/k$ (amount inactivated).

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