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Transformation of Porphobilinogen into Porphyrins by Preparations from Human Erythrocytes

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The conversion of porphobilinogen into porphyrin has been studied in preparations from various sources, including avian erythrocytes, rabbit reticulocytes, *Rhodopseudomonas spheroides*, *Chlorella* and higher plants. The literature has been reviewed by Rimington (1957, 1958, 1959) and Margoliash (1961). The primary tetrapyrrole formed is uroporphyrinogen. It is as porphyrinogens, rather than as their oxidized derivatives, porphyrins, that uroporphyrinogen and coproporphyrinogen (and presumably those intermediates with seven, six, five and three carboxyl groups/molecule respectively) can be used as substrates for subsequent steps in the biosynthesis of protoporphyrin and haem. The porphyrinogens can be readily oxidized to porphyrins and it is in the porphyrin form that they are detected and estimated.

The synthesis of uroporphyrin III, pseudouroporphyrin [now recognized as heptacarboxylic porphyrin belonging to the III series (Falk, 1955; Lockwood & Davies, 1961; Cornford & Benson, 1963)] and coproporphyrin III, with only traces of protoporphyrin, from δ-aminolaevulic acid by human haemolysates incubated in air was reported by Rimington & Booij (1957). Fallot, Canivet, Mondet & Poidatz (1956) reported that when this system was incubated with δ-aminolaevulic acid in nitrogen uroporphyrin I was formed.

In the present study both crude and partially purified preparations derived from human erythrocytes gave rise to a mixture of porphyrin isomers I and III from porphobilinogen. The amounts and percentages of these isomers were functions of certain incubation conditions. Because of these properties, the preparations seemed ideal material for a study of the enzymic control of uroporphyrinogen III synthesis from porphobilinogen.

MATERIALS

AnalaR (British Drug Houses Ltd.) HCl was found to be 11·6 N (42·3%, w/v). All dilutions were prepared on this basis and are recorded as weight of acid/100 ml. The heparin solution manufactured under the trade name Liquemin by Roche Products Ltd. was used. δ-Aminolaevulic acid was a generous gift from Dr J. Falk, Division of Plant Industry, C.S.I.R.O., Canberra, Australia. Porphobilinogen, kindly supplied by Professor C. Rimington, was derived from human acute-porphyria urines and purified by the method of Cookson & Rimington (1954). Solutions were prepared either in 0·1 M-phosphate buffer, pH 7·4, or in water to which sufficient HCl was added to effect solution of the porphobilinogen. Uroporphyrinogens I and III were prepared from the free porphyrins of the octamethyl esters described by Cornford & Benson (1963), by the procedure of Hoare & Heath (1959). The concentration of uroporphyrinogen was determined as follows: a 0·1 ml. sample was taken, 2 drops of ethanolic 0·2% iodine were added to oxidize it to porphyrin, followed by 2 drops of aq. 0·4% sodium thiosulphate ($Na_2S_2O_3,5H_2O$), and the solution was made up to 20 ml. with 5% (w/v) HCl. The concentration of uroporphyrinogen was calculated by eqn. (A) below.

METHODS

Enzyme preparations

Blood. Except where chicken blood was used for comparison, all preparations were derived from normal adult human blood. The blood (90 ml.) was collected immediately before commencement of enzyme preparations. It was taken, by intravenous puncture, into 4 ml. of 0·9% NaCl

containing 1820 i.u. of heparin. All manipulations after collection of the blood and before incubation were carried out at 4°.

Intact washed erythrocytes. The blood was centrifuged, plasma and leucocytes were removed, and the cells were washed twice with 0·9% NaCl. For studies with intact cells, the packed cells were made up to their original whole blood volume with 0·9% NaCl and mixed well.

Haemolysates. The packed cells from 90 ml. of whole blood were mixed thoroughly with 75 ml. of water, previously chilled to 0°, and stirred for 5 min. in an ice bath. Iso-osmoticity was then restored by adding 19 ml. of 4·5% (w/v) KCl.

Partially purified preparations. The human haemolysate was dialysed against 10 l. of 3 mM-sodium phosphate buffer, pH 7·4, overnight. The dialysed haemolysate was placed on a column (2·2 cm. × 12 cm.) of diethylaminoethylcellulose (DEAE-cellulose) previously equilibrated with the same buffer. The column contained 6 g. of DEAE-cellulose which had been packed by suction. The column was washed thoroughly with the same buffer and then the porphyrin-synthesizing system eluted with 0·1 M-sodium phosphate buffer, pH 7·4. A flow rate of 10 ml./5 min. was maintained, with the aid of negative pressure, during washing and elution, and the entire run was completed within 3 hr. to minimize enzyme inactivation. All attempts to gain a higher degree of purification by eluting with a rising ionic gradient, instead of batchwise, were unsuccessful. In such attempts severe losses ensued as a result of tailing, even when very small columns were employed.

In some instances the partially purified preparations were concentrated by pressure filtration through Viscose tubing as demonstrated by Grant, Rowe & Stanworth (1958) and described by Wildy, Nizet & Benson (1961).

Preparations from acetone-dried powders. Acetone-dried powders were prepared by adding 12 vol. of acetone, previously chilled to −10°, to the haemolysate and filtering by suction. After washing three times with chilled acetone and three times with chilled ether, the powder was stored *in vacuo* at −10° over NaOH pellets and wax shavings. A 100 ml. sample of human haemolysate yielded an average of 8·39 g. of powder. Extracts were prepared by grinding the acetone-dried powder with a volume of water equal to half the volume of fluid removed during preparation of the powder. After standing for 1 hr. with occasional grinding, the slurry was centrifuged and the supernatant collected. The residue was extracted again with the same volume of water and, after centrifugation, the extracts were combined and their volume adjusted to that of the original haemolysate.

