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PORPHYRIN BIOSYNTHESIS

VIII. AVIAN ERYTHROCYTE PORPHOBILINOGEN DEAMINASE–UROPORPHYRINOGEN III COSYNTHETASE, ITS PURIFICATION, PROPERTIES AND THE SEPARATION OF ITS COMPONENTS

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SUMMARY

1. A method for the isolation and purification of porphobilinogenase, porphobilinogen deaminase and uroporphyrinogen isomerase from avian erythrocytes is described.

2. Some properties of the isolated enzymes were studied. The optimal pH for porphobilinogenase and deaminase was 7.4. Purified porphobilinogenase was resolved into three bands on starch gel electrophoresis. The molecular weight of the purified enzymes was determined by gel filtration. The presence of porphobilinogen or NH_4^+ at certain concentrations afforded protection against heat inactivation of isomerase, the heat labile enzyme. Initial porphyrin formation by porphobilinogenase was linear with time.

3. The action of various compounds added to the system was studied. Thiol reagents inhibited both porphobilinogenase and deaminase, indicating the presence of thiol groups essential for activity. NH_4^+, hydroxylamine, adenine, ADP, ATP, some dicarboxylic acids and 2-methoxy-5-nitrotropone inhibited deaminase.

INTRODUCTION

The enzymic condensation of porphobilinogen into uroporphyrinogens has been described in various tissues[1–12]. It has been established that two enzymes are involved in this reaction, porphobilinogen deaminase and uroporphyrinogen III cosynthetase (also called isomerase). The two enzymes can be distinguished by their susceptibility to heat inactivation, porphobilinogen deaminase being heat stable whereas isomerase is unstable to heat. Deaminase alone converts porphobilinogen into uroporphyrinogen I, in the presence of both deaminase and isomerase, uroporphyrinogen III being formed, but isomerase alone has no action on either uroporphyrinogen I or porphobilinogen.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

The isolation and purification of porphobilinogenase[1] and the separation of porphobilinogen deaminase from isomerase have been previously reported[2–12]. LOCKWOOD AND BENSON[7], working with avian erythrocytes, found no evidence of more than one enzyme in such systems, as they failed to separate deaminase from isomerase by paper electrophoresis. However they postulated that the synthesis of uroporphyrinogen III is catalysed by two enzymes or by a single enzyme possessing both forms of activity. The present work describes the isolation and purification from avian erythrocytes of porphobilinogenase and the separation of its components deaminase and isomerase from each other. Some properties of the enzymes are also reported.

MATERIALS AND METHODS

Porphobilinogen was biosynthetically obtained[13] and assayed by the method of MOORE AND LABBE[14]. DEAE-cellulose for chromatography was obtained either from Sigma Chemicals or Schuchart (München). DEAE-cellulose columns were prepared as described by CORNFORD[8]. Calcium phosphate gel was prepared according to KEILIN AND HARTREE[15]. Sephadex or Sepharose gels were obtained from Pharmacia, Uppsala, Sweden, and starch gel for electrophoresis from Connaught Medical Research Lab., Toronto, Canada. Tris–HCl and Na_2HPO_4–NaH_2PO_4 buffers were used throughout. All other reagents used were of A.R. grade obtained from several commercial sources. All solutions were made up in ion-free glass-distilled water.

Source material of enzymes. Chicken blood was obtained and collected as already described[16].

Protein concentrations. Protein concentrations were determined using the Folin Ciocalteu reagent[17].

