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Studies on the Biosynthesis of Blood Pigments

4. THE NATURE OF THE PORPHYRINS FORMED ON INCUBATION OF CHICKEN ERYTHROCYTE PREPARATIONS WITH GLYCINE, δ -AMINOLAEVULIC ACID OR PORPHOBILINOGEN

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(Received 5 July 1955)

In the preceding papers of this series (Dresel & Falk, 1954, 1956*a*, 1956*b*), the incubation with certain substrates of chicken erythrocytes or of preparations obtained by haemolysis has been shown to result in the formation of various porphyrins. The substrates used have been glycine, δ -aminolaevulic acid (ALA) or porphobilinogen (PBG). The constitution of these porphyrins was defined as closely as possible. Some paper-chromatographic techniques that have proved very useful for studies of uroporphyrin and coproporphyrin are now described in detail. As well as making possible some definition of the isomeric types of the uroporphyrin and coproporphyrin formed, these methods have led to the recognition of a material which we have called *pseudouroporphyrin* and which may have significance in the biosynthesis of protoporphyrin. The 'protoporphyrin' formed has been studied by a variety of techniques, including countercurrent distribution. The instability of protoporphyrin in aqueous HCl under certain conditions is well known, and some aspects of the acid-catalysed hydration of its vinyl groups have also been investigated, with particular reference to methods for the hydrolysis of protoporphyrin ester.

EXPERIMENTAL

Materials

The samples of uroporphyrins I and III and coproporphyrins I and III used for markers in the paper-chromatographic studies have been specified previously (Falk &

Benson, 1953). Coproporphyrins II and IV were synthetic materials from the collection of the late Professor Hans Fischer, kindly made available to us by Professor S. Goldschmidt. Three samples of synthetic uroporphyrins were kindly supplied by Dr S. F. MacDonald. Samples *a* and *c* were uroporphyrins II and IV respectively, prepared by rational syntheses (MacDonald & Michl, 1955); sample *b* was uroporphyrin II, separated from a synthetic mixture of uroporphyrins (MacDonald, 1952).

The 2:6-lutidine (Hopkin and Williams, London) was used as received. The purification of the other solvents has been described by Falk & Benson (1953). The CaCO_3 used for column chromatography was very kindly given to us by Professor C. J. Watson of Minnesota; we have not found a satisfactory sample in England. All other reagents were of A.R. grade.

Methods

The methods of extraction of the 'uroporphyrin', 'coproporphyrin' and 'protoporphyrin' fractions from the tissue preparations have been described (Dresel & Falk, 1956*a*). These materials were prepared for chromatography by the following method: The HCl solutions in which the 'copro' and 'uro' fractions were determined spectrophotometrically, were evaporated to dryness *in vacuo*. The 'proto' fraction was transferred from the HCl solution to ether, the ether solution was washed with water and evaporated to dryness *in vacuo*. All samples were then esterified by standing for 20 hr. in HCl-methanol (cf. Rimington & Miles, 1951) or in 5% (v/v) H_2SO_4 -methanol at 20°, except protoporphyrin samples, which were esterified at -10°. When uroporphyrin samples were esterified by standing in HCl-methanol for 20 hr. at -10°, some material remained on and near the base line when the product was chromatographed by the method of Falk & Benson (1953). When, however, the esterification was carried out for 20 hr. at 20° in either HCl-methanol or H_2SO_4 -methanol, little or no material remained near the base line. The slow-running material was apparently unesterified porphyrin; esterification of the molecule was

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incomplete at -10° , but virtually complete at 20° . After esterification the esters were transferred to chloroform, the chloroform solution was washed twice with water, once with $2N\text{-NH}_4\text{OH}$, and three times with water and filtered through three filter papers folded together and moistened with chloroform; the filtrates were evaporated to dryness. Portions of these preparations of the porphyrin esters were hydrolysed, for chromatography by the 'lutidine' method (see below), by standing in $6.85N\text{-HCl}$ (25%, w/v) for 42 hr. at room temperature; the solutions were then evaporated to dryness at room temperature *in vacuo* over solid NaOH.

Decarboxylations were carried out *in vacuo*, as described by Edmonson & Schwartz (1953).

Paper chromatography. Chromatography of the porphyrin esters by the 'dioxan' method was done as described by Falk & Benson (1953) for separation of uroporphyrins I and III.

The 'lutidine' method is a modification of the method of Eriksen (1953), which employs a 2:6-lutidine-water mixture. The porphyrins were spotted on Whatman no. 1 paper, and descending chromatograms were allowed to run overnight at 22° . The moving phase was 2:6-lutidine-water (5:3.5, v/v), and separate beakers of 2:6-lutidine, $7N\text{-NH}_4\text{OH}$ and water were placed in the bottom of the tank. These were renewed daily; the volumes necessary for particular tanks had to be found by trial and error. For analysis of coproporphyrin-isomer mixtures, about $0.5\text{--}1.0\text{ }\mu\text{g.}$ of porphyrin, and for other purposes about $3\text{ }\mu\text{g.}$ of porphyrin, was applied at each spot on the paper. Reliance was not placed on R_F values, but on the correspondence of experimental spots with those given by markers; the analysis was always repeated at least once.

