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PORPHYRIN BIOSYNTHESIS

VI. SEPARATION AND PURIFICATION OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN ISOMERASE FROM COW LIVER. PORPHOBILINOGENASE AN ALLOSTERIC ENZYME

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SUMMARY

1. Porphobilinogenase has been isolated and purified from cow liver and its components, porphobilinogen deaminase and uroporphyrinogen isomerase, have been separated from each other and purified.

2. The effect of NH_4^+ was studied. The deaminase exhibited classical Michaelis–Menten kinetics in the absence or presence of NH_4^+, which at high concentrations behaved as a noncompetitive inhibitor of the deaminase. As expected from Hill plots, $n = 1$ both in the absence or presence of NH_4^+. Instead, when activity of porphobilinogenase is plotted *versus* porphobilinogen concentration, sigmoid curves are obtained; but the presence of NH_4^+ at different concentrations altered the kinetic parameters of this enzymic system, again showing normal kinetics. In addition, n values were found to be 2 for porphobilinogen per porphobilinogenase molecule and 1 in the presence of NH_4^+ which behaves as a competitive inhibitor of the isomerase. Results are discussed in relation to the allosteric theories of MONOD *et al.*[1,2] and liver porphobilinogenase is proposed to be an allosteric protein.

3. The presence of an ultrafiltrable factor which stimulates uroporphyrinogen formation from porphobilinogen has been revealed.

INTRODUCTION

FALK *et al.*[3] demonstrated that porphobilinogen could be used for the enzymic synthesis of porphyrins, and the conversion of porphobilinogen into uroporphyrinogens by the enzymic system called porphobilinogenase[4] has been studied in preparations from various sources, including bacteria, algae, higher plants and avian and mammalian red cells[5–12]. Evidences[3–12] show that porphobilinogenase contains at least two enzymes: the relatively heat-stable porphobilinogen deaminase (porphobilinogen ammonia-lyase) which catalyzes the synthesis of uroporphyrinogen I from porpho-

bilinogen and uroporphyrinogen isomerase or uroporphyrinogen III cosynthetase which directs the formation of uroporphyrinogen III from porphobilinogen when deaminase is also present in the system. The mechanism of the enzymic condensation of porphobilinogen to uroporphyrinogen is still unknown, as it is one of the substrates for the isomerase. We intend to study the reaction using purified preparations of the porphobilinogenase as well as those of deaminase and isomerase. Either the isolation or purification of porphobilinogenase or the separation of deaminase from isomerase have been previously reported[5–12]. The present work describes the isolation and purification from cow liver of the porphobilinogenase, that is the deaminase–isomerase complex which converts porphobilinogen into uroporphyrinogen III and the separation and purification of the deaminase and isomerase from each other. The presence of an ultrafiltrable factor which stimulates uroporphyrinogen formation from porphobilinogen has been revealed. Kinetic analyses have been conducted on the deaminase and the porphobilinogenase preparations both in the absence and presence of NH_4^+. This paper reports that porphobilinogenase displays cooperative interactions, suggesting that it is an allosteric protein.

MATERIALS AND METHODS

Porphobilinogen was obtained according to SANCOVICH *et al.*[13] and was assayed as described by MAUZERALL AND GRANICK[14]. Fresh cow liver was kindly supplied by Lab. Asoc. I.F.F.A., Estrella (Buenos Aires) and was stored frozen in solid CO_2. Sephadex was obtained from Pharmacia (London) (N.13), and Bio-Gel P was a generous gift from Bio-Rad Laboratories (Richmond, Calif.). Calcium phosphate gel was prepared using the method of KEILIN AND HARTREE[15].

Estimation of enzyme activity

The standard incubation system, unless stated otherwise, contained the enzyme preparation (usually 5 ml) together with 0.05 M Tris buffer (pH 7.4), 120 μg of porphobilinogen, 0.5 ml of 0.6 M NaCl and 0.5 ml of 0.12 M $MgCl_2$ in a final volume of 10 ml (pH 7.4). Incubations were carried out aerobically in conical 25-ml flasks in the dark with mechanical shaking at 38° for 3–4 h. For kinetic studies, the incubation system contained, in the same final volume and at the same pH, only enzyme, substrate and buffer, with or without the addition of other reagents such as NH_4^+. Blanks were run with porphobilinogen and without enzyme preparation. After incubation, 1.2 ml of concentrated HCl were added to each flask to precipitate the protein; the mixture was then exposed to light and air for 25–30 min to oxidize porphyrinogens to porphyrins. The precipitated protein was removed by filtration, and total porphyrins were determined in a 5% solution[16]. Porphyrins were then fractionated and esterified using the usual procedures[17]. Identification and quantitative determination of porphyrins formed were made using the method of BATLLE AND GRINSTEIN[18], and isomeric composition of uroporphyrin fractions was determined using the method of CORNFORD AND BENSON[19]. Porphobilinogen was estimated by the usual procedure[14]. Protein content was calculated by the method of LOWRY *et al.*[20]. An enzymic unit was defined as the amount of enzyme that catalyzes the formation of 1 nmole of uroporphyrinogen per h, under the standard conditions, the activity being units of enzymes per mg of protein.