Estimation of enzyme activity. The standard incubation system contained, unless otherwise stated, the enzyme preparation (usually 2 ml) together with 0.05 M Tris or phosphate buffer (pH 7.4), 30 μg of porphobilinogen, 0.35 ml of 1.7 M NaCl and 0.15 ml of 0.12 M $MgCl_2$, in a final volume of 3 ml and at pH 7.4. Incubations were carried out aerobically, in the dark, with mechanical shaking, at 38° for 90 min. When the effect of additives was studied, the incubation system contained only enzyme, substrate and buffer, with or without the addition of other reagents, in the same final volume and pH. Blanks were run with porphobilinogen and without enzyme. After incubation, trichloroacetic acid was added (to a final concentration of 5%, w/v) to precipitate the protein, the mixture was exposed to light and air for 20–25 min to oxidize porphyrinogens, the precipitated protein was removed by filtration, and porphyrins formed or the remaining porphobilinogen were estimated in acid solution[18]. Porphyrins were fractionated and esterified by usual procedures[16]. Identification, quantitative determination of porphyrins formed and isomeric composition of uroporphyrin fractions were made by published methods[19–21].

An enzyme unit was defined as the amount of enzyme that catalyses the formation of 1 nmole of uroporphyrinogen per h, under standard conditions, the specific activity being units of enzyme per mg of protein. For estimating isomerase activity, the reaction mixture was the same as described above, except that deaminase was also added in the ratio 1 mg of deaminase to 6 mg of the isomerase preparation.

NH_4^+ that inhibit these enzymes[1,3,22–24] should be removed from the enzyme preparations before the activity is estimated. This was carried out by molecular

sieving with 2 cm × 30 cm columns of Sephadex G-25, using 0.05 M buffer (pH 7.4) as eluant. When other gel columns were used, protein was eluted with the same buffers, and they were prepared as described by BATLLE *et al.*[25].

Fractions were automatically collected with an LKB collector (Stockholm). Molecular weights were estimated by gel filtration methods[26–29].

The procedure employed in starch gel electrophoresis was based on methods described by BODMAN[30].

Separation of the enzymic protein fraction from haemoglobin was carried out by following essentially the procedure described by HENNESSEY *et al.*[31] but using a column of DEAE-cellulose instead of the treatment of the supernatant in tubes.

RESULTS

Enzyme preparations

All operations were carried out in the cold room at 4°, unless otherwise stated. The specific activities of the enzymes varied greatly from one experiment to another, but the relative results shown here were reproducible.

Porphobilinogenase (Table I)

The avian blood (approx. 1 l) was centrifuged, plasma and leucocytes were removed, and the cells were washed with 0.9% NaCl. The packed cells were mixed thoroughly with an equal volume of water, previously chilled to 0°, and periodically stirred for 60 min in an ice-bath (Table I, Step 1). The avian erythrocyte haemolysate was centrifuged at 25 000 × *g* for 90 min, and the sediment was discarded (Step 2). The 25 000 × *g* supernatant was filtered through filter paper and then placed on a column (2 cm × 50 cm) of DEAE-cellulose previously equilibrated with 0.003 M phosphate buffer (pH 7.4). The column was washed thoroughly with the same buffer, the DEAE was then removed mechanically and the porphyrin-synthesizing system was desorbed from DEAE-cellulose as follows: 80–90 ml of 0.134 M phosphate buffer

TABLE I

ISOLATION AND PURIFICATION OF PORPHOBILINOGENASE FROM AVIAN ERYTHROCYTES

Incubation conditions were as described in MATERIALS AND METHODS. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out by usual methods[19–21]. Protein concentrations were determined using the Folin Ciocalteu reagent[17].

Fraction	*Total protein (mg)*	*Specific activity* × 10^3	*Purification (-fold)*	*Porphyrins formed (% type III)*
1. Haemolysate	65 000	1.2	1	100
2. 24 000 × *g* (supernatant)	15 600	4.8	4	100
3. DEAE-cellulose column	700	100	83.3	100
4. 0–90% satd. $(NH_4)_2SO_4$ fraction	125	542	451.6	100
5. Supernatant from dialysis	72	830	690	100
6. 30–70% satd. $(NH_4)_2SO_4$ fraction	58	1020	850	100
7. $Ca_3(PO_4)_2$ gel treatment	38	1500	1250	100
8. Sephadex G-100 column (peak)	3.5	5900	4910	100

