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PORPHYRIN BIOSYNTHESIS

VII. PORPHYRINOGEN CARBOXY-LYASE FROM AVIAN ERYTHROCYTES. PURIFICATION AND PROPERTIES

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SUMMARY

1. Uroporphyrinogen carboxy-lyase (EC 4.1.1.d), the enzyme catalysing the decarboxylation of uroporphyrinogen to coproporphyrinogen, has been isolated from normal chicken erythrocytes. The enzyme was purified 220-fold with a yield of 24% from haemolysate supernatant by DEAE-cellulose batch treatment, $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose.

2. The purified material appears to be homogeneous in polyacrylamide gel disc electrophoresis.

3. The enzyme was heat labile and inhibited by sodium salt; the activity was enhanced by EDTA, GSH and boiled rat-liver extract.

4. The influence of these chemical and physical agents on the removal of the first and second carboxyl groups from uroporphyrinogen was compared; the second group was more susceptible to these agents.

5. The possibility that one or several enzymes were involved in the stepwise decarboxylation of uroporphyrinogen is discussed.

6. The general name of porphyrinogen carboxy-lyase for the enzyme system is proposed because of the different porphyrinogens it can decarboxylate.

INTRODUCTION

Evidence that the porphyrinogens are the real intermediates in the metabolic pathway of haem was derived from the works of BOGORAD[1,2] and NEVE *et al.*[3] and was supported by many others[4–8].

Enzymatic decarboxylation of uroporphyrinogen I and III has been shown

Nomenclature: uroporphyrinogen, 8-COOH porphyrinogen; coproporphyrinogen, 4-COOH porphyrinogen; phyriaporphyrinogen, 7-COOH porphyrinogen.

using a crude preparation from Chlorella[1-2], avian erythrocytes[6,7] and *Rhodopseudomonas spheroides*[5], the biosynthetic products observed being phyriaporphyrin (7-COOH porphyrin), coproporphyrin and small amounts of 5- and 6-COOH porphyrins.

Previous works in this laboratory using phyriaporphyrinogen[7] and 5- and 6-COOH porphyrinogen[8] as substrates have confirmed that the transformation of uroporphyrinogen to coproporphyrinogen is a stepwise decarboxylation. Uroporphyrinogen carboxy-lyase which catalyses the removal of four carboxyl groups from uroporphyrinogen has been partially purified from rabbit reticulocytes[9-10] and from *Rhodopseudomonas spheroides*[11].

Studies on decarboxylating activity of a partially purified enzyme system were later carried out using porphobilinogen[12-13].

The present work describes the preparation of a purified form of porphyrinogen carboxy-lyase from chicken erythrocytes as well as some of its properties.

MATERIALS AND METHODS

δ-Amino laevulinic acid, cysteine, sodium thioglycollate, β-mercaptoethanol, GSH and DEAE-cellulose (0.83 mequiv/g), were purchased from the Sigma Chemical Co. and the Cianogum 41 from American Cyanamid Co. All other reagents were of analytical grade and obtained commercially. Porphobilinogen was obtained biosynthetically[14] or from urine[15].

Uroporphyrin (8-COOH porphyrin) I and III, 7-COOH porphyrin III, 6-COOH porphyrin III, 5-COOH porphyrin III, and coproporphyrin (4-COOH porphyrin) III were isolated by methods previously described[8]. The porphyrins used as substrates were further purified by chromatography on MgO columns[16]. Free porphyrins were prepared by hydrolysing the methyl esters in 25% (w/v) HCl for 24 h at room temperature.

Porphyrinogen preparations

Solutions of the porphyrins (60–75 μM) in 25 mM NaOH were reduced with freshly ground 3% sodium amalgam according to MAUZERALL AND GRANICK[9]. The porphyrinogen solutions were adjusted to pH 7.0 with 40% (w/v) H_3PO_4 and were immediately used. The yield of porphyrinogen determined after reoxidation to porphyrin was about 95%.