For the estimation of isomerase activity, the reaction mixture was the same as described above, except that an excess of deaminase was also added along with varying amounts of the isomerase preparation. NH_4^+ is known to inhibit the porphobilinogenase[21,22]; therefore, they must be removed from the enzyme preparation before estimating enzymic activity. This was carried out using the method of molecular sieving with 1.8 cm × 30 cm columns of Sephadex G-25 or Bio-Gel P-20, using 0.05 M Tris buffer (pH 7.4) as eluent. This method was chosen because recovery of enzyme units was total and because it was more rapid than dialysis. When Sephadex G-100 columns were used, protein was eluted with 0.05 M Tris buffer (pH 7.4) containing 0.1 M NaCl, unless otherwise stated. Fractions were automatically collected with an LKB collector (Stockholm). Protein content and enzymic activity were determined in each fraction, and those fractions containing activity were pooled.

Sephadex columns were prepared as described by BATLLE *et al.*[23].

RESULTS

Preparations of enzymes

All operations were carried out in the cold room at 4°, unless otherwise stated.

Preparation of porphobilinogenase (deaminase–isomerase complex)

Homogenate (10%, w/v) of liver was prepared in 0.25 M sucrose (Table I, Step 1); and the homogenate thus obtained was centrifuged at 11 000 × *g* for 10 min and the sediment was discarded (Step 2). Glacial acetic acid was added to the supernatant, to adjust it to pH 5.0; and after 20 min, the material was centrifuged for 10 min at 11 000 × *g*, and again the sediment was discarded (Step 3). The supernatant solution was then fractionated with solid $(NH_4)_2SO_4$; the fraction precipitating at 35–50% satn. was collected by centrifugation, dissolved in a small volume of 0.05 M Tris buffer (pH 7.4) and passed through a Sephadex G-25 or Bio-Gel P-20 column (Step 4).

TABLE I

ISOLATION AND PURIFICATION OF PORPHOBILINOGENASE FROM COW LIVER

Incubation conditions were as described in the MATERIALS AND METHODS section. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out by usual methods[18,19].

Fraction	*Units/mg* $\times 10^{-3}$	*Purification*	*Porphyrin formed (%)*		
			Uroporphyrin III	*Uroporphyrin I*	*Others*
1. Homogenate	28.3	1	15	—	85
2. 11 000 × *g* (supernatant)	59.4	2.1	15	—	85
3. Treatment with glacial acetic acid to pH 5.0 (supernatant)	102.0	3.6	35	—	65
4. 35–50% satd. $(NH_4)_2SO_4$ fraction	234.9	8.3	75	—	25
5. $Ca_3(PO_4)_2$-gel treatment	469.8	16.6	75	25	—
6. Second 50% satd. $(NH_4)_2SO_4$ fraction	736.0	26.0	75	25	—
7. Sephadex G-100 column (pooled fractions)	3090.4	109.2	100	—	—
7a. Sephadex G-100 column (peak activity)	5162.0	182.4	100	—	—

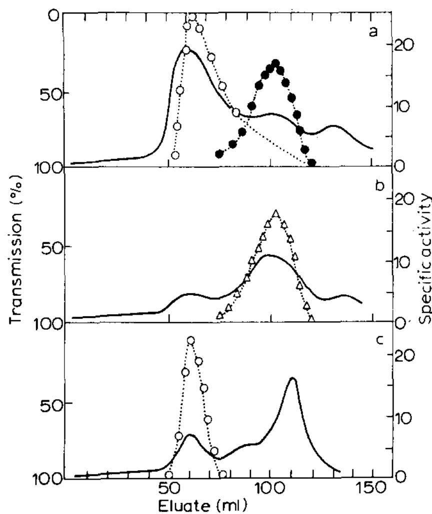

Fig. 1. Elution diagram in a 2 cm × 40 cm column of Sephadex G-100 of (a) porphobilinogenase, (b) deaminase and (c) isomerase preparations. ———, ultraviolet absorption as recorded, with a Uvicord I; ○·····○, isomerase activity; △·····△, porphobilinogenase activity; ●—●, deaminase activity. Activities were determined as described in MATERIALS AND METHODS.