(pH 7.4) was added to the adsorbent, and the mixture was stirred magnetically for 30 min in an ice-bath; the fluid supernatant was collected by centrifugation at 2000 × *g* for 10 min; this step was repeated three times, and the supernatants were combined to yield the enzyme protein fraction. Buffer concentration was made 0.05 M before the activity was estimated (Step 3). To the protein solution from Step 3, solid $(NH_4)_2SO_4$ was added to 90% saturation, and the pH was kept close to pH 7.4 by the addition of concentrated ammonia. After the suspension had been stirred for 1 h, the protein precipitate was collected by filtration and then dissolved in a small volume of 0.05 M phosphate buffer (pH 7.4) (Step 4). Dialysis of the protein solution against glass-distilled water for 24 h produced a flocculent precipitate, which was centrifuged and discarded (Step 5). This supernatant was then fractionated with $(NH_4)_2SO_4$; the fraction precipitating at 30–70% satn. was collected by centrifugation, dissolved in a small volume of 0.05 M phosphate buffer (pH 7.4) and passed through a Sephadex G-25 column (Step 6). The protein eluted was treated with calcium phosphate gel (3 mg gel; 1 mg protein). After being stirred for 10 min, the mixture was centrifuged at 2000 × *g* for 5 min, the supernatant was discarded, and the enzyme adsorbed on the gel was eluted successively with two portions of 3 ml of 0.134 M phosphate buffer. The two eluates were combined. Buffer concentration was made 0.05 M before the activity was estimated (Step 7). The protein solution was concentrated by $(NH_4)_2SO_4$ precipitation and applied to a Sephadex G-100 column (Step 8). Fig. 1a shows a typical elution diagram. Porphobilinogenase activity was associated with the second protein band.

Deaminase (Table II)

Steps 1–6 were the same as those described for porphobilinogenase. In Step 7 the protein solution from Step 6 was concentrated by $(NH_4)_2SO_4$ precipitation and desalted with Sephadex G-25. The eluate was heated with continuous stirring at 60° for 15 min, immediately cooled in an ice-bath and centrifuged; the precipitate was

TABLE II

ISOLATION AND PURIFICATION OF DEAMINASE FROM AVIAN ERYTHROCYTES

Incubation conditions were as described in MATERIALS AND METHODS. Identification and quantitative determination of porphyrins formed and isomer analysis of uroporphyrin fraction were carried out by usual methods[19–21]. Protein concentrations were determined using the Folin Ciocalteu reagent[17].

Fraction	*Total protein (mg)*	*Specific activity* × 10^3	*Purification (-fold)*	*Porphyrins formed (%)*	
				Type I	*Type III*
1. Haemolysate	65 000	1.2	1		100
2. 24 000 × *g* (supernatant)	15 600	4.8	4		100
3. DEAE-cellulose column	700	100	83.3		100
4. 0–90% satd. $(NH_4)_2SO_4$ fraction	125	542	451.6		100
5. Supernatant from dialysis	72	830	690		100
6. 30–70% satd. $(NH_4)_2SO_4$ fraction	58	1020	850		100
7. Heat treatment	25	4350	4100	85	15
8. Second 70% satd. $(NH_4)_2SO_4$ fraction	16.7	6300	6050	90	10
9. Sephadex G-100 column (peak)	3	8900	8550	100	

discarded. In Step 8 the supernatant was made to 70% satn. with $(NH_4)_2SO_4$, the precipitate was collected by centrifugation and dissolved in a small volume of 0.05 M phosphate buffer (pH 7.4). In Step 9 the protein solution was applied to a Sephadex G-100 column. Fig. 1b despicts a typical elution diagram. Deaminase was associated with the second protein band.

