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ISOLATION AND PROPERTIES OF WHEAT GERM UROPORPHYRINOGEN III COSYNTHETASE

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SUMMARY

1. A reproducible method for the isolation of uroporphyrinogen III cosynthetase from wheat germ is described.

2. Some properties of the isolated enzyme were studied. The pH optimum was 8.2. The enzyme was very sensitive to heat and was inhibited by several salts.

INTRODUCTION

The enzyme system which catalyzes the formation of uroporphyrinogen III from porphobilinogen is ubiquitous in plants and animals and has been isolated from different biological sources by a number of workers[1–5]. The system has been shown to consist of at least two enzymes: porphobilinogen deaminase, which catalyzes the formation of uroporphyrinogen I from porphobilinogen and is heat-stable; and uroporphyrinogen isomerase or uroporphyrinogen III cosynthetase, which catalyzes the formation of uroporphyrinogen III from porphobilinogen when present in a system containing porphobilinogen deaminase. Evidence for the existence of the cosynthetase has been mainly indirect, and is based on its destruction by preheating at temperatures that vary between 55 and 63°, according to its biological source[1,2,4,5].

The mechanism by which these enzymes direct the formation of uroporphyrinogen III from porphobilinogen is unknown, and it was our intention to study the reaction using porphobilinogen deaminase and uroporphyrinogen III cosynthetase prepared from wheat germ, as described by BOGORAD[3]. However, repeated attempts to isolate the latter free of deaminase activity were unsuccessful, and we were obliged to search for a different method, which is described in the present paper, together with several properties of the isolated enzyme.

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MATERIALS AND METHODS

Wheat germ was the generous gift of Molinos del Rio de la Plata (Buenos Aires). Porphobilinogen and [^{14}C]porphobilinogen (1 mC/mmole) were synthesized according to the method of FRYDMAN, DESPUY AND RAPOPORT[6]. Alumina C_γ gel was prepared according to the procedure of WILLSTÄTTER AND KRAUT[7] and heated at 100° during 4 h, or left to age for at least 5 months. Solvents for chromatography were purified according to FALK[8]. All other reagents were of analytical grade.

Assay

Unless otherwise indicated, the reaction mixture contained $3 \cdot 10^{-2}$ μmoles of unlabelled or labelled porphobilinogen (30 000 counts/min), 40 μmoles of Tris–HCl buffer (pH 8.2), and enzyme preparation (Fractions 40, 45 or 50, see below) or a mixture of porphobilinogen deaminase (0.5 mg of protein) and uroporphyrinogen III cosynthetase (0.7 mg of protein). The final volume was 200 μl. Incubations were carried out at 37° for 45 or 60 min. Blanks were run by adding either porphobilinogen or porphobilinogen deaminase after incubation.

Porphobilinogen was assayed in the usual way with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid–$HClO_4$ (84:16, v/v)), by determining the absorbance at 552 mμ[10].

Total porphyrins were assayed by determining the absorbance at 405 mμ (Soret band) of the incubation mixture oxidized with 10 μl of a 1% iodine solution and diluted to 3 ml with 2% HCl. As the enzyme system to be described formed neither coproporphyrin nor protoporphyrin, the absorbance thus determined was proportional to the total amount of uroporphyrin formed (isomer I + isomer III).

Protein was determined by the method of LOWRY *et al.*[11].

Isomer estimation

The proportion of uroporphyrinogens I and III formed was determined by a modification of the method of FALK AND BENSON[9], which comprises paper chromatographic separation of the octamethyl esters of the previously oxidized uroporphyrins I and III, elution of the spots, and spectrophotometric evaluation of the amount of porphyrin in each. The method can be made more accurate by the use of empirically determined correction factors[12]. In the present work, spectrophotometric determination of the uroporphyrin esters was replaced by measurement of the radioactivity of ^{14}C-labelled esters, and a different correction method was used.

Determinations were carried out as follows: after incubation with [^{14}C]porphobilinogen and oxidation of the porphyrinogens with 10 μl of a 1% iodine solution, the mixture was evaporated to dryness *in vacuo* at room temperature. The residue was mixed with 0.25 ml of a 5% H_2SO_4 solution in anhydrous methanol and shaken overnight. After diluting with water, the octamethyl esters of the uroporphyrins were extracted with ether. The extract was washed twice with distilled water and evaporated to dryness. The usual method for paper chromatographic separation of the octamethyl esters[9] was used. The fluorescent spots of the uroporphyrin esters were then eluted from the paper with a dioxan–chloroform mixture (4:1, v/v) and radioactivity of the evaporated residue was counted with a gas-flow counter.