The protein eluted was then treated with calcium phosphate gel (1 mg protein: 2 mg gel); after being stirred for 10 min, the mixture was centrifuged at 1000 × *g* for 5 min and the sediment was discarded (Step 5). $(NH_4)_2SO_4$ was added to the supernatant to 50% satn., and the protein precipitate was dissolved in a small volume of Tris buffer (Step 6). The enzyme preparation from the previous stage was then applied to a Sephadex G-100 column (Step 7). Fig. 1 shows a typical elution diagram where porphobilinogenase activity was associated with the protein shoulder and the specific activity of the peak of activity was 7 times higher than that of the preparation from the previous

TABLE II

ISOLATION AND PURIFICATION OF DEAMINASE FROM COW LIVER

Incubation conditions were as described in the MATERIALS AND METHODS section. Identification and quantitative determination of porphyrins formed and isomeric analysis of uroporphyrins fraction were carried out by usual methods[18,19].

Fraction	*Units/mg × 10⁻³*	*Purification*	*Porphyrin formed (%)*		
			Uroporphyrin III	*Uroporphyrin I*	*Others*
1. Homogenate	28.3	1	15	—	85
2. 11 000 × *g* (supernatant)	59.4	2.1	15	—	85
3. Supernatant from dialysis	141.5	5.0	45	55	—
4. 45–70% satd. $(NH_4)_2SO_4$	325.5	11.5	40	60	—
5. $Ca_3(PO_4)_2$-gel treatment	750.0	26.5	30	70	—
6. Heat treatment	2549.8	90.1	20	80	—
7. Second 70% satd. $(NH_4)_2SO_4$ fraction	3056.4	108.0	20	80	—
8. Sephadex G-100 column (pooled fractions)	9474.8	334.8	—	100	—

stage. It must be emphasized, however, that the main protein peak, as we suspected, had very high isomerase activity, which was extended towards the shoulder, where, in fact, both activities were measured. It has also been observed, by using approximate values of molecular weights (unpublished results), that the isomerase is a relatively more active enzyme than the deaminase, small amounts of the former being sufficient to restore the uroporphyrinogen III forming activity of deaminase.

Preparation of deaminase

Steps 1 and 2 (Table II) were the same as those described for porphobilinogenase. Dialysis of the supernatant against glass-distilled water for 24 h caused a heavy flocculent precipitate to form, which was centrifuged and discarded (Step 3). This supernatant was then fractionated with $(NH_4)_2SO_4$; the fraction precipitating at 45–70% satn. was collected by centrifugation, dissolved in a small volume of 0.05 M Tris buffer (pH 7.4) and passed through a Sephadex G-25 or Bio-Gel P-20 column (Step 4). The protein eluted was treated with calcium phosphate gel (1 mg protein: 2 mg gel), and the sediment was discarded (Step 5). The supernatant was heated at 65–70° for 20 min, was immediately cooled in an ice bath and was centrifuged; the precipitate was discarded (Step 6). The supernatant was made to 70% satn. with $(NH_4)_2SO_4$, and the precipitate was dissolved in 0.05 M Tris buffer (pH 7.4) (Step 7). The protein solution was applied to a Sephadex G-100 column (Step 8). Fig. 1b shows a typical elution diagram where deaminase activity was associated with the second protein band.

Preparation of isomerase

Steps 1, 2 and 3 (Table III) were the same as those described for porphobilinogenase. In Step 4, the supernatent was fractionated with $(NH_4)_2SO_4$ to 70–90% satn., and this precipitate was dissolved in 0.05 M Tris buffer (pH 7.4). In Step 5 the protein solution was then applied to a Sephadex G-100 column. Fig. 1c shows a typical elution

TABLE III

ISOLATION OF ISOMERASE FROM COW LIVER

For the estimation of isomerase activity (Steps 4a and 5a), incubation conditions were the same as described in MATERIALS AND METHODS section, except that purified deaminase was used (1 mg of deaminase: 3–6 mg isomerase). As the exact isomerase/deaminase ratio in the homogenate and supernatant is not known as yet, results cannot be expressed in terms of specific activity of isomerase. Identification and quantitative determination of porphyrins formed and isomeric analysis of uroporphyrin fraction were carried out by usual methods[18,19].

