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STUDIES ON PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN III COSYNTHASE FROM HUMAN ERYTHROCYTES

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SUMMARY

1. Porphobilinogen deaminase and uroporphyrinogen III cosynthase from human erythrocytes were isolated and partially purified.

2. The deaminase had a mol.wt of 25 000 ± 5000 and behaved as a single protein. It was found to be inactivated by photooxidation and chemical oxidations, by sulphydryl reagents and by divalent metals such as Ca^{2+}, Cd^{2+} and Mg^{2+}.

3. The cosynthase was inhibited by sulphydryl reagents, and was not inhibited by the oxidation procedures which inhibited the deaminase. Dithiothreitol was an effective protecting agent of cosynthase.

4. Deaminase and deaminase–cosynthase showed Michaelian kinetics. Uroporphyrinogen III formation increased with time and porphobilinogen concentration.

5. A number of porphobilinogen analogues were examined as substrates of the system. None of them behaved as such, while several were effective inhibitors.

6. Deaminase was bound to a Sepharose support and the immobilized enzyme associated with cosynthase in the absence of porphobilinogen. The whole complex formed uroporphyrinogen III.

INTRODUCTION

Uroporphyrinogen III is the universal precursor of haem, the chlorophylls and porphyrins. It is formed by the enzymatic polymerization of four units of porphobilinogen in a reaction mediated by two enzymes: porphobilinogen deaminase and uroporphyrinogen III cosynthase [1]. Several years ago we described the first non-destructive separation of both enzymes using human erythrocytes as enzyme source [2]. A similar non-destructive separation had also been achieved with the enzymatic system of wheat germ [3]. The non-destructive separation and recombination of both enzymes from animal origin allowed an estimation of the amount of cosynthase present in the whole system and indicated that it was present in a great excess over the deaminase. We then proposed that this excess assured the sole formation of uroporphyrinogen III under normal metabolic conditions [2]. This was later indirectly confirmed by Levin and Romeo who found a decrease in the normal cosynthase levels in erythropoietic porphyria both in animals and men [4], and attributed to it the formation of uroporphyrinogen I in this disease.

In the present paper we will discuss the properties of uroporphyrinogen III cosynthase and of porphobilinogen deaminase isolated from human erythrocytes. This study was carried out by analyzing the isomer composition of the formed products at the coproporphyrin stage. The direct analysis of the products as uroporphyrin octamethyl esters by either of the proposed methods [8] gave erroneous results on the isomer composition of the mixture, and as a consequence misleading properties for the enzymes were obtained.

MATERIALS AND METHODS

Human blood discarded for transfusion purposes was used. The wheat germ enzyme was the AS III fraction [5] which contained the deaminase plus cosynthase activities. Porphobilinogen (1) and [^{14}C]porphobilinogen (2500 cpm/nmole), 2-aminomethyl-3-pyrroleacetic acid (2), 2-aminomethyl-4-ethyl-3-pyrroleacetic acid (3), 2-methylopsopyrroledicarboxylic acid (4), its dimethyl ester (5), and isoporphobilinogen (6) were synthetic products ([6] and the references therein). DEAE-cellulose was obtained from Eastman Kodak and used after treatment by the method of Peterson and Sober [7]. Sephadex and Sepharose 4B were obtained from Pharmacia. All the solvents were previously purified and distilled. All other reagents were of analytical grade.

Assay

Unless otherwise indicated, the incubation mixture contained in a final volume of 100 μl:10 μmoles of phosphate buffer (pH 7.4), 13 nmoles of porphobilinogen or [^{14}C]porphobilinogen and enzyme. When uroporphyrinogen III cosynthase was assayed, porphobilinogen deaminase was added to the incubation mixture in order to form 0.8–1.5 nmoles of total uroporphyrin. Incubations were usually run at 37 °C for 60 min. Porphobilinogen was assayed with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid–perchloric acid (84:16, v/v)), by determining the absorbance at 552 nm. When sodium dithionite or sulphydryl compounds were present, Hg^{2+} was previously added. Total porphyrins were estimated by the method of Rimington and Sveinsson modified as indicated elsewhere [3]. Uroporphyrin isomers were estimated by the method of Falk and Benson (see ref. 8) when the purification sequence for uroporphyrinogen III cosynthase was examined. When the properties of the cosynthase were studied the isomer analysis was carried out by decarboxylating the uroporphyrin octamethyl esters with acid to coproporphyrins by the method of Edmonson and Schwartz [9]. The latter were extracted and separated by thin-layer chromatography on cellulose-coated plates (E. Merck, DC-Fertigplatten, Schichtdicke 0.10 mm) using 2,6-lutidine–0.7 M NH_4OH (40:28, v/v) as a solvent. Coproporphyrin isomers were located by fluorescence and eluted with a 0.7 M NH_4OH solution. The eluates were then plated on aluminium planchets and counted in a gas-flow counter. Protein was estimated by the method of Lowry et al. [10] or alternatively from its absorption at 260–280 nm.

Preparation and partial purification of porphobilinogen deaminase

300 ml of erythrocytes were diluted with 300 ml of distilled water and kept at −15 °C for 48 h. The haemolyzed erythrocyte suspension was then thawed and cen-

trifuged at 20 000 *g* for 20 min and the supernatant was filtered through several layers of nylon cloth. Part of the supernatant (150 ml) was dialized overnight against 8 l of 0.003 M phosphate buffer (pH 7.4). The supernatant could be kept at −15 °C.