Incubation conditions of carboxy-lyase

The reaction system contained 67 mM potassium phosphate buffer (pH 7.0), 1 mM GSH, 0.1 mM EDTA, enzyme preparation, 2.5–3.5 μM porphyrinogen and distilled water to 4 ml, unless otherwise indicated. Assays were carried out anaerobically in Thunberg tubes at 37° in the dark with mechanical shaking.

Incubation times are indicated in the figures and tables. For the control assays, the enzyme fraction was replaced by a boiled enzyme preparation.

Isolation and quantitative determination of porphyrins

After incubation, the reactions were stopped in two different ways according to the enzyme preparation:

(a) Haemolysate supernatant: by the addition of 5 vol. of ethyl acetate–acetic

acid (3:1, v/v), the mixtures being set aside overnight. The porphyrins were quantitatively extracted into 5% (w/v) HCl as previously described[8].

(b) Purified enzyme preparations: by the addition of concentrated HCl to give a final concentration of 5%. The mixtures were allowed to stand for 30 min at 0°. The proteins were centrifuged and washed with 5% HCl until no fluorescence was observed in the supernatant. The recovery of total free porphyrins after incubation was about 90%, except in the case of the haemolysate supernatant where the recovery was 70%.

The acid extracts were evaporated to dryness in a dessicator under vacuum, and the residues were esterified by the ordinary method employing methanol–sulphuric acid.

Quantitative determinations of the different porphyrins were performed as follows: 3–6 nmoles of methyl ester porphyrins were applied as a narrow strip 5 cm long on Whatman No. 1 paper, and the chromatogram was developed by the FALK AND BENSON[17] system.

After chromatographic separation, the porphyrins with different number of carboxyl groups were identified under ultraviolet light and isolated by cutting the corresponding strips and by eluting them with chloroform. The porphyrin content of the chloroformic eluates were spectrophotometrically determined and expressed as per cent of the total amount recovered. The amount of porphyrins in acid and chloroform were determined by measuring the absorption at the peak of the Soret band and by using the reciprocal extinction coefficient given by SALUM *et al.*[18] or the correction formula quoted by RIMINGTON AND SVEINSSON[19].

The reciprocal extinction coefficients in chloroform of 5-COOH and 6-COOH porphyrins (expressed in μg/ml) were interpolated between those of 7-COOH and 4-COOH porphyrins, assuming a linear function between the number of carboxylic groups and the reciprocal extinction coefficient. These coefficients are further expressed in nmoles/ml. The reciprocal extinction coefficients used in the determination of total free porphyrins were calculated on the basis of the individual coefficients, taking into account the percentage of the different porphyrins present in the mixtures as revealed by chromatography.

Protein concentrations

Proteins were determined by the method of LOWRY *et al.*[20] and by measuring the absorbance at 260 and 280 mμ (ref. 21). Crystallised bovine serum albumin was used as a standard.

Units

One unit of porphyrinogen carboxy-lyase activity is expressed as the amount of enzyme required to catalyse the decarboxylation of one nmole of uroporphyrinogen per h under the experimental conditions. Decarboxylation of uroporphyrinogen is expressed as nmoles of 7- + 6- + 5- + 4-COOH porphyrins or as percentage of these products formed, and that of phyriaporphyrinogen is expressed as nmoles of 6- + 5- + 4-COOH porphyrins or as percentage of these products formed.

Boiled rat-liver extract

Rat liver was homogeneised with 0.15 M KCl in a Potter–Elvehjem glass homo-

geniser. The mixture was kept at 100° for 3 min, rapidly cooled and centrifuged at 11 000 × *g*. The supernatant was stored at −10°.

Polyacrylamide disc gel electrophoresis

Electrophoresis in 5% polyacrylamide gel was performed essentially by the techniques of RAYMOND AND WEINTRAUB[22]. The electrophoretic runs were carried out on small cylinders (0.8 cm × 8 cm) in veronal buffer (pH 8.6), *I* 0.1, over a period of 4 h with constant current of 6 mA per tube.

All the manipulations were performed at room temperature and, after each run, the gels were removed and stained by immersing them in a solution of 0.25% Amido Schwarz with 7% acetic acid for 5 min, followed by repeated washing with 5% acetic acid to remove excess dye.