Fraction	*Porphyrin formed (%)*		
	Uroporphyrin III	*Uroporphyrin I*	*Others*
1. Homogenate	15	—	85
2. 11 000 × *g* (supernatant)	15	—	85
3. Treatment with glacial acetic acid to pH 5.0 (supernatant)	35	—	65
4. 70–90% satd. $(NH_4)_2SO_4$ fraction	—	—	—
4a. 70–90% satd. $(NH_4)_2SO_4$ fraction + deaminase (from Stage 6 of purification)	90–100	10–0	—
5. Sephadex G-100 column (pooled fractions)	—	—	—
5a. Sephadex G-100 column (pooled fractions) + deaminase (from Stage 6 of purification)	90–100	10–0	—

diagram, isomerase activity was associated entirely with the first protein peak, the main protein band being hemoglobin.

Properties

Optimum activities of all preparations were observed at pH 7.4 in 0.05 M Tris buffer or 0.067 M phosphate buffer, under the standard conditions described. The rate of disappearance of porphobilinogen is constant for at least 6 h and shows that about 4 moles of substrate are consumed for each mole of uroporphyrinogen which appears and is proportional to enzyme concentration.

Isomer analysis of reaction products

Preparations from different stages of purification of porphobilinogenase predominantly formed uroporphyrinogen III. The only product of the deaminase preparation obtained after treatment on a Sephadex G-100 column was uroporphyrinogen I, and the effect of dialysis was similar to the effect of heating, as to the isomeric type of uroporphyrinogens formed. Isomerase obtained either after 70–90% satn. with $(NH_4)_2SO_4$ or after treatment on a Sephadex G-100 column, consumed no porphobilinogen, and only uroporphyrinogen III was formed when deaminase was added to the system.

Effect of temperature

As previously shown in other tissues, liver isomerase is a heat-labile enzyme[3–6, 9–12,14,24,26] and loses 80% of its activity when heated for 20 min at 65–70° in the presence of deaminase; heat inactivation is slightly greater when isomerase is heated in the absence of deaminase.

TABLE IV

EFFECT OF ANAEROBIOSIS ,AEROBIOSIS AND CYSTEINE ON UROPORPHYRINOGEN RATE

Assay conditions were as described in MATERIALS AND METHODS section, except that incubations were anaerobic in 1, 2 and 3, and aerobic in 4, 5 and 6; cysteine at concentrations indicated was included in 2, 3, 5 and 6 at the time incubation. 15–20-Fold purified porphobilinogenase was used as enzyme preparation.

Conditions	*Uroporphyrin formed (nmoles/h)*
1. Anaerobic	5.9
2. Anaerobic + cysteine (0.1 mM)	6.1
3. Anaerobic + cysteine (1 mM)	5.9
4. Aerobic	5.7
5. Aerobic + cysteine (0.1 mM)	6.0
6. Aerobic + cysteine (1 mM)	6.0

Effect of anaerobiosis, aerobiosis and cysteine

The rate of uroporphyrinogens formed was the same whether the reaction mixture was incubated in air or anaerobically (Table IV). In either case, only uroporphyrinogens were present at the end of incubation; the isomer composition of the reaction product was unchanged. Cysteine at 1 and 0.1 mM did not modify either the isomer type or the porphyrinogen yield.

Effect of dialysis

Dialysis of the supernatant, obtained after Step 2 of all preparations, against

glass-distilled water for 24 h produced a heavy precipitate, which was separated by centrifugation. Activity was determined in both the supernatant and the precipitate. As already stated, the supernatant had deaminase activity (Step 3, Table II), while the precipitate had neither porphobilinogenase nor deaminase activity. As we suspected, however, it had isomerase activity; thus, on addition of deaminase to different amounts of precipitate dissolved in 0.05 M Tris buffer (pH 7.4), porphobilinogenase activity was completely restored. Therefore, dissociation of isomerase from deaminase by dialysis is a reversible process different from heat treatment, which is not reversible. Moreover, other activities were investigated in such a precipitate, which also was shown to have δ-aminolaevulinic acid dehydratase and decarboxylase(s) activities.

Stimulating factor for uroporphyrinogen formation

We have evidence for the presence of a factor which seems to be necessary and also stimulates uroporphyrinogen formation from porphobilinogen (Table V). When supernatant of $11\,000 \times g$ (A) was subjected to ultrafiltration for 3 h using an LKB ultrafilter, the ultrafiltrate residue (C) had less or no porphobilinogenase activity,

TABLE V

ULTRAFILTRATION EXPERIMENT

Activity of system A was taken as 100%. Incubation conditions were as those described in the MATERIALS AND METHODS section; that is 5 ml of A were used as "Enzyme preparation", but volumes of B and C, either incubated alone or mixed, were equivalent to 5 ml of A.