DEAE-cellulose chromatography

The dialyzed supernatant (150 ml) was applied on a DEAE-cellulose column (5 cm × 20 cm) previously equilibrated with 0.003 M phosphate buffer (pH 7.4). Most of the haemoglobin was eluted with 8 l of the same buffer. The deaminase and the cosynthase were eluted with 800 ml of 0.1 M phosphate buffer (pH 7.4). Fractions of 40 ml were collected. A typical elution profile of deaminase and cosynthase is shown in Fig. 1. The most active deaminase fractions (Fig. 1A) were pooled and concentrated to one-tenth of their original volume with Carbowax. This enzyme was stable for more than three months when kept at 4 °C. A second DEAE-cellulose exchange could be performed as described in our preliminary note [2].

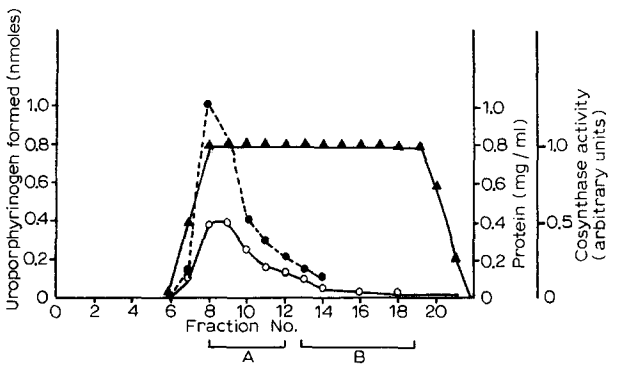

Fig. 1. Elution profile of deaminase and cosynthase from DEAE-cellulose. The procedure was as described in Materials and Methods. An arbitrary unit of 1 was assigned to a cosynthase preparation which formed 50% of uroporphyrinogen III when 0.8 nmole of total prophyrin was formed in the incubation under the standard assay conditions. (▲—▲), Cosynthase activity; (●---●), Deaminase activity; (○—○), Protein concentration.

Sephadex G-100 filtration

The concentrated enzyme preparation obtained from the first DEAE-cellulose (3 ml) was filtered through a 3 cm × 35 cm Sephadex G-100 column previously equilibrated with 0.05 M phosphate buffer (pH 7.4) and eluted with the same buffer. Fractions of 3 ml each were collected. The active fractions were pooled and concentrated with Carbowax to one-tenth of their original volume. The concentrated enzyme could be kept for 8 days at 4 °C without appreciable loss in its activity. This preparation was routinely used as source of porphobilinogen deaminase. Higher purifications were obtained when a long (1 m) Sephadex G-100 column was used but the obtained enzyme was more labile. A summary of the purification sequence of porphobilinogen deaminase can be seen in Table I.

Preparation of uroporphyrinogen III cosynthase

The preparation and purification of cosynthase is an arduous task due to the laborious estimation method for uroporphyrinogen III and the instability of the

TABLE I

PURIFICATION OF PORPHOBILINOGEN DEAMINASE FROM HUMAN ERYTHROCYTES

Fraction	Activity recovered (%)	Specific activity (units[a]/mg protein $\times 10^{-3}$)	Type of uroporphyrin isomer
Hemolysate	100	0.1	III
1st DEAE-cellulose eluate	83	1.6	III[c]
Sephadex G-100 eluate (concentrated)	33	15.2	I–III[c]
2nd DEAE-cellulose eluate[b]	60	16	I

[a] One unit of enzyme was defined as the amount which catalyzed the formation of 1 μmole of uroporphyrinogen/h under the conditions described in Methods.

[b] The enzyme used for this step was an $(NH_4)_2SO_4$ precipitate (45–60%) from the first DEAE-cellulose pooled fractions [2].

[c] The formation of isomer III disappeared after storage at 4 °C.

enzyme. No total separation of deaminase and cosynthase could be achieved during the first DEAE-cellulose purification step (Fig. 1). Nevertheless, a number of fractions contained exclusively cosynthase activity (Fig. 1B) and those were used as a subsequent source of the enzyme. The deaminase-cosynthase activities could be separated using a second DEAE-cellulose exchange column [2]. The fractions containing the cosynthase activity (Fig. 1B) were pooled and precipitated between 0–80% $(NH_4)_2SO_4$ The precipitate was dissolved in the minimum volume of 0.003 M phosphate buffer (pH 7.4) and dialyzed overnight against the same buffer. This preparation was stable at −15 °C for six months. The enzyme (20 μl containing 4 mg protein/ml) formed 0.5 nmoles of uroporphyrinogen III (measured as coproporphyrin) under the standard incubation conditions and was routinely used. A further purification could be obtained by a Sephadex G-100 filtration, but the activity was eluted over a large range of fractions and a very poor improvement with respect to the original preparation was achieved once concentrated, probably due to the enzyme's instability. When deaminase was added during this Sephadex G-100 filtration step the cosynthase was eluted before the deaminase, although part of its activity remained associated with the latter.