Incubation of enzyme preparations

The crude and the partially purified preparations were assayed for porphyrin-synthesizing activity by incubation with porphobilinogen or δ-aminolaevulic acid. Intact erythrocytes and haemolysates were incubated within 4 hr. of blood collection, and partially purified preparations within 27 hr., or 50 hr. if they had been concentrated. Extracts of acetone-dried powders were incubated within 4 hr. of commencement of extraction. Except when Thunberg tubes were employed, incubations were carried out in 100 ml. conical flasks plugged loosely with cotton wool. Incubation mixtures varied and are described below. Except where otherwise indicated, incubations were at pH 7·4 for 4 hr. at 38° in air in the dark. Where oxygen or nitrogen atmospheres were employed, incubation was in Thunberg tubes filled with commercial nitrogen passed through a solution of pyrogallol in 2 N-NaOH or with commercial oxygen; the tubes were evacuated and filled with gas three times before sealing. During incubation the flasks were agitated back and forth over 4·5 cm. in a horizontal plane at 120 cycles/min. For studies with intact cells the speed was decreased considerably to prevent damage to the cells. After incubation, intact cells were haemolysed before the addition of iodine, as described by Dresel & Falk (1956*a*).

The reaction was terminated and porphyrinogens were oxidized to porphyrin by the addition of iodine (1 ml. of ethanolic 0·2% solution/33 ml. of incubation mixture) followed by a solution of sodium thiosulphate (1 ml. of 0·4% $Na_2S_2O_3,5H_2O$/33 ml. of incubation mixture) to reduce the excess of iodine. Finally, protein was precipitated by the addition of 12 vol. of ethyl acetate–acetic acid (3:1, v/v) and the mixture set aside in the dark overnight.

Determination of porphyrins

Uroporphyrin, coproporphyrin and protoporphyrin were fractionated by the methods of Dresel & Falk (1956*a*). Their cyclohexanone procedure was employed and extraction of uroporphyrin into 5% (w/v) HCl facilitated by the addition of 2 vol. of ether to the cyclohexanone. Since preparations from human erythrocytes form very little protoporphyrin, coproporphyrin was usually determined in the presence of traces of protoporphyrin by extracting it from the ether into 5% (w/v) HCl. When separate determinations were made the coproporphyrin and protoporphyrin were extracted into 0·36% and 10% (w/v) HCl respectively as described by Dresel & Falk (1956*a*). The amounts of porphyrin were calculated from the following expressions, derived by the method of Rimington & Sveinsson (1950), by using values for ϵ and k quoted by Rimington (1960):

$$\mu\text{m-moles of uroporphyrin in 5\% HCl} = [2E_{405-6} - (E_{380} + E_{430})] \times 1{\cdot}008 \times v \quad \text{(A)}$$

$$\mu\text{m-moles of coproporphyrin in 5\% HCl} = [2E_{401-2} - (E_{380} + E_{430})] \times 1{\cdot}287 \times v \quad \text{(B)}$$

$$\mu\text{m-moles of coproporphyrin in 0·36\% HCl} = [2E_{401-2} - (E_{380} + E_{430})] \times 1{\cdot}121 \times v \quad \text{(C)}$$

$$\mu\text{m-moles of protoporphyrin in 10\% HCl} = [2E_{408-9} - (E_{380} + E_{430})] \times 2{\cdot}182 \times v \quad \text{(D)}$$

where v is the volume of solution, and E is the extinction at the wavelength indicated by the subscript.

The results are expressed as the amount of porphyrin produced/21 ml. of haemolysate, since this gave a direct comparison of the activities of various preparations. Values in terms of unit weight of nitrogen are also reported where applicable.

Identification of porphyrin isomers

The coproporphyrin fractions were returned to ether, after adjustment of the pH to 4 with saturated sodium acetate, and the ether was washed three times with small volumes of water. The combined washings were re-extracted completely with fresh ether and the combined ether fractions were finally washed with a very small volume of water. The ether was allowed to evaporate spontaneously overnight at room temperature in the dark. The dry samples were

dissolved in 2N-NH_3 and the isomeric composition of each was determined by lutidine chromatography (see below).

The uroporphyrin fractions were evaporated to dryness by vacuum distillation at 40–50°. The octamethyl ester of the uroporphyrin fractions was prepared and taken into washed chloroform as described by Falk, Dresel, Benson & Knight (1956). The amount of ester was calculated by eqn. (1) of Cornford & Benson (1963). Portions of known porphyrin content were taken for isomer analysis by decarboxylation–lutidine chromatography and by dioxan chromatography.

Chemical decarboxylation of uroporphyrin fractions. Samples (5–20 μg.) of the uroporphyrin esters were decarboxylated by the method of Edmondson & Schwartz (1953), as described by Cornford & Benson (1963). The free coproporphyrin thus produced was prepared as described above for lutidine chromatography.

Lutidine chromatography. The isomeric composition of the enzymically produced coproporphyrin fraction and also the coproporphyrin formed by chemical decarboxylation of the enzymically produced uroporphyrin fraction was determined by lutidine chromatography. The method was essentially that described by Eriksen (1958). On each chromatogram a range of standard mixtures (0·5 μg.) of coproporphyrins I and III was included. The isomer ratio of each sample was assumed to be the same as that of the standard mixture that had a similar chromatographic pattern.