When porphobilinogen deaminase was incubated with labelled porphobilinogen

in the absence of cosynthetase, *i.e.*, when uroporphyrin I only was formed, a varying amount of radioactivity, ranging from approx. 400 to 700 counts/min, was always found in the position corresponding to uroporphyrin III. This amount was determined for each experimental condition, and deducted from the corresponding uroporphyrin III activity. Typical values for this correction are presented in Table I. Table I also shows the amount of radioactivity appearing in the uroporphyrin I

TABLE I

CORRECTION VARIABLES FOR ESTIMATION OF [^{14}C]UROPORPHYRIN I AND III OCTAMETHYL ESTERS
The paper chromatogram was developed with the FALK AND BENSON solvents[9]. For further details, see the text.

[^{14}C]Uroporphyrin I octamethyl esters (counts/min)		*[^{14}C]Uroporphyrin III octamethyl esters (counts/min)*	
Eluted from uroporphyrin I position	*Eluted from uroporphyrin III position*	*Eluted from uroporphyrin III position*	*Eluted from uroporphyrin I position*
557	563	109	0
1607	499	162	9
2991	609	449	41
4014	482	851	99
6068	552	1526	127
7853	608	1891	187
8891	667	2169	142
10763	589	2405	286
		3519	325

position when enzymatically formed [^{14}C]uroporphyrin III (obtained with porphobilinogen deaminase in presence of an excess of uroporphyrinogen III cosynthetase) was run alone. This correction, which amounts to approx. 10% of the uroporphyrin III activity, has not been applied to uroporphyrin I values reported in this paper.

An experiment with mixtures of [^{14}C]uroporphyrin esters, reported previously by other workers[13], had led to the conclusion that the two distinct spots found after paper chromatographic separation contained mixtures of both isomers. In order to evaluate this error, standard mixtures of the radioactive isomers were prepared, in amounts comparable to those expected under varying incubation conditions. Some results are shown in Table II. The expected ratio of isomers was calculated from the amounts of radioactivity found at the uroporphyrin III and uroporphyrin I positions when uroporphyrins III and I, respectively, were run separately, with the radioactivity left by uroporphyrin III at the uroporphyrin I position also being considered as uroporphyrin I. In calculating the experimentally found ratios, isomer III values were corrected for the amount of radioactivity contributed to the uroporphyrin III spot by isomer I when the latter was run alone (see Table I for typical correction values).

The data summarized in Table II show that the method could safely be used for the determination of several properties of uroporphyrinogen III cosynthetase. It could not be used to draw conclusions from results where the percentage of uroporphyrin III in the mixture with uroporphyrin I was very low.

TABLE II

PERCENTAGE OF ERROR IN THE ESTIMATION OF UROPORPHYRIN ISOMERS

The [^{14}C]octamethyl esters were purified by paper chromatography prior to mixing. Paper chromatograms were developed with the FALK AND BENSON[9] solvents. Mixtures were run against individual [^{14}C]uroporphyrin I octamethyl ester standards in the amount present in each mixture.

Total uroporphyrin esters (counts/min)	*Uroporphyrin III/uroporphyrin I ratio*		*% Error in estimation*	
	% Expected	*% Found*	*Uroporphyrin III*	*Uroporphyrin I*
5106	3 : 97	2 : 98	33	1
3888	4 : 96	1 : 99	75	3
5350	17 : 83	13 : 87	23.5	5
4707	21 : 79	19 : 81	10	2.5
6563	41 : 59	38 : 62	7.5	5
3935	47 : 53	50 : 50	6.5	5.5
5022	52 : 48	49 : 51	6	6.5
6386	66 : 34	62 : 38	6	12
3333	67 : 33	69 : 31	1.5	6
3999	70 : 30	75 : 25	7	17
2929	86 : 14	90 : 10	4.5	29
707	89 : 11	88 : 12	1	9
2276	90 : 10	91 : 9	1	10
1747	92 : 8	92 : 8	0	0
2681	94 : 6	93 : 7	1	16.5

Enzyme preparation

Operations were performed at 0–4°. The steps prior to adsorption on alumina C_γ gel were modified from BOGORAD[3].