Homogenate
↓
Supernatant (A)
↓
Ultrafiltration
↓ ↓
Ultrafiltrate (B) Ultrafiltered residue (C)

Test system	Deaminase–isomerase activity (%)
A	100
B	0
C	0–50
B + C	110–130

while the ultrafiltrate (B) did not have activity; on addition of the ultrafiltrate to the ultrafiltrate residue, porphobilinogenase activity was not only restored but also slightly stimulated. A similar stimulating effect was reported to be found by HEATH AND HOARE[7,8] in extracts of various tissues.

Kinetics of deaminase

Plots of velocity against porphobilinogen concentration and their reciprocal, in the absence and presence of NH_4^+ followed classical Michaelis–Menten kinetics. Concentration of NH_4^+ was varied from 0.001 to 0.1 M; at 0.1 M deaminase was inhibited about 30% under the assay conditions described above. Nonetheless, the Michaelis constant, $K_m = 5$ μM, for porphobilinogen was found to be essentially independent of the NH_4^+ concentration (Table VI), which behaved as a noncompe-

TABLE VI

HILL COEFFICIENTS AND APPARENT K_m FOR PORPHOBILINOGEN

Activities were assayed as described in MATERIALS AND METHODS. Deaminase preparation used was 300-fold purified and porphobilinogenase preparation was 180-fold purified. "Heated enzyme" was porphobilinogenase heated 20 min at 65–70° and "dialyzed enzyme" was the supernatant obtained from a preparation of porphobilinogenase dialyzed 24 h against glass-distilled water, as described in Step 5 for purification of deaminase.

Enzyme and addition	n*	*Apparent K_m or $[S_{\frac{1}{2}}]$ (μM)*
1. Deaminase		
None	1.0	5.0
0.01 M NH_4^+	1.0	5.0
0.1 M NH_4^+	0.9	5.0
2. Porphobilinogenase		
None	1.7–1.95	11–14
0.0001 M NH_4^+	2.0	10
0.001 M NH_4^+	1.85	10
0.01 M NH_4^+	1.77	7
0.1 M NH_4^+	1.0	5
Heated enzyme	1.0	5
Dialysed enzyme	1.0	5.5

* n was calculated from the slopes of the plot of log $(v/v_{max} - v)$ against log [porphobilinogen].

titive inhibitor of the deaminase. The estimated K_i for NH_4^+ obtained from the Lineweaver–Burk plots and by using the graphical method of DIXON[27] was 0.172 M. However, it is interesting to add that NH_4^+ at concentrations that inhibited formation of uroporphyrinogen I has only a very slight effect upon the rate of porphobilinogen consumption (Table VII); that is, porphobilinogen was consumed without the corresponding production of porphyrinogen as if NH_4^+ in some way produce an accumulation of one intermediate between porphobilinogen and uroporphyrinogen which, under normal conditions, could be identified with the substrate for the isomerase. Similar observations in plant tissues were reported by BOGORAD[21,22].

TABLE VII

EFFECT OF NH_4^+ ON THE DEAMINASE

Porphobilinogen consumption of control was taken as 100%. Deaminase 150-fold purified was used.

Addition	*Porphobilinogen consumption (%)*	*Porphyrin yield (%)*
None	100	100
0.01 M NH_4^+	100	78
0.05 M NH_4^+	95	70
0.1 M NH_4^+	92	66
0.2 M NH_4^+	85	57