RESULTS

Enzyme purification

The procedure is summarised in Table I. All steps were carried out at 0–4°.

Step 1. Haemolysate supernatant. Fresh chicken blood with heparin as anticoagulant was used in all experiments. The plasma and buffy coat were separated by centrifugation at 11 000 × *g* for 15 min, and the cells were washed 3 times by suspension and centrifugation with 2 vol. of 0.9% NaCl. The cells were suspended in 1 vol. of water, and the haemolysate was obtained by twice freezing and thawing the preparation. After centrifugation at 35 000 × *g* for 60 min, the supernatant was carefully poured off and the packed stroma were discarded.

Step 2. DEAE-cellulose batch eluates. The procedure for removing haemoglobin from the haemolysate supernatant by differential adsorption on DEAE-cellulose was similar to that used by HENNESSEY *et al.*[23], with slight modifications. In each batch 6 ml of haemolysate supernatant were added to 5 ml of DEAE-cellulose suspension (pH 7.0). After standing 20 min, the mixtures were centrifuged for 15 min at 1000 × *g*, and the adsorbent was washed with 3 mM phosphate buffer (pH 7.0) until the supernatants were colourless.

The proteins were desorbed from DEAE-cellulose as follows. After the batches were pooled, 4 vol. of 0.134 M potassium phosphate buffer (pH 7.0) were added for each volume of haemolysate supernatant. The mixture was magnetically stirred for 45 min in the cold room and centrifuged at 1000 × *g* for 15 min.

These eluates contained δ-amino laevulinic acid dehydratase, porphobilinogenase and decarboxylase, all the soluble enzymes of the haem biosynthetic pathway (Table II) as revealed by coproporphyrin formation using δ-amino laevulinic acid as substrate. Further extraction with the same buffer allows one to obtain a practically quantitative recovery of these enzymes.

Step 3. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was slowly added with stirring in the cold room, and the preparations were maintained at pH 7.0 by the addition of diluted KOH as needed. To the eluates from Step 2, $(NH_4)_2SO_4$ was added to 40% saturation. The suspension was allowed to stand for 30 min with stirring before being centrifuged at 20 000 × *g*. The precipitates showed only slight activity and were discarded. The supernatant was then brought to 75% saturation. After stirring for

45 min, the precipitate was collected as above. The 75% supernatant was discarded because of its negligible activity.

The 40–75% pellet was suspended in 0.134 M potassium phosphate buffer (pH 7.0) to give a final concentration of about 7 mg/ml and was dialysed against three changes of 3 mM potassium phosphate buffer (pH 7.0) for 3 h. There was no lose of activity during this dialysis.

Step 4. DEAE-cellulose chromatography. The enzyme preparation from Step 3 was applied to a DEAE-cellulose column (1.6 cm × 18 cm) which had been previously equilibrated with 3 mM potassium phosphate buffer (pH 7.0) and 1 mM GSH at 5°.

Elutions were started with 1 column vol. of 3 mM potassium phosphate buffer containing 1 mM GSH and followed by 5 column vol. of a stepwise gradient of KCl in the same buffer containing GSH. The concentrations of KCl used were 0.1, 0.15, 0.2 and 0.25 M. The flow rate was maintained at about 1 ml/min, and 3.5-ml fractions were collected.

After determination of the absorbance profile of the eluted fractions, porphyrinogen carboxy-lyase activity was measured on some fractions. Three peaks of protein were eluted, but decarboxylase activity only appeared at the first protein peak. The elution pattern for the separation of the enzyme on DEAE-cellulose is presented in Fig. 1.

The final purified enzyme solution had a specific activity of 78 units/mg and showed a 220-fold purification (Table I). Approx. 70% of the activity put on the column was recovered. The highest specific activity so far obtained in any single fraction was 120 units/mg, representing a 340-fold purification. In such a fraction no activity of either δ-amino laevulinic acid dehydratase or porphobilinogenase was observed, as measured by the usual methods[24].

The enzyme preparations obtained in each step of fractionation were subjected to polyacrylamide gel disc electrophoresis to test the extent of purification. The purified enzyme preparation (Step 4) migrated as a single band on disc gel electrophoresis under conditions described in MATERIALS AND METHODS.