100 g of wheat germ were extracted with 400 ml of water. The slurry was centrifuged at 15 000 × *g* for 10 min and the supernatant solution was brought to pH 5 with 1 M acetic acid and allowed to stand for 30 min to 2 h. After 10 min centrifugation at 15 000 × *g*, the supernatant solution was fractionated with solid $(NH_4)_2SO_4$. The fractions precipitating at 35–40%, 40–45% and 45–50% saturation were dissolved separately in a small volume of 0.01 M Tris buffer (pH 7.6) and dialyzed overnight against 4 l of the same buffer. (These fractions are referred to hereafter as Fractions 40, 45 and 50, respectively). All three fractions presented porphobilinogen-consuming activity. Fractions 40 and 45 formed a mixture of uroporphyrinogens I and III; Fraction 50 formed only uroporphyrinogen III. Under the conditions described, it was not possible to obtain a fraction entirely lacking in porphobilinogen deaminase activity.

Uroporphyrinogen III-forming activity decreased sharply in all fractions after prolonged storage at −20°, whereas porphobilinogen-consuming activity was only slightly affected. Fraction 50, used as a source of uroporphyrinogen III cosynthetase, was processed as described below within 1 month of its preparation from wheat germ.

In a typical run, 1 ml of Fraction 50 was shaken with alumina C_γ gel in a ratio of between 1 and 1.75 mg of dry residue of the gel to 1 mg of protein. After 10 min, the slurry was centrifuged and the sediment washed successively with 0.05 M Tris buffer (pH 7.6) and with 0.05 M, 0.1 M and 0.2 M NaCl added to the same buffer. To the pooled NaCl supernatants, free of porphobilinogen-consuming activity (0.2–0.3 mg protein/ml), crystalline bovine serum albumin was added (1 mg/ml) and the

solution was dialyzed against 0.01 M Tris buffer (pH 7.6) and freeze-dried. The residue was dissolved in 1/10 of its original volume of water, and used not later than 7 to 10 days after preparation.

In order to prepare porphobilinogen deaminase, an aliquot of Fraction 40 or 45 was diluted to a protein concentration of 10 mg/ml after addition of sufficient 2 M Tris buffer (pH 8.2), to make the solution 0.1 M in buffer. The solution was heated at 75° for 15 min and cooled immediately in an ice bath.

RESULTS

Separation of uroporphyrinogen III cosynthetase from porphobilinogen deaminase

When a uroporphyrinogen III-forming fraction was adsorbed on alumina as described, no deaminase activity could be recovered. However, if a lower gel/protein ratio was used, both enzymatic activities were recovered separately. The results are summarized in Table III.

TABLE III

SEPARATION OF UROPORPHYRINOGEN III COSYNTHETASE FROM PORPHOBILINOGEN DEAMINASE

Reaction carried out under conditions of routine assay. When the 0.05 M NaCl wash was tested, deaminase was added in order to consume $8 \cdot 10^{-3}$ μmole of porphobilinogen.

Ratio protein/ alumina C_γ (mg)	*Supernatant solution or wash*	*Porphobilinogen consumption as % of activity of original extract*	*% of total porphyrins formed*	
			Uroporphyrin I	*Uroporphyrin III*
1 : 0.25	Supernatant	68	75	25
	0.05 M buffer	31	25	75
	0.05 M NaCl	0	25	75

Effect of cosynthetase concentration on uroporphyrinogen III formation

The amount of uroporphyrinogen III formed when the deaminase and porpho-

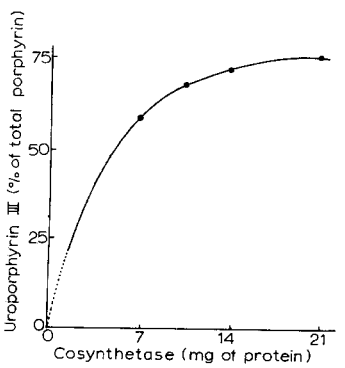


Fig. 1. Effect of cosynthetase concentration on uroporphyrin III formation. Incubation as indicated in the assay method. ———, error less than 10%; ....., error exceeds 10% (see Table II).

bilinogen were not limiting was proportional to the amount of cosynthetase present (Fig. 1). In order to determine the effect of cosynthetase on total porphyrin formation, this enzyme was incubated in amounts ranging from 0 to 0.32 mg with standard incubation mixtures containing 0.5 mg of deaminase. The mean absorbance at 405 mμ for a series of 6 determinations was 0.220±0.008. This led to the conclusion that the cosynthetase had no effect on the total amount of porphyrin formed.