From the ratio of coproporphyrin I to coproporphyrin III in the two fractions the amounts of the I and III isomers contained in the uroporphyrin and coproporphyrin fractions were calculated as μm-moles. The ratios and quantities of isomers found by this method are recorded in the Results section.

Dioxan chromatography. This method of determining the isomeric composition of uroporphyrin fractions was employed for comparison with the decarboxylation–lutidine chromatography method. It is useful for identifying pseudo-uroporphyrin and also porphyrins with six and five carboxyl groups/molecule, which, because of their decarboxylation together with uroporphyrin to coproporphyrin in the decarboxylation–lutidine chromatography method, are not detected by that method.

The esters derived from the uroporphyrin fraction were chromatographed as described by Cornford & Benson (1963); when the pseudo-uroporphyrin content was low (determined by a preliminary chromatogram) then 1 μg. of the sample was applied, but when the pseudo-uroporphyrin was high then 1·5 μg. was applied. For the second run 4 ml. of kerosene plus 1 ml. of dioxan were employed. After chromatographic development, the spots were each eluted with 3 ml. of washed chloroform, the eluates were measured spectrophotometrically and the amounts (μg.) of ester in the uroporphyrin I, uroporphyrin III and pseudo-uroporphyrin positions were determined by eqn. (1) of Cornford & Benson (1963). The ratio of uroporphyrin I to uroporphyrin III was determined and corrected by use of the nomograph (Fig. 4) or eqn. (2) of Cornford & Benson (1963). The uroporphyrin I:uroporphyrin III:pseudo-uroporphyrin proportions were then calculated.

Determination of enzyme activities

At the present state of knowledge, the porphyrin-synthesizing system includes four enzyme activities that are collectively responsible for the conversion of porphobilinogen into protoporphyrin. The systematic and trivial names of the enzymes set out below are suggested as those complying with the recommendations of the Commission on Enzymes of the International Union of Biochemistry (1961). The reactions that these enzymes catalyse and the measurement in the present work of their activities are as follows:

(*a*) *Porphobilinogen ammonia-lyase (joining four units and cyclizing).* This enzyme catalyses the joining of four porphobilinogen molecules, with the liberation of four molecules of ammonia, and the subsequent cyclization of the tetrapyrrole to yield uroporphyrinogen. The trivial name 'porphobilinogen cyclodeaminase' is suggested. Its activity is measured in the present work as μm-moles of total porphyrin formed under the conditions of the experiment. In the present studies of the effects of certain variables on the total porphyrin produced, only differences of more than 20% from the control were considered significant.

(*b*) *Uroporphyrinogen isomerase.* Acting in the presence of porphobilinogen cyclodeaminase, this enzyme catalyses the production of uroporphyrinogen III from porphobilinogen. In its absence, uroporphyrinogen I is formed by porphobilinogen cyclodeaminase. Since the details of its catalytic action are not known, no systematic name can be given to it and it must therefore tentatively remain known only by the above trivial name. Its activity is measured in the present work as μm-moles of series III porphyrin formed under the conditions of the experiment.

(*c*) *Uroporphyrinogen carboxy-lyase.* This enzyme catalyses the removal of four of the eight carboxyl groups on the side chains of the uroporphyrinogen molecule to yield coproporphyrinogen. It is best known as 'uroporphyrinogen decarboxylase' and this name is retained as the trivial form. Its activity is measured in the present work as μm-moles of coproporphyrin (I plus III) plus protoporphyrin formed under the conditions of the experiment.

(*d*) *Coproporphyrinogen-(acceptor) oxidoreductase (decarboxylating).* This enzyme catalyses the removal of two carboxyl groups and two H^+ ions from the side chains of the coproporphyrinogen molecule to yield protoporphyrin. It is well known as 'coproporphyrinogen decarboxylase', and this name is retained as the trivial form. Its activity is measured in the present work as μm-moles of protoporphyrin formed under the conditions of the experiment. The activity in preparations from human erythrocytes was always very low.

The total nitrogen content of enzyme preparations was determined by the micro-Kjeldahl method.

RESULTS

Experiments with the crude porphyrin-synthesizing system of human erythrocytes

Human haemolysates produced a mixture of uroporphyrin and coproporphyrin, with small amounts of protoporphyrin, from porphobilinogen. Dioxan chromatography of the methyl esters of the uroporphyrin fraction revealed a significant proportion of pseudo-uroporphyrin and also traces of porphyrins containing six and five carboxyl groups/molecule.

Table 1. *Porphyrins formed by crude preparations obtained from human and chicken erythrocytes*

The incubation mixture contained: 21 ml. of iso-osmotic haemolysate or extract of acetone-dried powder, 2 mg. of porphobilinogen (final concn. 268 μM), 1 ml. of 0·1 M-$MgCl_2$ and sufficient KCl to maintain iso-osmoticity of the final volume of 33 ml. Incubations were at 38° for 4 hr. in air. U, Uroporphyrin; C, coproporphyrin; P, protoporphyrin; T, total porphyrin.

	Porphyrin formed (μm-moles/21 ml. of haemolysate)								Percentage of total porphyrin formed	
		Series I			Series III					
Enzyme	Total	U	C	T	U	C	P	T	C+P	Series III
Human haemolysate (1)	273	36	—	36	109	113	15	237	47	87
Human haemolysate (2)	648	154	—	154	231	246	17	494	41	76
Extract of human-erythrocyte acetone-dried powder	544	300	5	305	128	103	8	239	21	44
Chicken haemolysate	727	10	—	10	127	138	452	717	81	99
Extract of chicken-erythrocyte acetone-dried powder	299	43	—	43	128	80	48	256	43	86

Table 2. *Comparison of the porphyrin-synthesizing activities of crude and partially-purified human-erythrocyte preparations*

The incubation mixtures contained: appropriate volume of enzyme preparation, 0·5 mg. of porphobilinogen (final concn. 67 μM) and KCl solution or sodium phosphate buffer to give a final volume of 33 ml. Incubations of the crude system were iso-osmotic with respect to KCl whereas those of the partially purified system contained 0·1 M-sodium phosphate buffer, pH 7·4. Incubations were at 38° for 4 hr. in air. C, Coproporphyrin; P, protoporphyrin.