Isomerase (Table III)

Steps 1–4 were the same as those described for porphobilinogenase. The protein solution from Step 4 was desalted with Sephadex G-25, the eluate was fractionated with $(NH_4)_2SO_4$ to 80–90% satn., and the precipitate was then dissolved in a small volume of 0.05 M phosphate buffer (pH 7.4) (Step 5). The protein solution was applied

TABLE III

ISOLATION OF ISOMERASE FROM AVIAN ERYTHROCYTES

For the estimation of isomerase activity (Steps 5a and 6a), incubation conditions were the same as described in MATERIALS AND METHODS except that 6000-fold purified deaminase was used (1 mg of deaminase/5 mg of isomerase). As the exact isomerase/deaminase ratio in the haemolysate and supernatant is not known, 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[19–21].

Fraction	*Porphyrins formed*	
	Type I	*Type III*
1. Haemolysate	—	100
2. 24 000 × *g* (supernatant)	—	100
3. DEAE-cellulose column	—	100
4. 0–90% satd. $(NH_4)_2SO_4$ fraction	—	100
5. 80–90% satd. $(NH_4)_2SO_4$ fraction	—	—
5a. 80–90% satd. $(NH_4)_2SO_4$ fraction + deaminase	10–0	90–100
6. Sephadex G-100 column (pooled fractions)	—	—
6a. Sephadex G-100 column (pooled fractions) + deaminase	10–0	90–100

to a Sephadex G-100 column (Step 6). Fig. 1c shows a typical elution diagram; isomerase was associated with the first protein peak.

Alternatively, isomerase was prepared by a simpler procedure in the following manner. The 25 000 × *g* supernatant obtained after Step 2 of the general purification, was applied to a Sephadex G-100 column (2.5 cm × 80 cm). The first protein peak, free of haemoglobin, had isomerase activity (Step 3). The pooled enzyme solution was fractionated with $(NH_4)_2SO_4$ to 80–90% satn. (Step 4), and rechromatographed on the same Sephadex G-100 column (Step 5). Fig. 1d shows the elution diagram, where again isomerase was associated with the first protein peak.

Gel filtration on Sephadex G-200, or Bio-gel P-300 instead of Sephadex G-100, also purified the enzymes but did not further improve the purification, so Sephadex G-100 was the material adopted.

Attempts were also made to purify the enzymes by treatment with glacial acetic acid to pH 5.0 and/or by the use of protamine sulphate, but these procedures were abandoned since they caused some inactivation of the enzymes.

Sometimes enzymes were prepared from acetone-dried powders of haemoly-

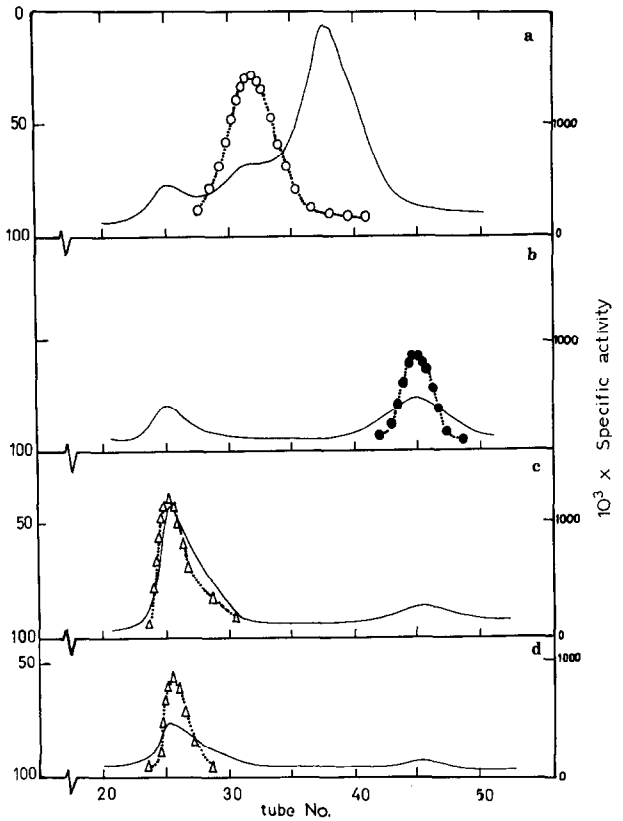

Fig. 1. Elution diagram in a 2.5 cm × 80 cm column of Sephadex G-100 of (a) porphobilinogenase, (b) deaminase, (c) and (d) isomerase preparations. (———) ultraviolet absoption as recorded with a Uvicord I; (○·····○) porphobilinogenase activity; (●·····●) deaminase activity; (△·····△) isomerase activity. Fractions of 4.3 ml were collected. Activities were determined as described in MATERIALS AND METHODS.