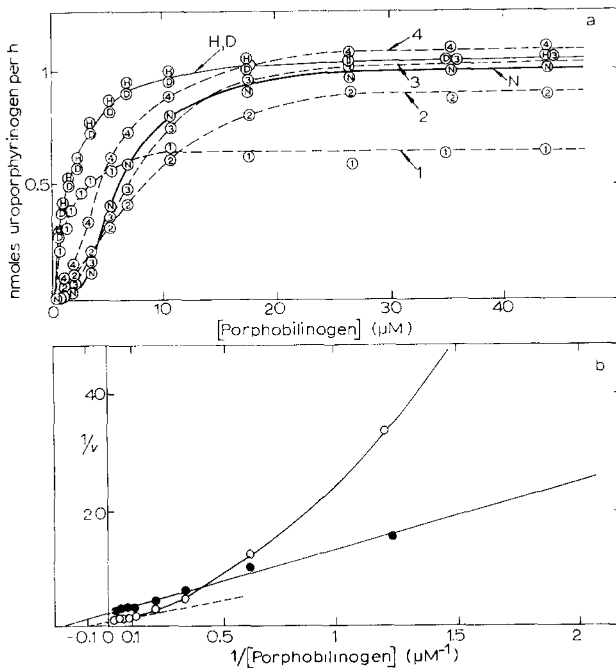

Fig. 2. Effect of porphobilinogen concentration on the activity of the porphobilinogenase under various conditions. Activities were measured as described. (a) With different concentrations of porphobilinogen: alone (Curve N); in the presence of NH_4^+, 0.1 M (Curve 1); 0.01 M (Curve 2); 0.001 M (Curve 3) and 0.0001 M (Curve 4); with porphobilinogenase heated 20 min at 65–70° (Curve H) and with supernatant of porphobilinogenase dialyzed against glass-distilled water (Curve D) (as described in Step 5 of deaminase purification). (b) Double reciprocal plots of velocity against porphobilinogen concentration without additions (○—○) ;in the presence of 0.1 M NH_4^+ or with heated or dialyzed porphobilinogenase (●—●).

Kinetics of porphobilinogenase

Plots of activity of porphobilinogenase *versus* porphobilinogen concentration at all enzyme concentrations studied were sigmoid, and the reciprocal plots were not linear (Fig. 2). The $[S_{\frac{1}{2}}]$ values for porphobilinogen, calculated from several different experiments, were between 11 and 14 μM (Table VI). It can also be seen from Fig. 2 and Table VI that heat treatment or dialysis against glass-distilled water, or addition of different concentrations of NH_4^+, alters the shape of the substrate saturation curve from a sigmoid to an hyperbolic pattern and also increases the affinity of the enzyme system for porphobilinogen; thus the apparent K_m values for porphobilinogen in the presence of high concentrations of NH_4^+, or with the heated or dialyzed enzyme, was again 5 μM.

It is also very interesting to note that if porphobilinogen consumption was taken as a measure of enzyme activity, plots of velocity of porphobilinogenase against porphobilinogen concentration showed normal kinetics pattern.

It is well known that nonlinear double reciprocal plots or sigmoid substrate saturation curves can result from a variety of causes such as allosterism[1,2] and random substrate–enzyme interactions. The possibility of a random mechanism, according to the description of CLELAND[28], cannot be completely excluded on the basis

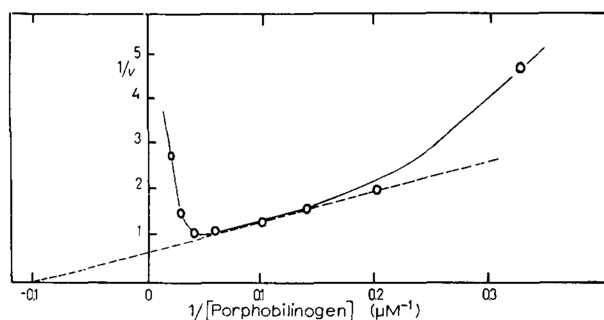

Fig. 3. Effect of porphobilinogen concentration on the formation of uroporphyrinogen III. Assay conditions were as described. Enzyme preparation was porphobilinogenase 150-fold purified. Velocity was measured as nmoles of uroporphyrinogen III formed per h.

of the kinetic results obtained, and further experiments will have to be performed to resolve this issue.

Velocity plots of any such allosteric reaction can be accounted for by the relation empirically deduced by HILL[29]. A plot of log $(v/v_{max}-v)$ against the log $[S]$ (Hill plots) gives a straight line with a slope of n. The value of n is an approximate[30] measure of the probable binding sites for the substrate. When such manipulations were carried out (Table VI), n values of about 2 were obtained for the porphobilinogenase, while either the addition of high concentrations of NH_4^+, heat treatment or dialysis appreciably changes the slope of the Hill plot to $n = 1$, which was the value obtained for the deaminase both in the absence or presence of NH_4^+.

The effect of porphobilinogen concentration

The action of substrate concentration on the activity of porphobilinogenase was also studied, and inhibition of isomerase by high substrate concentration was found,

TABLE VIII

EFFECT OF NH_4^+ ON THE ENZYMIC SYNTHESIS OF UROPORPHYRINOGEN III

Assay conditions were as described in MATERIALS AND METHODS. Porphobilinogenase preparation was 150-fold purified. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out using usual methods[18,19].