RESULTS

Properties of porphobilinogen deaminase

When the DEAE-cellulose enzyme was assayed the expected stoichiometric relation (4:1) among porphobilinogen consumption and porphyrin formation was usually found. However, a number of blood samples afforded DEAE-cellulose deaminase preparations which had lower porphyrin yields. When those preparations were carried through the Sephadex G-100 filtration step, a correlation between porphyrin yields and inactivation with time was found in the eluted fractions, (Table II), The fractions which had lower porphyrin yields were the more labile and lost activity when assayed after 48 h. The fractions which had higher porphyrin yields had higher protein concentrations (although they were not necessarily the most active fractions), and were the most stable with time. This effect, also observed with the wheat germ enzyme,

TABLE II

CORRELATION BETWEEN PORPHYRIN YIELDS AND DECREASE OF DEAMINASE ACTIVITY WITH TIME

100 μl of the Sephadex G-100 eluted enzyme were assayed immediately and after 48 h storage at −15 °C. The incubation mixture contained in a final volume of 120 μl: 9 nmoles of porphobilinogen (PBG), 10 μmoles of Tris–HCl buffer (pH 8.2) and Sephadex-purified erythrocyte deaminase. Incubations were performed for 1 h at 45 °C.

Fraction No.	Original activity			Activity after 48 h		
	PBG (nmoles)	Porphyrin (nmoles)	Yield (%)	PBG (nmoles)	Porphyrin (nmoles)	Inactivation (%)
8	1.35	0.34	100	1.35	0.33	0
12	10.8	2.3	85	8.37	1.76	23
14	10.8	2.08	77	4.05	0.72	63
16	7.29	1.36	74	2.20	0.16	70
17	4.86	0.80	66	1.62	0.1	67
18	2.63	0.40	60	0	0	100
19	1.08	0.16	58	0	0	100

could be due to the stabilizing effect of proteins on the uroporphyrinogen cyclization site of the deaminase [5].

The purified and concentrated Sephadex G-100 enzyme was stable at 4 °C for more than a month, due mainly to the protective effect of the high phosphate concentrations. When the concentrated enzyme was dialyzed against 0.003 M phosphate buffer (pH 7.4) a loss of 50% of its activity took place after 24 h.

The deaminase lost very rapidly its activity when kept at −15 °C. It could be heated up to 65 °C for 15 min without loss of activity. Preheating at 70 °C for 5 min enhanced its activity, however, after 15 min of heating, a drop of 20–30% in its original activity took place. The activity was entirely lost after the enzyme was heated at 100 °C for 1 min. The enzymatic activity increased with temperature. At 26 °C the activity was only 20% of the activity at 37 °C, while at 45 °C it was twice the activity at 37 °C. At 45 °C no chemical polymerization of porphobilinogen was observed under the conditions described in Assay.

Addition of glycerol (15%) inhibited 40% of the deaminase activity, and this effect was enhanced by preheating the enzyme at 70 °C.

Molecular weight

A Sephadex G-100 column (0.9 cm × 95 cm) was equilibrated with 0.05 M phosphate buffer (pH 7.4) at a flow rate of 6 ml/h. The protein markers used were: bovine serum albumin, ovalbumin, bovine growth hormone and ribonuclease. Porphobilinogen deaminase had a V/V_0 ratio of 1.80 corresponding to a molecular weight of about 25 000 ± 5000 when compared with the marker proteins.

Optimum pH

High concentrations of phosphate buffer had a stabilizing effect on the erythrocyte deaminase. An optimum pH of 7.4 with phosphate buffer was found for the Sephadex G-100 purified, concentrated and dialyzed enzyme. An optimum pH of 8.2

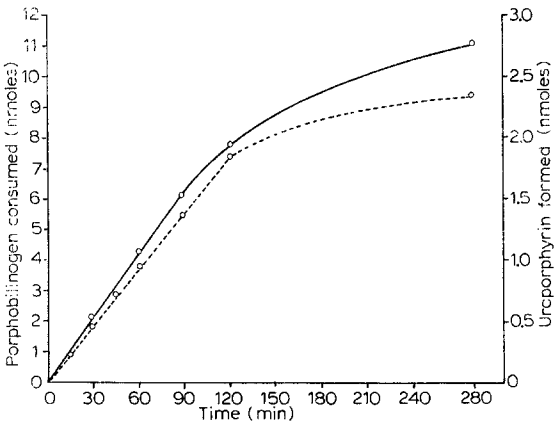

Fig. 2. Rate of substrate consumption. The standard assay procedures were used. Deaminase was a Sephadex G-100-purified enzyme. (○—○), Porphobilinogen consumed; (○---○), Uroporphyrin formed.

was determined in Tris–HCl buffer. The enzyme was stable above pH 9 and unstable below pH 5. The enzymatic activity was lost after preincubation of the enzyme in acetate buffer (pH 3.9) for 15 min followed by dialysis.

Effect of dissociating agents

8 M urea inhibited the deaminase activity, but this inhibition was totally reversed by either dilution or dialysis. A 75 % loss of deaminase activity took place in the presence of 0.025 % of dodecylsulphate, while at a 0.03 % concentration the activity was entirely lost.

Kinetics

The activity of porphobilinogen deaminase increased linearly with time for 2 h. After this period the increase in activity slowed down (Fig. 2). The effect of substrate concentration on substrate consumption and product formation (Fig. 3a) showed