	Porphyrin formed		Percentage of total porphyrin formed	
Enzyme preparation	(μm-moles/21 ml. of original haemolysate)	(μm-moles/mg. of nitrogen)	C+P	Series III
Human haemolysate (3)	117	0·44	61	93
Human haemolysate (4)	254	0·96	46	87
Human haemolysate (5)	166	0·63	61	92
Partially purified system (6)	190	55·2	17	87
Partially purified system (7)	185	54·9	18	92
Partially purified system (concentrated) (8)	172	50·2	25	89

Both isomers I and III were formed. The formation of series I was apparently not due to partial loss during the haemolysing process of the ability to form the III isomer. Essentially the same proportion of uroporphyrin I was formed from δ-aminolaevulic acid by intact cells as by a haemolysate prepared from the same blood sample. The ratio of the two isomers was the same after 1·5 hr. of incubation as after 4 hr.

The porphyrins produced by human haemolysates and acetone-dried powders thereof are compared in Table 1 with those produced by similar preparations from chicken erythrocytes.

Most of the I isomer formed by human haemolysates remained fully carboxylated as uroporphyrin. It appeared therefore that the uroporphyrinogen decarboxylase of human erythrocytes was very much more active towards uroporphyrinogen III than towards uroporphyrinogen I. This was confirmed by showing that in a 27:23 (I:III) mixture uroporphyrinogen III is converted into coproporphyrinogen III at 7·5 times the rate that uroporphyrinogen I is converted into coproporphyrinogen I.

Experiments with the partially-purified porphyrin-synthesizing system of human erythrocytes

Comparison of porphyrins produced before and after purification. Dialysis of human haemolysates followed by their partial purification on a DEAE-cellulose column resulted in little, if any, loss of total porphyrin-synthesizing activity (Table 2). The partially purified preparations had not lost their ability to produce the III isomer. On the other hand, a good deal of the coproporphyrin-forming activity was lost by the treatment. This was removed on the column and not during dialysis, for tests made on the crude dialysed haemolysate revealed no loss. The chromatography resulted in an approximately 80-fold purification, in terms of total nitrogen, of the series III-forming system.

Effects of substrate concentration. Intact washed erythrocytes, haemolysates, extracts of acetone-dried powders and partially purified preparations produced a mixture of series I and III porphyrins at all substrate concentrations. The ratio of the two isomers was a function of the concentration of δ-aminolaevulic acid (with intact cells) or porpho-

bilinogen (with other preparations). The amount of series I, as a percentage of the total porphyrin, formed by the partially purified system is plotted against porphobilinogen concentration in Fig. 1, together with similar plots for the crude haemolysate and an extract of an acetone-dried powder thereof. All curves flattened out as the porphobilinogen concentration approached saturation. The concentration of porphobilinogen (67 μM; 0·5 mg./33 ml.) chosen for all further experiments with the partially purified preparation gave a moderately high yield of series III porphyrin while keeping the series I formation low.

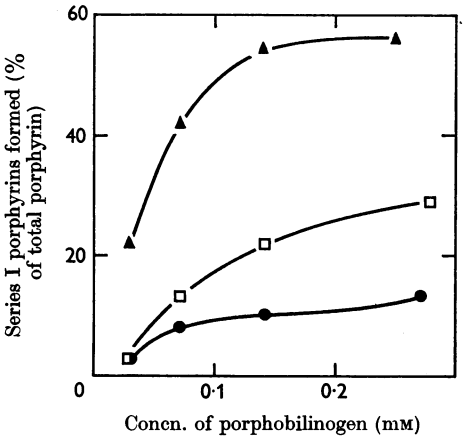


Fig. 1. Effect of porphobilinogen concentration on the amount of series I, as a percentage of the total porphyrin, formed by human-erythrocyte preparations. Incubation mixtures contained: appropriate volume of enzyme preparation, and KCl or sodium phosphate buffer to give a final volume of 33 ml. Incubations of haemolysates and extracts of acetone-dried powders were iso-osmotic with respect to KCl, whereas those of the partially purified system contained 0·1 M-sodium phosphate buffer, pH 7·4. Incubations were carried out at 38° for 4 hr. in air. ●, Haemolysate; ▲, acetone-dried-powder extract; □, partially purified preparation.

Effect of sodium salt concentration. The addition of either sodium phosphate buffer or sodium chloride to the incubation mixture, though having little effect on total porphyrin, markedly diminished the total coproporphyrin and the uroporphyrin I that was formed (Table 3). The net result was an increase in the percentage of the III isomer.

When the uroporphyrin fractions were examined by dioxan chromatography of their methyl esters, so affording a determination of pseudo-uroporphyrin, it was found that the sum of the pseudo-uroporphyrin and coproporphyrin was not significantly affected by the presence of sodium salts, the deficit in coproporphyrin being made up by an increase in pseudo-uroporphyrin. As the amounts of porphyrins with six and five carboxyl groups/molecule remained low in all cases, the decrease in coproporphyrin formation due to sodium salts appeared to result from an inhibition of further decarboxylation of pseudo-uroporphyrinogen, i.e. the step equivalent to the removal of a second carboxyl group from the uroporphyrinogen molecule.