Concn. of inhibitor NH_4^+ (M)	*Total porphyrinogen formed*		*Uroporphyrinogen III formed*	
	nmoles/h	*Inhibition (%)*	*nmoles/h*	*Inhibition (%)*
None	0.530	0	0.530	0
0.2	0.298	44.0	0.060	90.0
0.1	0.349	34.3	0.087	83.5
0.05	0.369	30.5	0.143	63.0
0.01	0.465	12.4	0.278	47.5
0.005	0.520		0.360	30.0
0.001	0.536		0.428	20.0
0.0005	0.537		0.537	0
0.0001	0.575		0.575	0
0.00001	0.530		0.530	0

as increased amounts of uroporphyrinogen I were found with increased porphobilinogen concentration, while total porphyrin formed remained practically unchanged. Similar observations were reported by CORNFORD[10].

The effect of NH_4^+

Concentrations ranging from 0.00001 to 0.2 M were tested on the porphobilinogenase at constant concentrations of substrate (Table VIII); it was found that NH_4^+ at concentrations up to 0.2 M inhibited 90% the formation of uroporphyrinogen III and only 40% that of uroporphyrinogen I. These values were taken as a measure of the effect of NH_4^+ on the isomerase and deaminase activities, respectively. From plots of activity of isomerase against NH_4^+ concentration, typical inhibition curves were obtained (Fig. 4). Moreover, it was found using the method of DIXON[27] for graphical determination of K_i that NH_4^+ inhibited competitively the isomerase, showing a K_i = 0.01 M. Either Sephadex G-25 or Bio-Gel P-20, using 0.05 M Tris buffer (pH 7.4) as eluent, removed inhibition of NH_4^+ both with the deaminase and the isomerase. It has been found that low concentrations of NH_4^+ (0.0001–0.001 M) slightly activited the enzyme (Fig. 2 and Table VIII). It has also been observed, working with partially purified preparations of porphobilinogenase, that the addition of sodium or magnesium salts at certain concentrations to the incubation mixtures does not appreciably change total porphyrins formed but that it seems to increase the amount of uroporphyrinogen III. Moreover, different behaviors of purified isomerase and porphobilinogenase preparations on calibrated Sephadex columns in the absence or presence of salts have

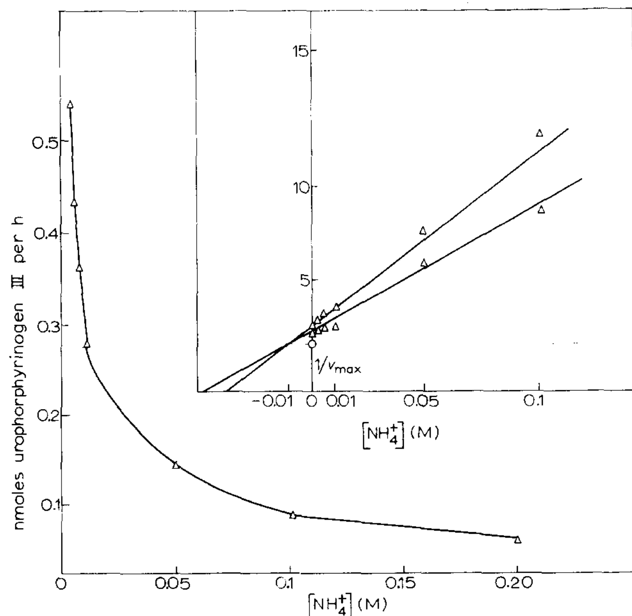

Fig. 4. Effect of NH_4^+ on porphobilinogenase. Assay conditions were as described. Velocity of porphobilinogenase as nmoles of uroporphyrinogen III per h was plotted against inhibitor concentration. Porphobilinogen concentration was held at a constant saturating level (5 times $[S_{\frac{1}{2}}]$. The enzyme preparation used was 150-fold purified. Inset: plots of reciprocal of velocity against inhibitor concentration for graphical determination of inhibitor at two porphobilinogen concentrations.

also been observed, and it has been found that protein fractions identified with deaminase and isomerase activities have very different molecular weights. These findings are being investigated further.

Evidences have also been found indicating that in crude liver homogenates there is a great excess of isomerase.