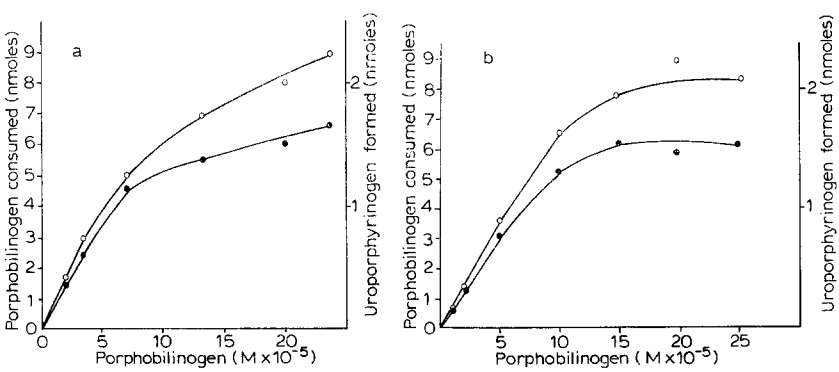

Fig. 3. Effect of substrate concentration on deaminase and deaminase cosynthase activities. The standard assay conditions were used, except for the substrate concentrations which were those indicated. (a) The enzyme used was the Sephadex G-100 deaminase. (b) The enzyme used was a whole system isolated from DEAE-cellulose (Fig. 1A). (●—●) Uroporphyrinogen formed. (○—○) Porphobilinogen consumed.

classical Michaelis–Menten kinetics. When urophorphyrinogen cosynthase was added in a sufficient amount to obtain 100% formation of uroporphyrinogen III, or when a original deaminase–cosynthase system (80% formation of uroporphyrinogen III) was used (Fig. 3b), the same classical Michaelis–Menten kinetics were obtained. This was at variance with the results obtained by Sancovich et al. [11], who found that the deaminase–cosynthase system isolated from cow liver behaved as an allosteric system when the formed products were estimated. From the values for porphobilinogen consumption by the deaminase a K_m of $1.3 \cdot 10^{-4}$ M was determined, and from the values for porphyrin formation a K_m of $7.7 \cdot 10^{-5}$ M was determined. The values $V = 15.3$ nmole/h and $V = 2.1$ nmole/h were found for porphobilinogen consumption and porphyrin formation, respectively. In the case of the deaminase-cosynthase system, values for porphobilinogen consumption gave a K_m of $4.6 \cdot 10^{-5}$ M, and values for porphyrin formation gave a K_m of $5.1 \cdot 10^{-5}$ M. $V = 7.0$ nmole/h for substrate consumption and $V = 1.4$ nmole/h for porphyrin formation were found when the same deaminase preparation was used in both experiments. These results indicated that there is an increased affinity for porphobilinogen in the deaminase–cosynthase system as compared with deaminase alone, while at the same time a decrease in the reaction rate takes place.

Effect of porphobilinogen analogues on deaminase activity

A number of synthetic pyrroles (Fig. 4) structurally related to porphobilinogen were assayed as substrates of the enzyme. None were found to act as such, even at the slowest rate. However, they exerted an inhibitory effect on the enzymatic activity. A free (non-esterified) propionic acid residue at C-4 was essential to obtain an efficient inhibition of the deaminase. Isoporphobilinogen and 2-methylopsopyrroledicarboxylic acid inhibited the activity by addition to the incubation mixture, while 2-aminomethyl-3-pyrroleacetic acid and 2-aminomethyl-4-ethyl-3-pyrroleacetic acid exerted their effect only by pre-incubation (Table III). When deaminase was preincubated with 2-aminomethyl-3-pyrroleacetic acid, and the mixture was latter dialyzed to eliminate the excess of pyrrole, the enzyme remained partially inhibited both for por-

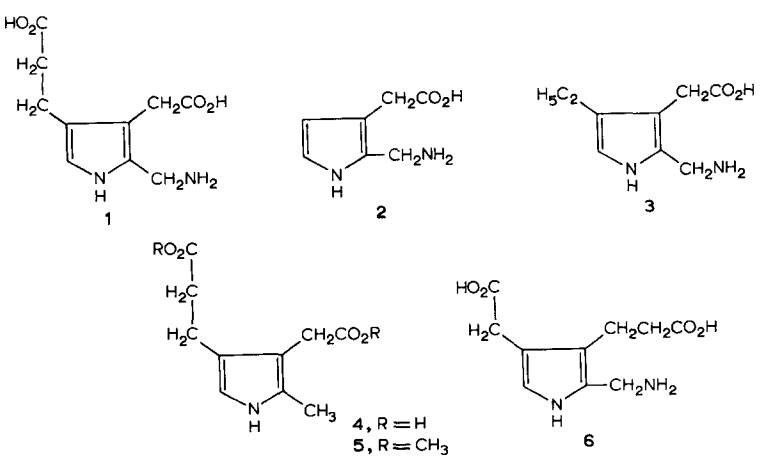

Fig. 4. Structures of synthetic pyrroles related to porphobilinogen.

TABLE III

EFFECT OF SYNTHETIC PORPHOBILINOGEN ANALOGUES ON DEAMINASE ACTIVITY

The incubation mixture contained in a final volume of 100 μl: 10 μmoles of phosphate buffer (pH 7.4), porphobilinogen (15 nmoles), deaminase (Sephadex G-100-purified enzyme) and the indicated pyrrole (40 nmoles of 2-aminomethyl,3-pyrroleacetic acid and 30 nmoles of the other pyrroles). Incubations were run for 60 min at 37 °C. Preincubations were carried out for 30 min at 37 °C omitting porphobilinogen, which was added at the start of the incubation.

Pyrrole	Preincubation	Inhibition of porphyrin formation
2-Aminomethyl-3-pyrroleacetic acid	−	8
2-Aminomethyl-3-pyrroleacetic acid	+	50
2-Aminomethyl-4-ethyl-3-pyrroleacetic acid	−	17
2-Aminomethyl-4-ethyl-3-pyrroleacetic acid	+	70
Isoporphobilinogen	−	50
Isoporphobilinogen	+	50
2-Methylopsopyrroledicarboxylic acid	−	50
2-Methylopsopyrroledicarboxylic acid	+	50
Methyl 2-methylopsopyrroledicarboxylate	−	0
Methyl 2-methylopsopyrroledicarboxylate	+	0

phobilinogen consumption and for porphyrin formation. The partially inhibited deaminase was more resistent to further inhibition by *p*-hydroxymercuribenzoate (0.04 mM) or photooxidation in the presence of methylene blue, at concentrations and conditions which inhibited the non-treated enzyme.