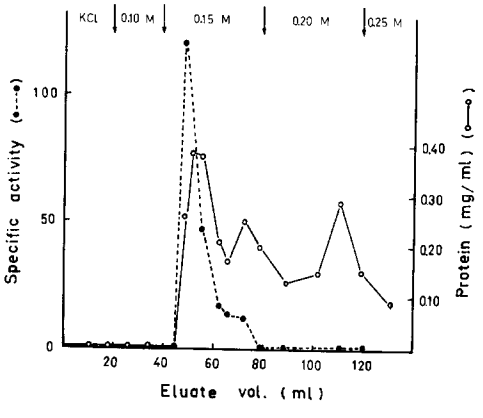

Fig. 1. DEAE-cellulose chromatography of porphyrinogen carboxy-lyase. 20 mg of protein were applied to the column (1.6 cm × 20 cm) equilibrated with 3 mM potassium phosphate buffer (pH 7.0) and eluted as described in the text. ○—○, protein; ●— — —●, specific activity (nmoles/h per mg).

TABLE I

PURIFICATION OF PORPHYRINOGEN CARBOXY-LYASE

Details of the procedure and assay methods are described in the text. Incubation time, 30 min.

Purification steps	*Total protein (mg)*	*Activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
1. Haemolysate supernatant	14 800	5240	0.35	100
2. DEAE-cellulose batch eluates	130	3420	26	65
3. 40–75% satn. $(NH_4)_2SO_4$ ppt.	77	1840	24	35
4. DEAE-cellulose column eluates	16	1260	78	24

Optimal pH

The activity of porphyrinogen carboxy-lyase at different pH values was measured in 67 mM potassium phosphate buffer ranging from pH 6.5 to 7.7. The pH of maximal activity was found to be 7.0, in good agreement with the value observed by MAUZERALL AND GRANICK[9].

Factors influencing the determination of enzyme activity

The influence of aero- and anaerobiosis during incubation as well as the effect of EDTA was studied. The results are shown in Table II.

When anaerobiosis was assayed with uroporphyrinogen as substrate, an increase in the uroporphyrinogen decarboxylation was found. This activation was enhanced by the addition of EDTA. The action of both factors was more striking on the phyria-porphyrinogen decarboxylation. Similar results were obtained whe n δ-aminolaevulinic acid and different enzyme preparations were used, as shown in Table II.

Effect of sulphydryl reagents

The anaerobic decarboxylation of the porphyrinogens was markedly activated by GSH at the concentration of 1 mM, while thioglycollate only slightly stimulated at

TABLE II

EFFECT OF ANAEROBIOSIS AND EDTA ON PORPHYRINOGEN DECARBOXYLATION

Assay mixtures were incubated at 37° for 2 h. Substrates and enzyme preparations are indicated in the tab Relative activation is expressed with respect to aerobic assay.

Substrate	*Enzyme prep.*	*Conditions*	*7- + 6- + 5- + 4-COOH porphyrins (%)*	*Relative activation*	*6- + 5- + 4-COOH porphyrins (%)*	*Relative activation*
δ-Amino laevulinic acid (0.5 mM)	Step 2	Aerobiosis	35	1	16	1.0
		Anaerobiosis	55	1.6	34	2.1
δ-Amino laevulinic acid (1.25 mM)	Step 3	Aerobiosis	30	1	11	1
		Anaerobiosis	62	2.0	44	4.0
Uroporphyrinogen III (5 μM)	Step 2	Aerobiosis	57	1	18	1
		Anaerobiosis	79	1.4	41	2.3
		Anaerobiosis + 0.1 mM EDTA	85	1.5	60	3.3

TABLE III

INTERMEDIATE ACCUMULATION IN UROPORPHYRINOGEN DECARBOXYLATION

Incubation conditions were the same as in Fig. 2. Porphyrins formed were measured as described in the text.