DISCUSSION

The formation of uroporphyrinogen III from porphobilinogen is one of the most complex steps in porphyrin metabolism. In some metabolic diseases, such as hepatic and erythropoietic porphyria, large amounts of Type I porphyrins are formed; accumulation of I isomers could then be attributed to a failure or absence of the isomerase[22] or perhaps to an inhibition of this enzyme by an excess of substrate; therefore it seemed worthwhile examining the conversion of porphobilinogen into uroporphyrinogens in liver at the enzymic level.

Procedures described here for the isolation and purification of porphobilinogenase, that is the system normally converting porphobilinogen into uroporphyrinogen III, and the separation of deaminase and isomerase activities from each other are very reproducible. Although both activities have been separated from various sources, with or without previous destruction or inactivation of one of them[4-12], we cannot be completely sure that they are, in fact, two distinct enzymes and not part of a complex protein formed by two or more different subunits having two activities. Thus, most of the methods described to separate deaminase from isomerase involved either inactivation of the isomerase by heating or reversible dissociation by dialysis against glass-distilled water, as in the present paper. On the other hand, isomerase free of deaminase has been obtained by treatment at high ionic strength, and indeed we have observed that high ionic strength greatly influences the behavior of isomerase on calibrated gel columns.

The kinetics of porphobilinogenase as a function of porphobilinogen concentration has been found to exhibit a sigmoid dependence as shown in Fig. 2. This type of relationship suggest that cooperative effects act to facilitate the binding of porphobilinogen to the isomerase as no cooperative effects were observed with the deaminase. The values of n from the Hill plots (Table VI) ranging from 1.7 to 1.95 for different porphobilinogenase preparations, and $n = 1$ for deaminase, would suggest the existence of two binding sites for porphobilinogen per porphobilinogenase molecule, one being on the deaminase unit and the other in the isomerase unit. The loss of homotropic interactions when ammonium ions were added to the system or when porphobilinogenase was heated or dialyzed against glass-distilled water was clearly shown (Fig. 2). The curve for reaction velocity against porphobilinogen concentrations in these conditions has now a Michaelis–Menten form; the reciprocal plot is linear, the Hill plot has a slope of unity and there is an increase in the affinity of the enzyme for porphobilinogen, so the enzyme system is converted to a form which is still fully active catalytically, but uroporphyrinogen I is formed instead of uroporphyrinogen III. We identified this form with the deaminase.

The action of NH_4^+ on deaminase and isomerase activities showed that they have a separate effect on the two activities as already suggested by BOGORAD[22], acting non-competitively on the deaminase and competitively on the isomerase; moreover, excess

porphobilinogen also acts inhibiting the isomerase (Fig. 3), and then it appears as if there is some kind of competition between the porphobilinogen–isomerase complex and porphobilinogen (or NH_4^+) for the deaminase–polypyrrol complex, as it was in some way proposed by CORNFORD[10]. Perhaps NH_4^+ could inhibit by binding at the same site of porphobilinogen on the isomerase or probably at a different site which could be that of the polypyrrolic substrate on the isomerase or, still, they could produce dissociation of the deaminase–isomerase complex. The inhibition of isomerase by an excess of porphobilinogen could explain the abnormal formation of uroporphyrinogen I in some porphyria. The deaminase–isomerase complex is thought to have special kinetic properties because it binds porphobilinogen at least at two sites which are able to interact. For porphobilinogenase, the affinity for porphobilinogen is variable, being poor when porphobilinogen is very low and good when saturation is approached. This explanation would account for the sigmoidal saturation curve and would fit the model proposed by MONOD *et al.*[1,2]. Some evidences, shortly to be published, have been obtained showing that excess of isomerase exists in a crude preparation of porphobilinogenase, which would suggest that probably under normal conditions several units of isomerase are associated with one unit of deaminase in an active complex, in which the excess of isomerase assures the formation of uroporphyrinogen III and presumably reversibly modifies the properties of the complex by altering its enzymic specificity as suggested by LEVIN AND COLEMAN[11]. Either destruction or dissociation by dialysis, heating, high ionic strength or deficiency of the isomerase would remove such control, the regulatory properties of the system being lost. In any case, the existence of cooperative effects produced by some kind of protein–protein interactions are suggested and, although speculative in many respects, one could also think that porphobilinogen could induce a conformational change in the isomerase, forming the isomerase–porphobilinogen complex, which in turn, assumed to be bound to the deaminase trypyrrol complex, could induce a change in the deaminase, thus reducing its binding with another porphobilinogen molecule. Although the mechanism of the enzymic synthesis of uroporphyrinogens from porphobilinogen is still unknown, our data would suggest that, at least in liver, the mechanism of the porphobilinogenase enzyme would be "allosteric" in nature.

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