sates, and these were obtained and extracted by the procedure of CORNFORD[8]; the extracts were then worked up as described above.

Properties

Studies were performed with different enzyme preparations, but the results recorded here correspond to those obtained with 1200-fold purified porphobilinogenase, 6000-fold purified deaminase, and isomerase after Step 5 of purification, unless otherwise stated.

Optimal activities of porphobilinogenase and deaminase were observed at pH 7.4 in 0.05 M phosphate buffer or 0.05 M Tris buffer. The shapes of the curves were the same for both enzymes, and for both porphyrin formation and porphobilinogen consumption. The maxima found agree with those reported by LOCKWOOD AND BENSON[7]. Under the standard conditions described, uroporphyrin formation increased linearly with time up to 6 h, and there was no evidence of any lag. Porphobilinogen consumption was also linear. The amount of porphyrins formed was pro-

portional to the concentration of enzyme over a wide range. The activity of both porphobilinogenase and deaminase was the same in the absence of oxygen as in its presence, as already reported[7]. Avian erythrocyte porphobilinogenase and deaminase were very stable at all stages of purification. $(NH_4)_2SO_4$ precipitated the enzyme in a form that was stable at $-15°$ for months.

Effects of heat

It is well known that isomerase is a heat-labile enzyme[2,3,7–9,11,12,32]: results of heat-treatment of the enzyme preparations from avian erythrocytes were similar to those previously reported. When heated at 60° for 15 min, porphobilinogenase appeared to be completely altered, giving only 15% of uroporphyrinogen III but significantly increasing total uroporphyrin formation (Table IV); this increase in activity

TABLE IV

EFFECT OF HEAT ON ISOMERASE ACTIVITY

Incubation conditions were as described in MATERIALS AND METHODS. Heated enzyme: the 30–70% satd. $(NH_4)_2SO_4$ fraction was desalted by gel filtration and then heated at 60° for 15 min; after cooling, the mixture was centrifuged at 10 000 × *g* for 10 min and the precipitate was discarded. In Expts. 3 and 4 heating was performed in the presence of porphobilinogen or ammonium at the concentrations stated. Before the enzyme activity was estimated these compounds were removed from the mixture by gel filtration.

System	*Addition*	*Specific activity* × 10^3	*Uroporphyrin formed (%)*	
			Type I	*Type III*
1. 30–70% satd. $(NH_4)_2SO_4$ fraction	None	1630	—	100
2. Heated enzyme	None	6769	85	15
3. Heated enzyme	0.044 mM porphobilinogen	7700	60	40
4. Heated enzyme	10 mM NH_4^+	6000	—	100

was invariably found, and it agrees with the findings of other authors[7,8,12,32]. The sensitivity of the system to heating was influenced by the presence of porphobilinogen (10 μg/ml) or 0.01 M ammonium which afforded protection against heat inactivation: ammonium gave 100% protection. At higher temperatures there was a significant loss of activity.

Estimation of molecular weights

The weight-average molecular weights determined by gel filtration at various enzyme concentrations were estimated to be 110 000 ± 1100 for porphobilinogenase, 40 000 ± 4000 for deaminase and 280 000 ± 28 000 for isomerase. The value obtained for isomerase is higher than that of the porphobilinogenase; therefore it is possible that, under the experimental conditions used, some association of various fractions of isomerase could have occurred. The use of calibrated Sepharose 6 B or 4 B columns in the presence of certain dissociating agents has provided some evidence of dissociation of the enzyme into fractions of mol. wt. 70 000 (unpublished results), and experiments to obtain further evidence are in progress.