Effect of inhibitors

A number of divalent metals inhibited the activity of porphobilinogen deaminase; Mg^{2+} (25 mM) and Ca^{2+} (25 mM) inhibited 90% of the enzymatic activity while Cd^{2+} (12 mM) inhibited the enzymatic activity completely. Pb^{2+} (25 mM) had no effect on deaminase activity. In all cases porphobilinogen consumption was measured. Preincubation of the enzyme with *N*-bromosuccinimide (0.07 mM) followed by dialysis inhibited 70% of its activity. Photooxidation of the enzyme in the presence of methylene blue (0.03%) and rose bengal (0.03%), over a 5 min period, inhibited 90% and 60% of the enzymatic activity, respectively. Sulphydryl reagents such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide were also inhibitors of the deaminase. These effects were similar to those described for the wheat germ enzyme [5, 12].

Properties of uroporphyrinogen III cosynthase. Effect of porphobilinogen concentration on uroporphyrinogen III formation

Increasing concentrations of porphobilinogen exerted no inhibition on the formation of uroporphyrinogen III when a deaminase cosynthase system was used (Fig. 5a). While the formation of total porphyrinogens reached a plateau, the formation of uroporphyrinogen III levelled off before the same effect could be detected for uroporphyrinogen I. The addition of dithiothreitol while not affecting the formation of total porphyrins, does affect the isomer distribution among the products. The formation of uroporphyrinogen I now levelled-off at lower substrate concentrations than

those required to obtain the same effect with uroporphyrinogen III (Fig. 5b). The influence of dithiothreitol must be attributed to its stabilizing effect on the cosynthase, since it allowed the formation of a larger percentage of uroporphyrinogen III. The inhibition of the formation of uroporphyrinogen III with increasing concentrations of porphobilinogen reported by Sancovich et al. [11] could not be confirmed by us,

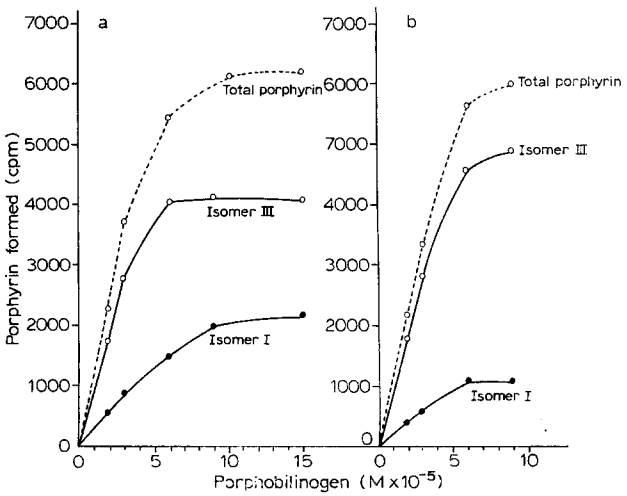

Fig. 5. Effect of porphobilinogen concentration on uroporphyrinogen III formation. (a) The incubation mixture was the described in Table IV, except for porphobilinogen concentrations which were those indicated. (b) The same as (a), but dithiothreitol (3 mM) was present during the incubation procedures. Isomers were estimated as coproporphyrins.

either with the described enzymes or with the wheat germ enzymes even at much higher substrate concentrations than those indicated in Fig. 5. The reported results must be attributed to the isomer estimation method used by the latter authors (Stevens, E. and Frydman, B., unpublished).

Rate of uroporphyrinogen III formation

The formation of uroporphyrinogen III increased linearly up to 30 min and then started to level-off. Simultaneously the increase in uroporphyrinogen I was enhanced after 30 min (Fig. 6). This could be due to the partial inactivation of the cosynthase after that period of time, while the deaminase was very stable even after a 2 h incubation period. Addition of dithiothreitol (3 mM) afforded the formerly described protective effect on the cosynthase (Fig. 5b); and after a 60 min incubation period a concentration ratio of 5:1 for uroporphyrinogen III–uroporphyrinogen I was obtained as compared to a concentration ratio of 2:1 obtained in the absence of dithiothreitol (Fig. 6). When the rate of uroporphyrinogen III formation was compared with the rate obtained with the wheat germ system [3] it became evident that the cosynthase from human erythrocytes was more stable than the enzyme from wheat germ, while the deaminase from human erythrocytes was more labile than the enzyme from wheat germ.

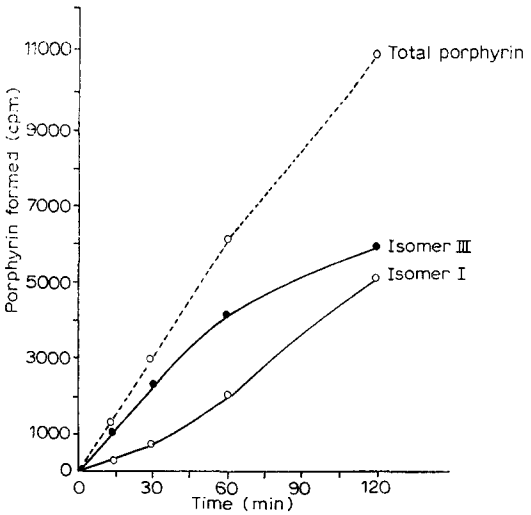

Fig. 6. Rate of uroporphyrinogen III formation. The incubation mixture was the described under Table IV, except for incubation times, which were those indicated. Isomers were estimated as coproporphyrins.