No direct inhibitory effect of sodium salts on the conversion of porphobilinogen into uroporphyrinogen I could be observed in an experiment with a preheated enzyme preparation that forms series I porphyrin exclusively.

The action of sodium salts observed under the experimental conditions employed in the present work is summarized in Scheme 1.

The effect of sodium chloride on incubations performed with dialysed extracts of acetone-dried powders was very marked. They normally yield a relatively low percentage of the III isomer. In one

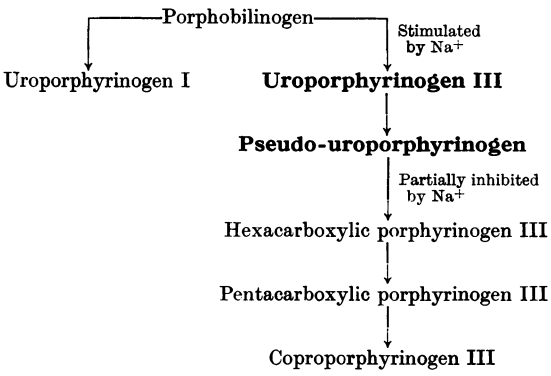
Table 3. *Effect of sodium salts on the activity of the partially-purified porphyrin-synthesizing system*

Incubation mixtures contained: 6 ml. of concentrated enzyme preparation (equivalent to 33 ml. of haemolysate) in 0·1 M-sodium phosphate buffer, pH 7·4, and 0·5 mg. of porphobilinogen (final concn. 67 μM). Sodium phosphate buffer, pH 7·4, tris buffer, pH 7·4, and sodium chloride were added to give the final concentrations indicated. Volumes were adjusted to 33 ml. with water. Incubations were at 38° for 4 hr. in air. U, Uroporphyrin; C, coproporphyrin; P, protoporphyrin; T, total porphyrin.

Concn. of Na^+ ion (mM)			Concn. of tris buffer (mM)	Porphyrin formed (μm-moles/21 ml. of haemolysate)						Percentage of total porphyrin formed	
					Series I			Series III				
As phosphate	As chloride	Total		Total	U	C	T	U	C+P	T	C+P	Series III
					Expt. 1							
33	—	33	25	187	71	2	73	36	78	114	43	61
78	—	78	—	195	52	—	52	79	64	143	33	73
181	—	181	—	172	19	—	19	110	43	153	25	89
					Expt. 2							
34	—	34	25	205	76	5	81	33	91	124	47	60
186	—	186	25	173	18	—	18	103	52	155	30	90
34	148	182	25	192	30	—	30	120	42	162	22	84
189	—	189	—	206	39	—	39	119	48	167	23	81

case an increase from 28% to 80% of the III isomer was effected by increasing the Na^+ ion concentration from 170 mM to 370 mM. This experiment illustrates the ability of sodium salts to partially reverse the inhibition of uroporphyrinogen isomerase caused by the acetone-dried powder preparation.

Effect of pH. The broad optimum between pH 7·2 and pH 8·1 for total porphyrin formation in the absence of sodium chloride (Fig. 2*a*) was sharpened to approx. pH 7·8 by the addition of an approximately iso-osmotic concentration of sodium chloride (Fig. 2*b*).



Scheme 1. Conversion of porphobilinogen into porphyrinogens. In the presence of sodium salts uroporphyrinogen III and pseudo-uroporphyrinogen are increased, and uroporphyrinogen I, hexacarboxylic porphyrinogen III, pentacarboxylic porphyrinogen III and coproporphyrinogen III are all decreased.

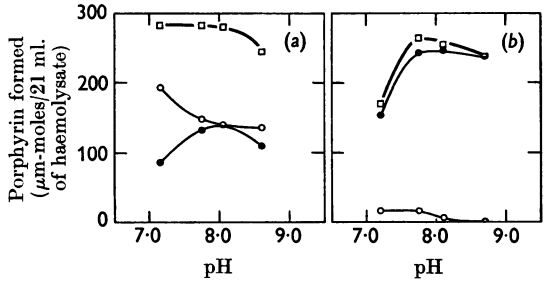


Fig. 2. Effect of pH on the activity of the partially-purified porphyrin-synthesizing system: (*a*) in the absence of NaCl; (*b*) in the presence of 148 mM-NaCl. Incubation mixtures contained: 0·5 ml. of concentrated enzyme preparation (equivalent to 6·7 ml. of haemolysate) in 0·1 M-sodium phosphate buffer, pH 7·4 (different preparations for *a* and *b*), 2·5 ml. of 0·1 M-tris buffer of the required pH, 0·125 mg. of porphobilinogen (final concn. 67 μM), 0·03 ml. of octanol and, for (*b*), 2·5 ml. of 2·86% (w/v) NaCl (final concn. 148 mM). Distilled water was added to give a final volume of 8·25 ml. Incubations were carried out under nitrogen in Thunberg tubes at 38° for 4 hr. ○, Series I porphyrin; ●, series III porphyrin; □, total porphyrin.

The effect of sodium salts on the ratio of series III to series I porphyrin was observable at all pH values studied. The pH optimum for series III formation was 8·1 both in the presence and absence of sodium chloride. The amount of series I was in both cases decreased by a rise in pH, but its formation was only completely suppressed by incubation at pH 8·7 in the presence of 148 mM-sodium chloride. The pH optimum for coproporphyrin formation was 7·2 or less.