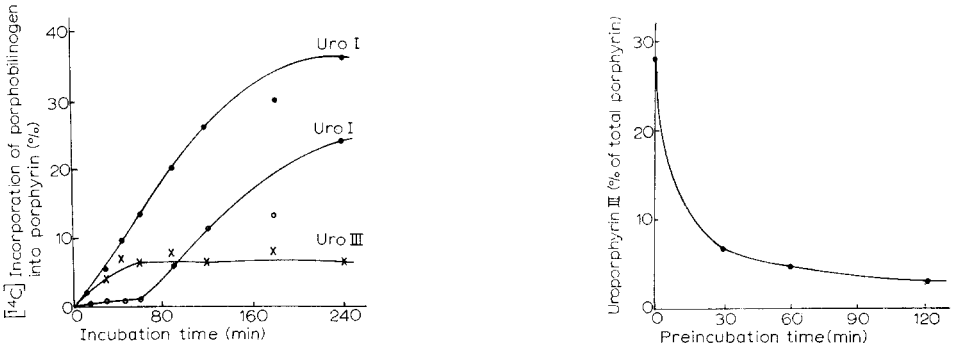


Fig. 2. Rate of uroporphyrin formation. Incubation mixtures: ●—●, 0.5 mg porphobilinogen deaminase, $3 \cdot 10^{-2}$ μmole [^{14}C]porphobilinogen, 40 μmoles Tris–HCl buffer (pH 8.2), final volume 200 μl; ×—× and ○—○, as ●—●, *plus* 0.7 mg uroporphyrinogen III cosynthetase. Isomers assayed as described in text. The % of uroporphyrin III in the mixture ranged from 20% at 240 min to 69% at 45 min.

Fig. 3. Influence of cosynthetase preincubation on uroporphyrin III formation. The enzyme was preincubated at 37° and assayed as usual.

Rate of uroporphyrinogen III formation

As can be seen in Fig. 2, the formation of isomer III increased linearly with time until 45 to 60 min and then ceased, while isomer I formation proceeded linearly for a much longer period of time. This could be due to instability of the cosynthetase, which became inactivated when preincubated at 37° for more than 45 to 60 min in the absence of porphobilinogen or porphobilinogen deaminase (Fig. 3). Although the

TABLE IV

EFFECT OF TEMPERATURE ON UROPORPHYRINOGEN III COSYNTHETASE

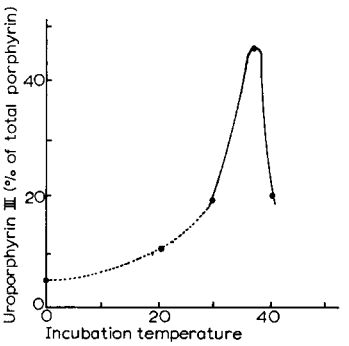
Enzyme preincubated for 15 min in the absence of deaminase and porphobilinogen. Incubation conditions as indicated for routine assay.

Preincubation temperature (°C)	*Uroporphyrin III*	
	counts/min	*% of total porphyrin*
19	2028	47.0
30	1711	43.5
40	2089	48.8
50	153	3.5
60	0	0
70	41	1.2

errors in uroporphyrinogen III estimation are rather large in this last determination (see Table II), the effect can easily be followed.

Effect of temperature

As already mentioned above, uroporphyrinogen III cosynthetase is very sensitive to heat and loses almost all its activity when preincubated for 15 min at 50° in the absence of porphobilinogen or porphobilinogen deaminase (Table IV). The enzyme shows maximum activity at 37° (Fig. 4).



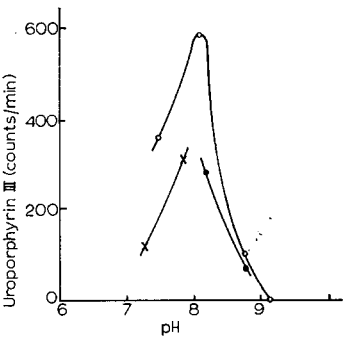


Fig. 4. Effect of temperature on uroporphyrin III formation. Incubation mixture as indicated for routine assay. Incubation period, 45 min at each temperature. ———, error less than 10%; ....., error exceeds 10% (see Table II).