Incubation time (min)	*Porphyrins formed (nmoles)*			
	7-COOH	*6-COOH*	*5-COOH*	*4-COOH*
15	2.9	0.5	0.5	1.2
30	3.5	0.6	0.5	2.2
45	3.7	0.6	0.6	3.6

the same concentration. However, when cysteine (0.1–1 mM) was tested, an opposite effect on activity was found. Further studies on these effects are in progress.

Cofactor requirements

In order to study the requirement of a cofactor for the porphyrinogen carboxy-lyase, pyridoxal phosphate was tested. There was no effect on the catalytic activity at the concentrations (0.2 and 0.5 mM) used.

On the other hand, when a boiled rat-liver extract was added to an incubation mixture, decarboxylating activity was stimulated about 13% for the first carboxyl group removal and 34% for the second.

Time-course of reactions

Uroporphyrinogen decarboxylation over a 45-min period was investigated, and the results are illustrated in Fig. 2. There was an initial rapid decarboxylation of the substrate, which increased more slowly after 15 min.

Phyriaporphyrinogen decarboxylation proceeded slowly with a linear increase over the 45-min period.

It is evident that phyriaporphyrinogen is accumulated during incubation (Table III). This accumulation increased over the first 15 min and then remained constant. Table IV shows the formation of the decarboxylation products from uroporphyrinogen and enzyme preparations from different purification steps. As it can be seen, accumulation of phyriaporphyrinogen occurred in all cases.

TABLE IV

INTERMEDIATES IN PORPHYRINOGEN DECARBOXYLATION

Reaction mixtures were the same as in Fig. 2 except for enzyme fractions, which are indicated. Incubation time, 30 min.

Enzyme prep.	*Protein incubated (mg)*	*Porphyrins formed (nmoles)*			
		7-COOH	*6-COOH*	*5-COOH*	*4-COOH*
Step 1	31	3.1	0.5	0.5	1.4
Step 2	0.44	3.2	0.5	0.6	1.5
Step 3	0.53	3.5	0.5	0.5	2.2
Step 4	0.13	3.9	0.6	0.5	2.8

TABLE V

EFFECT OF SODIUM SALT CONCENTRATION ON PORPHYRINOGEN DECARBOXYLATION

Expt. 1 contained 0.5 mM δ-amino laevulinic acid and enzyme fraction from Step 2. Expt. 2 contained 2.8 μ uroporphyrinogen III and enzyme fraction from Step 3. NaCl was present in the assays at the concentration indicated in the table. Other conditions are as described in the text. Incubation time, 30 min.

Expt. No.	*Substrate*	[*Na*$^+$] *(mM)*	*Total porphyrin (nmoles)*	*Uroporphyrinogen decarboxylation*		*Phyriaporphyrinogen decarboxylation*	
				7- + 6- + 5- + 4-COOH porphyrins (%)	*Inhibition (%)*	*6- + 5- + 4-COOH (%)*	*Inhibition (%)*
1	δ-Amino	0	15.3	49		26	
	laevulinic	200	10.1	35	28	15	42
	acid	400	6.3	15	69	5	81
2	Uro-	50	11.3	58	—	30	
	porphyrino-	100	11.3	55	5	20	33
	gen III	200	11.3	44	24	16	47
		400	11.3	20	66	6	80

Effect of NaCl

The effect of NaCl concentrations on the decarboxylation steps was examined in different enzyme fractions, using δ-amino laevulinic acid and uroporphyrinogen as substrates (Table V). With both substrates less decarboxylation of porphyrinogen was observed when NaCl was present in the concentrations assayed. In Expts. 1 and 2, at 200 mM NaCl the transformation of uroporphyrinogen to phyriaporphyrinogen was inhibited about 25%, the second step being 45% inhibited. At 400 mM, the effect was more striking, and twice as much inhibition was found. Furthermore, intermediates with 6- and 5-carboxyl groups were found to decrease with increasing sodium salt concentration. On the other hand, total porphyrins biosynthesised from δ-amino laevulinic acid were markedly diminished by the addition of such a salt.

TABLE VI

EFFECT OF HEATING ON PORPHYRINOGEN CARBOXY-LYASE ACTIVITY

Enzyme preparation from Step 2 was heated at 60° for the times indicated, immediately cooled in an ice ba and assayed for enzyme activity as described. Incubations were carried out at 37° for 2 h.