Effect of NH^+_4 and hydroxylamine on the deaminase cosynthase interaction

It was known that the addition of NH^+_4 and hydroxylamine to a deaminase cosynthase system resulted in a more pronounced inhibition of uroporphyrinogen formation than of porphobilinogen consumption [13, 3]. An examination of this effect with the erythrocyte enzymes indicated that increasing concentrations of NH^+_4 inhibited the formation of total porphyrins and also the formation of uroporphyrinogen III (Table IV). At a concentration of 100 mM NH^+_4 both inhibitions run approximately a parallel course and the formation of uroporphyrinogen I was inhibited to a smaller extent (Table IV, Expt 1). In the absence of large amounts of phosphate buffer the inhibition of uroporphyrinogen III formation was less pronounced, but the whole enzymatic system was evidently more unstable especially due to the lability of the cosynthase (Table IV, Expt 2). An examination of the effect on the wheat germ enzymes indicated a more pronounced inhibition of uroporphyrinogen III formation. At a 30 mM concentration of NH^+_4, the decrease in uroporphyrinogen III formation was simultaneous with an increase in uroporphyrinogen I formation (Table IV, Expt 3). The inhibition of the enzymes by NH^+_4 was then closely dependent on the enzyme's source and on their stability. No total inhibition of uroporphyrinogen III formation was achieved even at relatively high NH^+_4 concentrations, at variance with the strong inhibitory effect of NH^+_4 on isomer III formation described by other authors [11]. It has been suggested [13] that this inhibition was due to the lack of an hypothetical cosynthase substrate formed normally by the deaminase and not formed any more in the presence of NH^+_4. However, the simultaneous decrease in the formation of both isomers found with the erythrocyte enzymes (Table II, Expt 1) does not lend support to this suggestion.

The addition of hydroxylamine (30 mM) totally inhibited uroporphyrinogen III formation, allowing the sole formation of isomer I. This effect may be attributed to either an inhibition of the cosynthase activity or to an interference in the deaminase cosynthase interaction.

TABLE IV

EFFECT OF AMMONIA AND HYDROXYLAMINE ON UROPORPHYRINOGEN ISOMER FORMATION

The incubation mixture contained, in a final volume of 100 μl: 10 μmoles of phosphate buffer (pH 7.4); [^{14}C]porphobilinogen (9 nmoles, 2500 cpm/nmole) and enzyme. The erythrocyte enzymes used were the Sephadex-purified deaminase and the DEAE-cellulose-purified cosynthase. The wheat germ used was that indicated in Materials and Methods. Incubations were run for 60 min at 37 °C. Uroporphyrinogen formed and isomer composition were estimated as coproporphyrins.

Exp. No.	Enzymatic system	Ammonia (mM)	Hydroxylamine (mM)	Uroporphyrinogen formed					
				Isomer I		Isomer III		Total porphyrin	
				(cpm)	Inhibition (%)	(cpm)	Inhibition (%)	(cpm)	Inhibition (%)
1	Deaminase –	—		2000		4160		6160	
	cosynthase	30		2000		3850	7	5850	5
	from human	60		1600	20	3000	28	4600	25
	erythrocytes	100		1485	25	2510	40	3995	35
2	Deaminase –	—		3675		4450		8125	
	cosynthase	100		2600	29	2420	45	5020	38
	from human		30	4800		130	100	4930	39
	erythrocytes	—		4214		1512		5726	
		100*	30*	2550	40	1200	20	3750	35
				2100	Activation	135	100	2235	69
3	Wheat germ	—		3810		8600		12410	
	deaminase –	30		4500	18	4970	42	9470	24
	cosynthase	60		4220	11	4290	50	8510	31
		100		3900	2	2970	65	6870	45
			20	4870	28	200	100	5070	60
			30	3555		135	100	3690	70

* The addition of the phosphate buffer (10 μmoles) was omitted in the incubation mixture.

Effect of sulphydryl reagents

The cosynthase and the deaminase from human erythrocytes were unequally affected by sulphydryl reagents. As mentioned above, *N*-ethylmaleimide was an inhibitor of porphobilinogen deaminase. At a concentration where 50% of total porphyrinogen was inhibited, its effect on uroporphyrinogen III formation was more pronounced (Table V). Addition of dithiotreitol reverted the inhibition of deaminase by *N*-ethylmaleimide while it did not affect cosynthase inhibition. Similar results were obtained with the wheat germ enzymes. *p*-Hydroxymercuribenzoate inhibited both enzymes and the effect was reverted by the addition of dithiotreitol.

Effect of oxidations on cosynthase activity

The oxidation and photooxidation of erythrocyte deaminase was mentioned above. The effects of *N*-bromosuccinimide oxidation on cosynthase and the effect of the photooxidation by light in the presence of sensitizers are summarized in Table VI. Methylene blue itself exerted a protecting effect on the cosynthase. The cosynthase was more sensitive to photooxidation in the presence of rose bengal than the deaminase and was not affected by *N*-bromosuccinimide oxidation.