Inhibition and inactivation of uroporphyrinogen-isomerase activity. Preheating the preparation at 63° for 30 min. destroyed uroporphyrinogen-isomerase activity and resulted in a small increase in total porphyrin formation. Inhibition of the isomerase activity with hydroxylamine caused a more significant stimulation of total porphyrin formation (Table 4). Small decreases in total porphyrin and isomer III occurred in the presence of sodium cyanide; the percentage of III was unaltered. An atmosphere of oxygen decreased the III isomer and, to a lesser extent, the total porphyrin; only insignificant changes occurred when incubations were carried out in nitrogen rather than in air. Uroporphyrinogen-decarboxylase activity was markedly stimulated by lowering the oxygen concentration (Table 5).

Identification of uroporphyrin isomers and determination of pseudo-uroporphyrin by dioxan chromatography

Although the two methods did not always agree completely in their assessment of isomer proportions, the findings obtained by the dioxan method, under carefully controlled conditions, led in all experiments to the same overall conclusions as those obtained by decarboxylation–lutidine chromatography which are set out above.

Table 4. *Inhibition and inactivation of uroporphyrinogen-isomerase activity of partially purified preparations*

Incubation mixtures contained: appropriate volume of enzyme preparation, 30 mM-tris buffer, pH 7·4, plus 148 mM-NaCl (or 0·1 M-sodium phosphate buffer, pH 7·4) and 67 μM-porphobilinogen. Incubations were carried out in air, except where indicated, at 38° for 4 hr.

Factor	Porphyrin formed (% of control without factor)		Change in series III porphyrins (percentage of total porphyrin)
	Series III	Total	
Preheating at 63° for 30 min.	4	118	−79
Hydroxylamine (20 mM)	15	185	−75
Cyanide (1·0 mM)	82	83	− 1
Oxygen, in lieu of air	77	87	−11

Table 5. *Effects of oxygen and nitrogen on the activity of the partially-purified porphyrin-synthesizing system*

Incubation mixtures contained: 8 ml. of enzyme preparation (equivalent to 8·7 ml. of haemolysate) in 0·1 M-sodium phosphate buffer, pH 7·4, 0·125 mg. of porphobilinogen (final concn. 67 μM) and 0·03 ml. of octanol. The final volume was 8·28 ml. Each incubation was carried out in duplicate in Thunberg tubes and duplicates were combined after incubation. Incubations were at 38° for 4 hr. U, Uroporphyrin; C, coproporphyrin; P, protoporphyrin; T, total porphyrin.

	Porphyrin formed (μm-moles/21 ml. of haemolysate)							Percentage of total porphyrin formed	
		Series I			Series III				
Gas phase	Total	U	C	T	U	C+P	T	C+P	Series III
Oxygen	161	30	—	30	118	13	131	8	81
Air	185	15	—	15	136	34	170	18	92
Nitrogen	184	10	—	10	85	89	174	48	95

The pseudo-uroporphyrin represented between 0 and 30% of the total porphyrin formed, depending on the experimental conditions and the enzyme preparation. It increased at the expense of coproporphyrin with increased sodium salt concentration, but in experiments with other variables it rose or fell parallel with the coproporphyrin. The highest percentage was found by incubating the partially purified system at low substrate concentrations and relatively high sodium salt concentration.

DISCUSSION

The porphyrin-synthesizing activity of preparations derived from human erythrocytes varied with the blood donor and was significantly elevated in preparations obtained from a donor who had for many years been giving blood for Blood Bank purposes [haemolysate (2) in Table 1]. Control experiments revealed that the porphyrin formed spontaneously from porphobilinogen amounted to less than 2% of that produced enzymically and therefore made a negligible contribution to the total porphyrin yield. In general, the porphobilinogen-cyclodeaminase activities are not maximal for this enzyme, for the substrate concentrations employed were maximal for series III formation and fell considerably short of the saturation point of the porphobilinogen cyclodeaminase.

Uroporphyrinogen-decarboxylase activity. An ultrafiltrable factor was required by the *Rhodopseudomonas spheroides* enzyme (Hoare & Heath, 1958) and may be required by the chicken-erythrocyte enzyme (Urata & Kimura, 1960). The present studies indicated no such requirement by the human-erythrocyte uroporphyrinogen decarboxylase.

Increase in sodium salt concentration partially inhibited further decarboxylation of pseudouroporphyrinogen. This might suggest that two (or more) enzymes are required for the conversion of uroporphyrinogen into coproporphyrinogen, but no direct evidence on this point was obtained in the present investigation.

The porphyrins presumed, from their mobility on dioxan chromatograms (Cornford & Benson, 1963), to contain five and six carboxyl groups/molecule respectively were never found in more than trace amounts. It seems, therefore, that further decarboxylation of these porphyrins is very rapid compared with the rate of removal of the first two carboxyl groups of the uroporphyrinogen molecule.