Fig. 5. Effect of pH on uroporphyrin III formation. Incubation mixture as indicated for routine assay. The enzymes were previously dialyzed for 2 h against two changes of distilled water. Incubation period, 45 min. Buffers: ×, phosphate; ○, Tris–HCl; and ●, Tris–maleate.

pH maximum

The enzyme has a sharp maximum at pH 8.2, with activity decreasing rapidly at higher or lower pH values (Fig. 5).

Inhibitors

A number of salts were found to inhibit both porphobilinogen deaminase and uroporphyrinogen III cosynthetase, although the latter was much more sensitive to inhibitors at concentrations higher than $5 \cdot 10^{-2}$ M (Table V). The degree of inhibition appeared to be unrelated to ionic strength. These salts had no effect on cosynthetase activity when the enzyme was associated with porphobilinogen deaminase as in $(NH_4)_2SO_4$ Fraction 50.

Several nucleotides tested were found to have no effect on formation of either of the two isomers: AMP, ATP, UMP, UTP ($5 \cdot 10^{-2}$ M) and UDP ($4 \cdot 10^{-2}$ M). NaF ($5 \cdot 10^{-2}$–$2 \cdot 10^{-1}$ M), δ-aminolevulinic acid ($2 \cdot 10^{-3}$ M) and iodine ($3.2 \cdot 10^{-5}$ M) also had no effect.

Wheat germ cosynthetase was also tested together with porphobilinogen deaminase isolated from human erythrocytes[5]. The incubation period had to be extended to 2 h because of the low rate of porphobilinogen consumption of the blood deaminase, and consequently the cosynthetase was partially inactivated. Although uroporphyrin III formation was not optimal, enough was revealed by chromato-

TABLE V

INHIBITION OF UROPORPHYRINOGEN III COSYNTHETASE BY DIFFERENT SALTS

Assay performed as usual. Incubation was carried out at 37° during 45 min. (a) Measured by total elution of both octamethyl esters from the paper as described, pooling and counting. Error less than 3%. (b) Estimated as usual. For error of determination, see Table II.

Addition	*Concentration (M)*	*Total porphyrins formed (a)*		*Uroporphyrin III formed (b)*	
		%	*% Inhibition*	*% of total porphyrins*	*% Inhibition*
—	—	100	0	65	0
NaCl	0.05	90	10	52	20
	0.10	82	18	53	18
	0.20	71	29	18	72
KCl	0.05	91	9	62	4
	0.10	84	16	50	23
	0.20	68	32	30	54
$MgCl_2$	0.05	68	32	50	23
	0.10	45	55	28	57
	0.20	30	70	21	68
NH_4Cl	0.05	98	2	56	14
	0.10	96	4	22	66
	0.20	62	38	8	88
Na_2SO_4	0.05	95	5	60	8
	0.10	96	4	40	38
	0.20	82	18	30	54

graphic analysis to prove that the enzymes from such different sources can work together to form uroporphyrin III from porphobilinogen.

DISCUSSION

The procedure described for preparation of uroporphyrinogen III cosynthetase is reliable, although allowance must be made for variations in the adsorption pattern from one batch of alumina C_γ gel to another. As NaCl partly inhibits the formation of uroporphyrin III, the enzyme-containing eluates must be desalted prior to concentration. However, the extremely low protein concentration makes the enzyme unstable to desalting by dialysis or Sephadex G-25. The addition of serum albumin provides satisfactory stabilization, and the desalted, concentrated preparation can be preserved at −20° for at least a week without loss of activity.

It was not possible to determine the K_m of uroporphyrinogen III cosynthetase since the substrate for this enzyme is still unknown. The determination of an apparent K_m in the presence of the deaminase (*cf.* ref. 3) was not judged feasible, due to the rather large error inherent in the chromatographic assay method.

The isolated uroporphyrinogen III cosynthetase was inactivated at a lower temperature (40–50°) than the one reported for the whole deaminase–cosynthetase system (55°) (ref. 3). It was also observed that salts which had a marked inhibitory

effect on the isolated enzyme had no effect whatever on the whole system. This suggests that in the crude extracts the cosynthetase is associated with porphobilinogen deaminase in a way that affords it better protection.

Further studies on the purification and properties of the cosynthetase from plant and animal sources will be reported.

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