Expt. No.	*Substrate*	*Heating time (min)*	*Uro-porphyrinogen decarboxylated (nmoles)*	*Inactivation (%)*	*Phyria-porphyrinogen decarboxylated* (nmoles)*	*Inactivation (%)*
1	Uroporphyrinogen	0	8.7		5.2	
	III (22.8 nmoles)	5	4.1	53	0.0	100
		15	3.2	63	0.0	100
2	Uroporphyrinogen	0	4.5	—	3.5	
	I (24.8 nmoles)	5	0.8	82	0.0	100
3	Phyriaporphyrinogen	0			10.9	
	III (21 nmoles)	10			2.2	80

* Phyriaporphyrinogen decarboxylation was calculated as the sum of nmoles of 6-, 5- and 4-COO porphyrinogens formed from either phyriaporphyrinogen added (Expt. 3) or phyriaporphyrinogen formed fr uroporphyrinogen (Expts. 1 and 2).

Effect of preheating

The effect of heating on uroporphyrinogen carboxy-lyase and phyriaporphyrinogen carboxylyase activities is shown in Table VI. As can be seen, when uroporphyrinogen III was the substrate (Expt. 1), about 50% of the uroporphyrinogen carboxy-lyase activity remained after heating the enzyme solution at 60° for 5 min, while no activity of phyriaporphyrinogen carboxy-lyase was detected under the same condition. Comparative studies carried out with both uroporphyrinogen isomers indicated that the decarboxylation of Isomer I took place at half the rate of Isomer III, confirming the results of MAUZERALL AND GRANICK[9]. Moreover, higher inactivation of uroporphyrinogen I decarboxylation by heat was found (Expt. 2). In order to study more specifically the effect of heat on the second step of decarboxylation, phyriaporphyrinogen III was used as substrate (Expt. 3). The different percentage of inactivation in the second step of decarboxylation obtained in Expts. 1 and 3 could be due to the fact that, in Expt. 1, a small amount of phyriaporphyrinogen was formed because of the enzyme inactivation by heat. Therefore, the amounts of 6-, 5- and 4-COOH porphyrinogens formed from this diminished amount of phyriaporphyrinogen could not have been detectable by chromatography.

DISCUSSION

In order to study the overall decarboxylation of uroporphyrinogen to coproporphyrinogen and to determine whether one or more enzymes are involved in this process, we have purified the enzyme system from chicken erythrocytes and studied the influence of different agents on it.

Considering the high content of haemoglobin in red cells, the main problem was its elimination. A method of HENNESSEY *et al.*[23], when modified, was found to be efficient and rapid for the separation of the haem-soluble enzymes from the haemoglobin. Further purification of the enzyme system by different procedures, outlined in Table I, resulted in a 220-fold purification and a 24% yield. This purified enzyme system behaved electrophoretically as one component.

Since decarboxylation of uroporphyrinogen to coproporphyrinogen occurs in four steps (8-COOH $\xleftarrow{1}$ 7-COOH $\xleftarrow{2}$ 6-COOH $\xleftarrow{3}$ 5-COOH $\xleftarrow{4}$ 4-COOH porphyrinogen), one could assume the existence of more than one enzyme or one enzyme with several catalytic sites. A relatively high accumulation of phyriaporphyrinogen was reported in several papers[15,24–27] and also was observed in the present work (Table IV). Thus, we can consider two distinct metabolic stages: 8-COOH $\xrightarrow{1}$ 7-COOH $\xrightarrow{2}$ 4-COOH porphyrinogen. The enzyme activity corresponding to the second stage may be calculated by assuming that the amount of phyriaporphyrinogen accumulated is sufficient to saturate the enzyme system (Table IV). Furthermore, the intermediates of this stage, *i.e.* 6-COOH and 5-COOH porphyrinogens, were always present in small amounts, and these quantities did not change during incubation (Table III). One could assume, therefore, that a kind of steady state has been reached with the rates of appearance an disappearance of the intermediates being substantially the same. Consequently, the formation of coproporphyrinogen, under our particular experimental conditions, may be regarded as a measure of the enzyme activity corresponding to the second stage, and a comparison can be made between the first and second stages. It is interesting to emphasise that both the degree of purification and the yield were always the same