TABLE V

EFFECT OF SULPHYDRYL REAGENT ON THE ACTIVITIES OF DEAMINASE AND COSYNTHASE

The preincubations were run in a final volume of 50 μl in the presence of phosphate buffer (10 μmoles, pH 7.4) for 15 min at 37 °C. After preincubating either the cosynthase alone or in presence of *N*-ethylmaleimide (3 mM) or *p*-hydroxymercuribenzoate (0.06 mM), deaminase, [^{14}C]porphobilinogen and the indicated substances were added and the volume was completed to 100 μl. Dithiothreitol (3 mM) was added to the *N*-ethylmaleimide-treated cosynthase, and 0.3 mM dithiothreitol was added to the *p*-hydroxymercuribenzoate-treated enzyme. Incubations were carried out for 60 min at 37 °C. Isomers were estimated as coproporphyrins.

Preincubated system	Addition	Uroporphyrinogen formed				
		Isomer I (cpm)	Isomer III (cpm)	Inhibition (%)	Total porphyrin (cpm)	Inhibition (%)
Cosynthase	Deaminase	1700	3300		5 000	
Cosynthase + *N*-ethylmaleimide	Deaminase	2941	474	86	3 415	32
Cosynthase + *N*-ethylmaleimide	Deaminase + dithiothreitol	3715	653	80	4 370	12
Cosynthase	Deaminase + *N*-ethylmaleimide	1950	550	83	2 500	50
Cosynthase	Deaminase	2210	4300		6 510	
Cosynthase + p-hydroxymercuribenzoate	Deaminase	1000	500	88	1 500	77
Cosynthase + p-hydroxymercuribenzoate	Deaminase + dithiothreitol	2737	3005	30	5 842	10
Cosynthase	Deaminase + *p*-hydroxymercuribenzoate	1813	1550	64	3 365	48
Erythrocyte*	—	3675	4450		8 125	
Deaminase cosynthase Erythrocyte*	*N*-ethylmaleimide	3550	426	90	3 931	51
Deaminase cosynthase	*p*-hydroxymercuribenzoate	2710	1600	64	4 310	47
Wheat germ* deaminase cosynthase	—	3800	8600		12 400	
Wheat germ* deaminase cosynthase	*N*-ethylmaleimide	6880	720	91	7 600	38.5
Wheat germ* deaminase cosynthase	*p*-hydroxymercuribenzoate	4362	7925	7	12 287	

* The system was incubated without preincubation.

Association between porphobilinogen deaminase and uroporphyrinogen III cosynthase

The deaminase was bound to Sepharose 4B by mixing 2.5 ml of settled Sepharose with an equal volume of water to which 1 g of cyanogen bromide was added. The solution was immediately adjusted to pH 11, and was kept at this pH by titrating with a 6 M NaOH solution. After completion of the reaction (approx. 15 min) the Sepharose was filtered and washed with 20 ml of cold 0.1 M $NaHCO_3$ using a Buchner funnel. The washed Sepharose was suspended in 5 ml of a 0.1 M $NaHCO_3$ solution containing Sephadex G-100 purified deaminase (25 mg of protein) free of any cosynthase activity and the suspension was slowly stirred for 20 h at 4 °C. The solid was

TABLE VI

EFFECT OF PHOTOOXIDATION AND *N*-BROMOSUCCINIMIDE ON COSYNTHASE ACTIVITY

The photooxidation of the cosynthase was done in the presence of buffer and of 0.03% of the indicated dyes by illuminating small tubes immersed in an ice bath for 5 min. A 100 W lamp, placed 20 cm from the tubes was used as the light source. When light was omitted, the mixtures were kept in ice for the time of illumination and the incubations were started by adding the Sephadex-purified deaminase plus [^{14}C]porphobilinogen (9 nmoles, 2550 cpm/nmole). Incubations were carried out at 37 °C for 60 min. The cosynthase plus phosphate buffer were preincubated for 15 min at 37 °C either in the presence or absence of *N*-bromosuccinimide (0.7 mM) and then dialyzed against 0.003 M phosphate buffer (pH 7.4) for 4 h with two changes. Incubations were carried out as above.

Cosynthase treatment	Uroporphyrinogen formed			
	Isomer I (cpm)	Isomer III (cpm)	Inhibition (%)	Total porphyrin (cpm)
—	4875	3935	—	8810
hν	4870	3940	—	8810
Rose bengal	4830	4055	—	8885
Rose bengal + hν	5680	2950	27	8630
Methylene blue	3000	5335	—	8335
Methylene blue + hν	4540	4076	14	8616
No *N*-bromosuccinimide	6230	1916	—	8146
N-bromosuccinimide	6250	1800	—	8050

separated by centrifugation, and washed twice with 10 ml of 0.1 M acetic acid, followed by two consecutive washings with 0.05 M phosphate buffer (pH 7.4). A 15% yield of diaminase was recovered bound to the Sepharose when measured under the standard incubation conditions. The same results were obtained with wheat germ deaminase. The deaminase–Sepharose complex could be used for four consecutive incubations without loss in activity. It was stable at 4 °C for at least a month, but lost its activity after 3–4 months. The immobilized deaminase formed only uroporphyrinogen I. When incubated together with a deaminase-free cosynthase under the standard incubation conditions it formed uroporphyrinogen III.