Electrophoresis

Different enzyme preparations were subjected to electrophoresis on starch gel at different pH's. At alkaline pH's, the most highly purified porphobilinogenase resolved into three bands, while isomerase and deaminase migrated as a single band each (Fig. 2). Isomerase reached the same position as the second running band of porphobilinogenase, and deaminase behaved as the slower band. The electrophoretic migration rate of these preparations was compared with that of the same enzymes obtained from other sources; as is also shown in Fig. 2, some differences were observed.

Isomer analysis of reaction products

Preparations from different stages of purification of porphobilinogenase formed mainly uroporphyrinogen III, and the only product of the most highly purified deaminase preparations was uroporphyrinogen I. Isomerase obtained after 80–90% satn. with $(NH_4)_2SO_4$ or after Sephadex G-100, consumed no porphobilinogen and formed only uroporphyrinogen III when deaminase was added to the system.

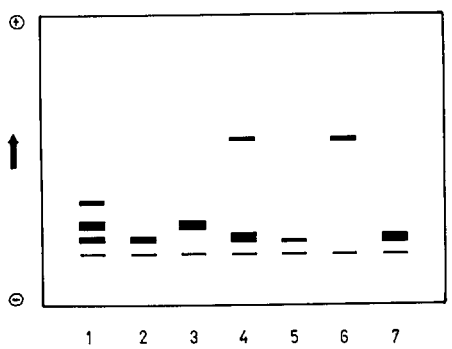

Fig. 2. Electrophoretic behaviour on starch gel of avian erythrocyte enzymes. (1) 4900-fold purified porphobilinogenase; (2) 8550-fold purified deaminase; (3) most highly purified isomerase; soybean callus enzymes[12]; (4) 70-fold purified porphobilinogenase; (5) 140-fold purified deaminase; (6) most highly purified isomerase; (7) 182-fold purified bovine liver porphobilinogenase (ref. 11 and H. A. SANCOVICH, A. K. ROSENBERG, A. M. DEL C. BATLLE AND M. GRINSTEIN, unpublished results). Borate buffer, pH 8.2. A current of 30 mA was passed for 4 h at room temperature.

Conversion of a new pyrrol intermediate into uroporphyrinogens

The formation of a pyrrolic intermediate resulting from the action of soybean callus porphobilinogenase on porphobilinogen has been reported[33]. It was found that this intermediate acted as the second substrate, with porphobilinogen, in the formation of uroporphyrinogen III by avian erythrocyte isomerase. When the intermediate was incubated with porphobilinogen and deaminase a mixture of 40% uroporphyrinogen I and 60% uroporphyrinogen III was formed. Soybean callus deaminase, acting on porphobilinogen, also produced a different pyrrolic intermediate[33] which still behaved as a substrate for avian isomerase but with the formation of 67% uroporphyrinogen I and 33% uroporphyrinogen III; while, in the presence of porphobilinogen and the avian deaminase, it formed only uroporphyrinogen I.

TABLE V

EFFECT OF VARIOUS SUBSTANCES ON PORPHOBILINOGENASE AND DEAMINASE

Incubation conditions were as described in MATERIALS AND METHODS. The activity of the system in the presence of other reagents is expressed on the basis of porphyrin formed or porphobilinogen consumed as per cent of the controls . The substrate was added 5 min after the addition of the compound tested. Metal ions were tested as chlorides. Identification, quantitative determination of porphyrins and isomer analysis of uroporphyrin fraction were carried out by usual methods[23–25].