throughout the purification steps, taking into account both the enzyme activity corresponding to the first stage and that corresponding to the second one. This suggests the existence of only one protein with catalytic activity toward the different porphyrinogens, since the decarboxylation yields coproporphyrinogen as the end product. Taking into account this multiple catalytic function, we call the enzyme system porphyrinogen carboxy-lyase instead of uroporphyrinogen carboxy-lyase. On the other hand, we have obtained other results indicating a differential behaviour of removal of the first and second carboxyl groups of uroporphyrinogen. The removal of the second carboxyl group was always more susceptible to a number of agents, namely enzyme preheating, anaerobiosis, EDTA, GSH, NaCl, boiled extract from liver (Tables II, V and VI). Another indication in favour of the difference in the two stages is the time-course of the reaction (Fig. 2). It is seen that two different rates (v_1 and v_2) exist for

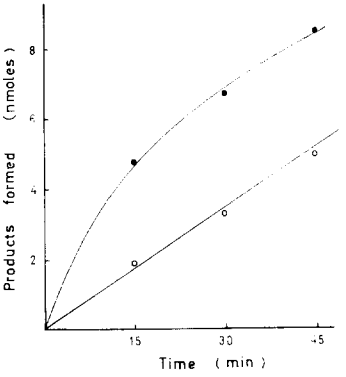

Fig. 2. Time-course of porphyrinogen decarboxylation by porphyrinogen carboxy-lyase. Enzyme from Step 3 was incubated with 2.9 μM uroporphyrinogen for the times indicated in the figure. ●—●, uroporphyrinogen decarboxylation (7- + 6- + 5- + 4-COOH porphyrins); ○—○, phyriaporphyrinogen decarboxylation (6- + 5- + 4-COOH porphyrins).

each of them: 8-COOH $\xrightarrow{v_1}$ 7-COOH $\rightarrow$ 6-COOH $\rightarrow$ 5-COOH $\xrightarrow{v_2}$ 4-COOH with $v_1 > v_2$. This is supported by the accumulation of phyriaporphyrinogen obtained using enzyme preparations of different purity (Table IV). Taking into account the stepwise character of the decarboxylation process, we express the uroporphyrinogen decarboxylating activity as the disappearance of uroporphyrinogen, *i.e.* the formation of all decarboxylation products. In this way, we are measuring v_1. The use of the amount of coproporphyrinogen formed as an expression of decarboxylating activity does not consider the presence of the 7-, 6- and 5-carboxyl intermediates observed. An increased formation of coproporphyrinogen was observed under anaerobic conditions (Table II) in agreement with the results reported by MAUZERALL AND GRANICK[9]. This may be due either to a greater enzyme protection against inactivation or to the oxidation of porphyrinogens in aerobiosis.

In confirmation of the findings of other workers[11-12,28-29], a thermostable co-factor from rat-liver homogenate was found to stimulate the decarboxylating activity. Preheating was found to inactivate both uroporphyrinogen carboxy-lyase and phyriaporphyrinogen carboxy-lyase, the latter being much more sensitive to heat. A similar effect was obtained using uroporphyrinogen I as substrate. Increasing NaCl concen-

tration diminished, in our system, the rate of elimination of the first carboxyl group of uroporphyrinogen as well ase that of the subsequent ones, unlike the conclusion reported by CORNFORD[13]. Moreover, a decrease in the amount of porphyrin biosynthesised from δ-amino laevulinic acid was observed when an high sodium concentration was used. This could be due to an inhibition of both δ-amino laevulinic acid dehydratase and/or porphobilinogenase (deaminase–isomerase).

Some of our experimental data thus far obtained indicate the presence of only one protein component with decarboxylase activity. However, the behaviour of this protein toward agents such as heat and NaCl would be more in accordance with the existence of at least two different catalytic sites. This assumption would agree with the experimental fact that two different stages do exist. It is hoped that further work now in progress will be able to shed more light on this problem.

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