The interaction of the Sepharose–deaminase complex and the cosynthase was determined by packing the former in a 0.5 cm × 10 cm column, while a similar column was packed with Sepharose treated as described above to which deaminase had not been added. The columns were equilibrated with 0.5 M phosphate buffer (pH 7.4) and 300 μl of cosynthase (Fig. 1, Peak B) were filtered through the columns. The Sepharose–deaminase complex retained part of the added protein, while the Sepharose preparation retained none (Fig. 7). No cosynthase activity was eluted in the former case, due to the retention of the cosynthase on the Sepharose–deaminase column while in the latter case most of the applied cosynthase activity was eluted as such, Similar results were obtained when the cosynthase preparation contained porphobilinogen (150 nmole/ml). No elution of cosynthase or deaminase cosynthase activities from the Sepharose–deaminase column was achieved with either buffer plus substrate (0.1 mM porphobilinogen) or with higher buffer or salt (2 M NaCl) concentrations. Elutions with 8 M urea or 6 M guanidine hydrochloride could not be performed due to their inactivating effect on the cosynthase activity.

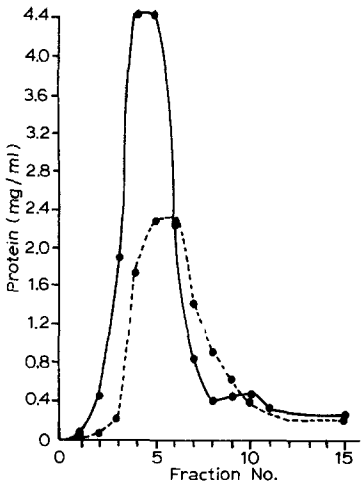

Fig. 7. Protein profile obtained by filtration of cosynthase through Sepharose 4B–deaminase (●---●), and Sepharose 4B (●—●).

When the whole Sepharose–deaminase cosynthase preparation was used as such for incubation purposes, it was found that it formed 70% of uroporphyrinogen III indicating that no inactivation of the enzymes took place. Controls prepared either by filtering heated cosynthase preparations on Sepharose–deaminase columns, or by filtering active cosynthase preparations on deaminase-free Sepharose did not afford isomer III forming systems. When the Sepharose–deaminase complex became inactive with time, no retention of the cosynthase took place and the latter emerged with the bulk of the protein (Fig. 7).

DISCUSSION

The properties of human erythrocyte deaminase were very similar to those described for the wheat germ enzyme [5], while the studies with the cosynthase afforded a number of novel properties of the latter. The deaminase was a low molecular weight enzyme and it was known [5, 14] that it behaved as a single protein which could not be dissociated even by strong dissociating agents. The erythrocyte cosynthase had apparently a higher molecular weight than the deaminase, although rigorous determinations must await a stabilizing procedure for the enzyme. As in the case of the wheat germ enzyme the yield of the product in some preparations of deaminase was less than the expected from the stoichiometric values. This effect can be explained if two active sites are accepted for the deaminase; one to which the porphobilinogen units became bound, and a second one more labile where the final cyclization step takes place. The enzyme was inhibited by oxidizing agents, especially by those which are known to oxidize tryptophanyl residues. We described elsewhere [12] the inhibitory effect of pyrrolooxygenase on erythrocyte deaminase due to the oxidation of essential tryptophanyl residues of the latter. The cosynthase was not affected either by chemical oxidation (*N*-bromosuccinimide) or by pyrrolooxygenase oxidation [12]. However, it was partially inactivated by a photooxidation more specific for histidine residues than for tryptophan residues (Table VI). These differential properties can afford a physio-

logical regulation mechanism for the deaminase cosynthase system, based on a deaminase inactivation by interaction with pyrrolooxygenase which does not affect the cosynthase present in the system. In this way uroporphyrinogens will not be formed while the cosynthase pool will remain unchanged.

Both deaminase and deaminase cosynthase showed classical Michaelian kinetics as was the case with the wheat germ enzymes [3, 5]. High porphobilinogen concentrations did not inhibit uroporphyrinogen III formation, but only formed higher amounts of uroporphyrinogen I. Since the physiological concentration of porphobilinogen is usually low, it could be predicted that in the case of a sudden rise in its concentration the sufficient amount of uroporphyrinogen III will always be formed while the excess of porphobilinogen will be diverted at least in part, to the formation of uroporphyrinogen I.

Cosynthase was more sensitive to sulphydryl reagents than the deaminase, and with *N*-ethylmaleimide the inhibition was irreversible. This explains the strong protective effect of dithiothreitol on the rate of uroporphyrinogen III formation (Fig. 5b). The well known unstability of cosynthase and its inhibition during the incubation procedures could then be attributed to its very sensitive sulphydryl groups. When the rates of uroporphyrinogen III and uroporphyrinogen I formation are compared (Fig. 6), its is obvious that they both seem to share a common intermediate. The common intermediate must be the deaminase–cosynthase complex. The results with the immobilized enzymes indicated that deaminase and cosynthase formed a strong association which was not dependent on the presence of substrate. It has already been demonstrated [15–17] that uroporphyrinogen III and uroporphyrinogen I do not share a common pyrrylmethane intermediate and are apparently originated from two differents dipyrrylmethanes. This leaves the deaminase–cosynthase association as the sole common intermediate between both uroporphyrinogens. The initial association forms mainly uroporphyrinogen III, and its formation decreases as the cosynthase becames progressively inactive, very likely due to its labile sulphydryl groups. We advanced the proposal [15–18], that the cosynthase acts as a "specifier protein" for the deaminase (similar to the role of lactoalbumin in the lactose synthetizing system [19]), changing the mechanism of the condensation of the porphobilinogen units on the deaminase surface. This proposal finds support in the experiments performed with the immobilized deaminase, which binds the cosynthase in the absence of any pyrrole giving rise to a uroporphyrinogen III forming system.

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