Additive	*Concn. (mM)*	*Porphobilinogenase rate*			*Deaminase rate*	
		Porphyrin formation	*Porphobilinogen consumption*	*Isomer III (%)*	*Porphyrin formation*	*Porphobilinogen consumption*
Cysteine	0.1	87	89	43	77	108
Iodosobenzoate	1	5	45	—	5	44
PCMB	0.1	1	57	—	5	45
	1	1	20	—	3	10
	1					
PCMB + Cysteine	1	2	32	—	23	88
PCMB	1					
PCMB + Cysteine	5	80	90	100	98	100
DTNB	1	58	81	13	18	74
CN^-	1	96	100	20	100	100
Thiourea	1	59	98	100	114	98
SO_3^{2-}	1	58	99	100	74	106
F^-	1	89	100	80	98	100
	10	85	96	—	92	95
NaN_3	1	108	69	100	105	64
	10	86	96	100	107	80
Hydroxylamine	1	51	82	100	100	100
	10	42	82	100	53	102
	100	8	80	100	41	100
NH_4^+	10	95	96	100	64	94
	100	38	80	100	98	94
	200	21	62	100	—	—
Adenine	1	62	92	100	71	97
	10	41	76	100	26	99
ATP	5	16	65	—	6	64
ADP	5	28	70	100	3	64
Succinic acid	1	80	80	100	77	94
	10	85	80	63	80	99
Phthalic acid	1	67	97	100	70	100
2-Methoxy-5-nitrotropone	1	70	90	60	0	100
Hg^{2+}	0.01	0	5	—	5	10
Zn^{2+}	0.01	15	35	—	20	60
	0.1	34	72	80	39	100
	1	11	98	40	63	93
Cd^{2+}	1	2	58	—	31	88
K^+	100	110	66	50	80	80
Mg^{2+}	50	68	90	100	67	96
	100	25	74	—	60	90

Effect of various substances on enzyme activities (Table V)

Experiments carried out with different enzyme preparations afforded essentially similar results. Those obtained with 6000-fold purified deaminase and 850-fold purified porphobilinogenase appear in Table V, which shows the final concentration of additives to the reaction mixture.

Cysteine at 0.1 mM produced slight inhibition of deaminase and isomerase, whereas 1 mM cysteine and other similar compounds tested were without effect.

Both enzymes were inhibited by some of the thiol reagents tested, and *p*-chloromercuribenzoate (PCMB) inhibition was reversed by cysteine. Thus the possibility of the existence of monothiol functional groups was clearly indicated.

Cyanide did not produce inhibition, but type I porphyrin was formed by porphobilinogenase, fluoride slightly inhibited isomerase, and thiourea and sulphite were also inhibitory, so the presence of vicinal dithiol groups was suggested.

Several nitrogen bases such as those listed in Table V were tested; 10 mM sodium azide slightly inhibited porphobilinogenase and at 1 mM it seems to inhibit porphobilinogen consumption both for porphobilinogenase and deaminase. Hydroxylamine inhibited both porphobilinogenase and deaminase activities; however only isomer III was produced by porphobilinogenase, as if the inhibitory action were on the deaminase. NH_4^+ at certain concentrations inhibited both deaminase and porphobilinogenase as expected; also ATP, ADP and adenine inhibited porphobilinogenase and deaminase but again porphobilinogenase formed only uroporphyrin III.

Deaminase activity was diminished by some dicarboxylic acids and by 2-methoxy-5-nitrotropone, a specific reagent for free amino groups of proteins[34]. Of these compounds, succinic acid at 10 mM and nitrotropone also affected isomerase activity, but it is seen that porphobilinogen consumption seems not to be altered.

Metal ions such as Hg^{2+}, Zn^{2+} and Cd^{2+} inhibited both enzymes. K^+ and Na^+ in a wide range of concentrations seem to have no great effect, only slight activation occurring at 100 mM, but with K^+, 50% less isomer III was formed. Mg^{2+} inhibited both porphobilinogenase and deaminase above 50 mM.