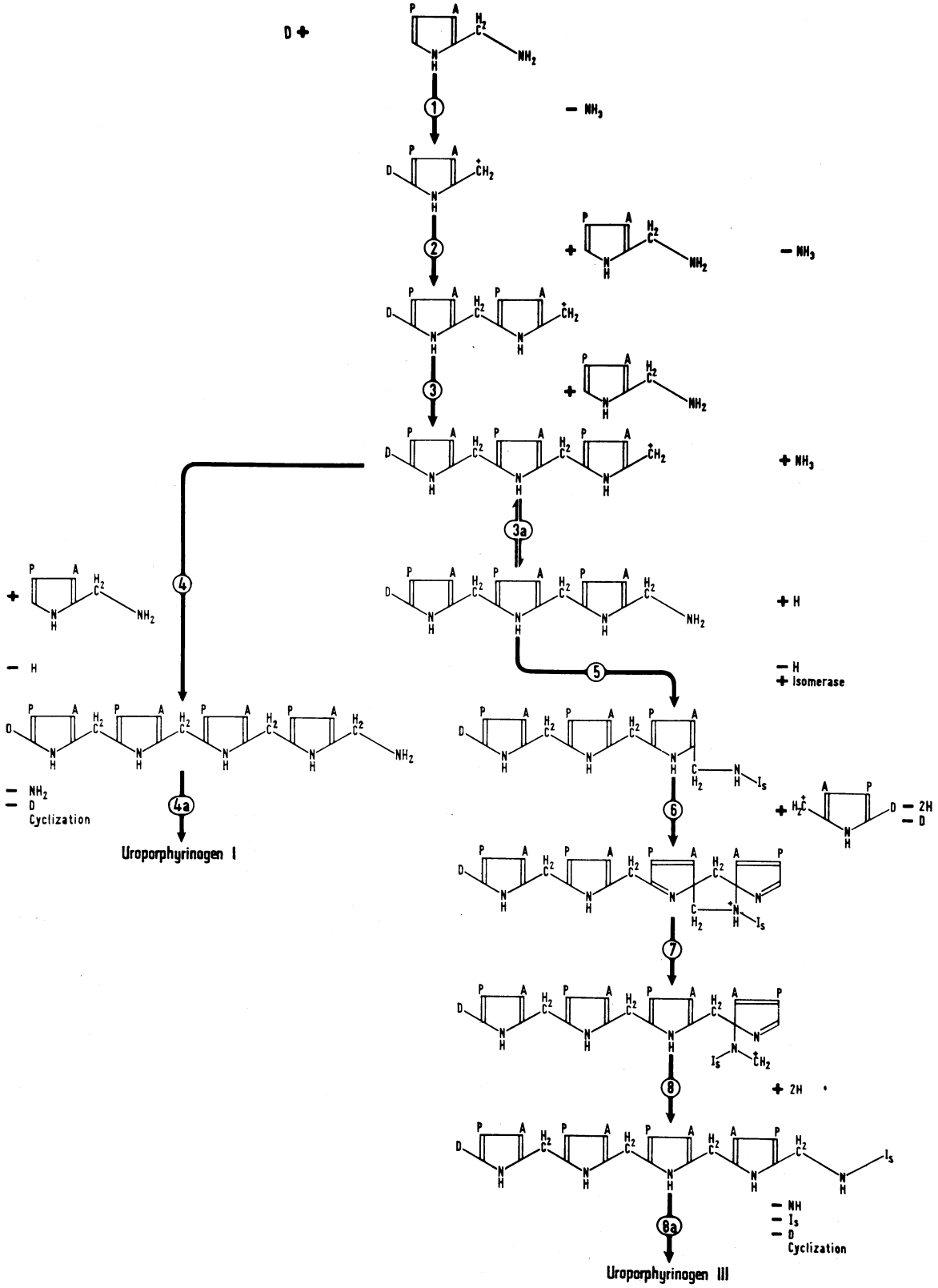
Possible mechanism for the enzymic formation of uroporphyrinogen from porphobilinogen (*Scheme* 2). It is suggested that porphobilinogen cyclodeaminase readily catalyses the formation of a linear tripyrrole from porphobilinogen by a series of reactions, possibly similar to reactions (1), (2) and (3), and that it adds a fourth porphobilinogen unit less readily. A competition between uroporphyrinogen isomerase and porphobilinogen is proposed to occur at this point (reactions 4 and 5). Uroporphyrinogen III formation could then perhaps result from the ability of uroporphyrinogen isomerase to catalyse reaction between the substituted α-positions of the tripyrrole and a porphobilinogen unit (reaction 6) (cf. Cookson & Rimington, 1954) and subsequent migration of a side chain (reactions 7 and 8) (cf. Robinson, 1955; Bullock, Johnson, Markham & Shaw, 1958).

The scheme is speculative in many respects, but takes into account the following experimental data on the enzymic reaction:

(*a*) Free formaldehyde is not a stoicheiometric by-product (Bogorad & Marks, 1960), nor is it utilized (Lockwood & Benson, 1960; Bogorad & Marks, 1960).

(*b*) 100% yield has been demonstrated (Dresel & Falk, 1956*a*, *b*; Granick & Mauzerall, 1958; Bogorad, 1958*a*, *b*; Lockwood & Benson, 1960), indicating that no porphobilinogen units are expended.

(*c*) Opsopyrroledicarboxylic acid is a com-



Scheme 2. Biosynthesis of uroporphyrinogens I and III from porphobilinogen. See the text for explanation. A, Acetic acid side chain; P, propionic acid side chain. D, Porphobilinogen cyclodeaminase; I_S, uroporphyrinogen isomerase.

petitive inhibitor (Bogorad, 1957; Carpenter & Scott, 1959). It therefore does not act as an intermediate but forms a complex with porphobilinogen cyclodeaminase, indicating that the formation of a complex of porphobilinogen with this enzyme is through its free α-position (cf. Rimington, 1962). Further, dipyrrylmethanes with all their α-positions free are also inhibitory, though not competitively (Hoare & Heath, 1960), whereas carboxyporphobilinogen, which contains no free α-position, is without effect (Hoare & Heath, 1959).

(*d*) Uroporphyrinogen isomerase is inhibited by hydroxylamine (Bogorad, 1958*b*; and the present paper). It is feasible therefore that this enzyme forms a complex with the aminomethyl side chain of porphobilinogen or a polypyrrole.