DISCUSSION

LOCKWOOD AND BENSON[7] have claimed that in avian erythrocytes there was no independent deaminase, such as an enzyme associated with a heat-labile inhibitor. However they considered the possibility that the enzyme system responsible for the conversion of porphobilinogen into uroporphyrinogen III might be formed by two distinct enzymes, although they failed to show any evidence of the presence of two protein components. In this paper a method is reported for the purification from avian erythrocytes of porphobilinogenase and the separation of its components isomerase and deaminase from each other.

Isomerase was inactivated by heating the intact system at 60° for 15 min: it is known to be more sensitive than the soybean isomerase[12] which is only destroyed after heating at 70° for 30 min. As also reported for the latter enzyme[12], heating increased 4-fold the uroporphyrin synthesis, and the addition of 0.01 M NH_4^+ or 0.044 mM porphobilinogen afforded protection against heat inactivation. These findings suggest that isomerase could associate with the amino-methyl group of porphobilinogen, so when porphobilinogen or some amino derivative is present, it could bind the enzyme at the same site, thereby stabilizing it.

As has been previously reported[7], the presence of monothiol groups in these enzymes is visualized although a mechanism for their participation cannot yet be postulated. They could stabilize the enzyme–substrate complex, or they might perhaps be indispensable to the enzymes by conferring on them a structural arrange-

ment necessary for activity. It has been postulated that porphobilinogenase contains two or more interacting catalytic sites[11,24], and that the conversion of porphobilinogen into uroporphyrinogens could occur in two steps[12,33], the first being the formation of an intermediate polypyrrol[33] and the second the final production of uroporphyrinogen from porphobilinogen and the polypyrrol. It may be that –SH groups are involved in these reactions. Thiol inhibitors affected to a different extent porphyrin formation and porphobilinogen consumption, being generally more inhibitory towards porphyrin formation, as if the second site were more sensitive. A study of the number and function of the –SH groups of these enzymes is being carried out.

The existence of dithiol groups essential for activity is also possible, because cyanide, sulphite or thiourea, reagents known to cleave disulphide bonds, sometimes caused partial inhibition. CN^-, as already reported[7], did not inhibit total porphyrin formation but in their presence, porphobilinogenase produced 80% uroporphyrinogen I, as if cyanide interferes or competes with the substrate for isomerase.

NH_4^+ and hydroxylamine inhibited porphobilinogenase and deaminase, and the same effect was observed with adenine, ATP and ADP. These results are different from those obtained for the same enzymes obtained from other sources (refs. 11, 12, 24, and H. A. SANCOVICH, A. K. ROSENBERG, A. M. DEL C. BATLLE AND M. GRINSTEIN, unpublished results). The results suggest that these compounds have separate effects on deaminase and isomerase being greater on the deaminase. The inhibitory action could be due to a competition between the enzymes and the bases for their substrates, as proposed by CORNFORD[8], SANCOVICH *et al.*[11,24] and LLAMBÍAS AND BATLLE[12]. These results also point to the idea that porphobilinogenase contains at least two active centres which are unequally affected by certain modifiers.

In addition, to see whether positively charged groups are involved in the attachment of the substrates to isomerase or deaminase, the effect of some dicarboxylic acids was examined. All of them inhibited deaminase between 10–30%, only succinic acid at 10 mM modified isomerase, but it is not known whether this inhibition has any significance. However, porphobilinogen consumption was not greatly affected, so it is also probable that dicarboxylic acids inhibit deaminase by competition with porphobilinogen or the polypyrrol intermediate for some positively charged groups on the enzyme, which could be amino groups as suggested by TAIT[35]. The possibility remains that the inhibitory effect was exerted on the substrate, but no evidence of such reaction could be obtained. However the great inhibition of deaminase by 2-methoxy-5-nitrotropone gave additional support to the first possibility.

Among several metals tested, Hg^{2+}, Zn^{2+} and Cd^{2+} inhibited both enzymes, which could be due to combination between metal and some thiol groups on the enzymes.

On the basis of these results and other experimental evidence, a hypothetical scheme has been suggested for the enzymic synthesis of uroporphyrinogen from porphobilinogen[12].

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