(*e*) Bogorad's (1958*b*) experiments led him to propose that uroporphyrinogen isomerase catalyses the synthesis of uroporphyrinogen III from porphobilinogen and some product of porphobilinogen cyclodeaminase. The substrate concentration–activity curves reported above are consistent with this concept. If uroporphyrinogen isomerase functioned after the formation of the tetrapyrrole by porphobilinogen cyclodeaminase, it would be expected that, in that portion of the curves where the uroporphyrinogen isomerase was unsaturated, all the uroporphyrinogen synthesized would belong to the III series. A proportion of uroporphyrinogen I is, however, produced at all substrate concentrations. Increase in the percentage of series I with increased porphobilinogen concentration suggests that a competition, such as that represented by reactions (4) and (5) in Scheme 2, may exist between uroporphyrinogen isomerase and porphobilinogen for a product of porphobilinogen cyclodeaminase. In a system where uroporphyrinogen isomerase was limiting, as appears to be the case in the preparations examined in the present work, increased substrate concentration would favour reaction (4) and a greater proportion of uroporphyrinogen I would be formed; if, on the other hand, the uroporphyrinogen isomerase was equivalent in its potential activity to porphobilinogen cyclodeaminase or if the porphobilinogen concentration was low, reaction (5) would be favoured and a greater proportion of uroporphyrinogen III would be formed. Uroporphyrinogen I formation *in vivo* would be suppressed, even if the uroporphyrinogen isomerase was potentially less active than porphobilinogen cyclodeaminase, by the low concentration of porphobilinogen which is normally available to the system.

(*f*) When uroporphyrinogen isomerase is inactivated by preheating (Booij & Rimington, 1957; Bogorad, 1958*b*; and the present paper) or by hydroxylamine (the present paper) porphyrin synthesis is increased. This fact also suggests that the isomerase functions before the completion of tetrapyrrole formation by porphobilinogen cyclodeaminase.

SUMMARY

1. The preparation employed for detailed studies of uroporphyrin formation from porphobilinogen was derived from haemolysates of normal human blood and had been purified approximately 80-fold by chromatography on diethylaminoethylcellulose without loss of uroporphyrinogen-isomerase or porphobilinogen-cyclodeaminase activities.

2. Human erythrocytes and partially purified preparations thereof produce a mixture of the I and III porphyrin isomers from δ-aminolaevulic acid or porphobilinogen. The amounts and ratios of these isomers were influenced by changes in pH, salt composition, substrate concentration and oxygen concentration, as well as the integrity of the enzyme system itself. The relationship between isomer composition and porphobilinogen concentration is compatible with the proposal that at some stage in uroporphyrinogen formation a competition exists between uroporphyrinogen isomerase and porphobilinogen. The proposal is the basis of a hypothetical scheme suggested for the enzymic catalysis of uroporphyrinogen III formation. The uroporphyrinogen isomerase is heat-labile and is inhibited by hydroxylamine, but not by cyanide.

3. The uroporphyrinogen decarboxylase of human erythrocytes decarboxylates uroporphyrinogen III to coproporphyrinogen III at 7·5 times the rate it decarboxylates uroporphyrinogen I to coproporphyrinogen I, when the two uroporphyrinogen isomers are present in equal concentrations. The decarboxylation of uroporphyrinogen III is partially inhibited by sodium salts, removal of the second carboxyl group being the stage that is apparently affected, i.e. the further decarboxylation of pseudo-uroporphyrinogen.

4. The uroporphyrinogen-decarboxylase activity was not dependent on a diffusible cofactor.

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The Metabolism of Tryptophan

3. THE METABOLISM OF 2-HYDROXYLAMINOBENZOIC ACID IN RELATION TO TRYPTOPHAN METABOLISM*

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Cramer, Miller & Miller (1960) and Nelson & Troll (1961) found that 2-acetamidofluorene and benzidine are metabolized by *N*-hydroxylation; Booth & Boyland (1964) and Uehleke (1963) showed that these oxidations proceed in the presence of rabbit-liver microsomes. As arylhydroxylamines are intermediates in the metabolism of arylamines, it seemed possible that similar processes might be involved in the oxidation of kynurenine and anthranilic acid, which are known metabolites of tryptophan (Dalgliesh, 1951; Charconnet-Harding, Dalgliesh & Neuberger, 1953). If this occurs the products would be 2-hydroxylaminokynurenine (I) and 2-hydroxylaminobenzoic acid (II) respectively, and these might, by analogy with other arylhydroxylamines, be carcinogenic. There are indications that some metabolic products of tryptophan can induce bladder cancer (Allen, Boyland, Dukes, Horning & Watson, 1957), and Ehrhart, Georgii & Stanislowski (1959) have shown that 3-hydroxyanthranilic acid induced leukaemia in mice of the RFH strain.

If anthranilic acid were to undergo *N*-hydroxylation *in vivo* the product would be 2-hydroxylaminobenzoic acid (II). Consequently the metabolic fate of these acids and tryptophan was studied in rats and rabbits. Attempts to prepare the hydroxylamines corresponding to kynurenine and *o*-aminoacetophenone were unsuccessful, as compounds with carbonyl groups in the *ortho*-position to the hydroxylamino group cyclize extremely readily. Thus *o*-nitrobenzaldehyde (Bamberger & Elger, 1906) and *o*-nitroacetophenone (Bamberger & Elger, 1903) give, on reduction, anthranil and *C*-methylanthranil respectively.

* Part 2: Boyland & Williams (1956).

EXPERIMENTAL

Animals. Animals were kept in cages designed for the separate collection of urine and faeces. Urine was collected in containers surrounded by solid CO_2 and removed at 4, 6,