Column chromatography on CaCO_3 . Columns were packed by tamping down small portions of dry CaCO_3 at a time; slight suction was maintained without change of pressure during the rest of the procedure. After 5 min. of such suction, benzene-chloroform (10:1, v/v) was run slowly and carefully on the top of the column until it was wet throughout and there was a slight head of liquid. By this method air-pockets and cracks in the column were largely avoided. The 'uroporphyrin' ester was then applied to the column in a small volume of the same solvent mixture, which was also used for the development. When development was complete, the column was dried by increasing the suction; after extrusion the separated bands were eluted with chloroform. Uroporphyrins I and III are not well separated on these columns, whereas 'pseudo-uroporphyrin' (see below) moves faster and is well separated from uroporphyrins I and III. As shown by Grinstein, Schwartz & Watson (1945), these columns are also useful for other separations; thus uroporphyrin I, coproporphyrin I and protoporphyrin separated very well from each other under the above conditions.

Countercurrent distribution. An all-glass apparatus containing twenty tubes, each with a lower-layer capacity of 10 ml., was used. The phases used were the two layers from bulk equilibration of ether and HCl of suitable normality. It was convenient to dissolve the porphyrin in 0.2 ml. of dioxan before making the volume up to 10 ml. with the aqueous phase. This had no effect on the partition coefficient, K , of protoporphyrin, and made it possible to work with much more concentrated solutions than could be obtained with the aqueous phase alone. Acetone has been used in a similar manner by Granick & Bogorad (1953).

This procedure has the added advantage of avoiding the use of more concentrated HCl to solubilize the porphyrin; when the porphyrin being handled is protoporphyrin this is important, because of its instability in aqueous acid (see below). After completion of the distribution, the amount of porphyrin in each tube was determined spectrophotometrically.

Free protoporphyrin as reference material for these studies was obtained as follows. Protoporphyrin dimethyl ester was prepared from blood by the method of Grinstein (1947), was recrystallized twice, and was hydrolysed by standing at room temperature in $6.85N\text{-HCl}$ for 42 hr. The resulting free porphyrin was fractionated by countercurrent distribution, using ether- $0.3N\text{-HCl}$; the free protoporphyrin occupied a concentration band centred in tube 12 of the apparatus. A considerable amount of material of much lower K value, which remained in the first few tubes, was discarded. Some material having a much higher K value than protoporphyrin was found in the last few tubes; this had a strong absorption band at about $670\text{ m}\mu$, and it was also discarded. The material occupying the main band at tube 12 was refractionated twice in the same way, when it occupied a symmetrical band centred between tubes 12 and 13, and was the sole porphyrin component detectable by spectrophotometry or by fluorescence in ultraviolet light. It was transferred into ether and the ether solution was evaporated to dryness. The absence of extra components after the third fractionation showed that no alteration of the protoporphyrin occurred under the conditions used for the countercurrent distribution. Even in the dry state, at about 20° and in the dark, this purified protoporphyrin slowly changed. After 2 months a considerable amount of material of low K value was again present. On such fractionation with ether- $0.3N\text{-HCl}$ we have consistently found protoporphyrin to occupy a band centred at tubes 12 and 13.

Reference protoporphyrin ester was prepared similarly by repeated countercurrent distribution of the recrystallized dimethyl ester. After several fractionations with ether- $1.0N\text{-HCl}$, some material of low K value being eliminated, the ester occupied a single symmetrical concentration band centred in tube 11. The absence of other components showed that these conditions of fractionation did not cause hydrolysis or other change in the ester.

It was also found that when free protoporphyrin, purified as above, was esterified by standing for 18 hr. at -10° in methanol containing 5% (v/v) H_2SO_4 , no material other than protoporphyrin ester was revealed by countercurrent distribution.

RESULTS

Chromatographic separation and identification of coproporphyrin isomers

Authentic coproporphyrins. As reported briefly elsewhere (Falk, 1954a, 1955), the 'lutidine' method described above is very sensitive for the detection of the coproporphyrin isomers I and III in mixtures. It is possible to identify with certainty isomers I and II, the third spot from a mixture of all four corresponding to isomer III or isomer IV or both. No micro-method yet available separates either coproporphyrins III and IV or uroporphyrins III and IV.

Porphyrins, in general, separate on the present 'lutidine' chromatograms according to the number of carboxylic side chains present. The straight-line relationship between R_F and the number of carboxyl groups, described for the older method (Nicholas & Rimington, 1949), cannot, however, be used with this method, since the reference curve varies with the coproporphyrin isomer used as marker. This makes the interpretation of 'intermediate' spots more difficult (cf. Falk, 1954*a*). In the present work, although 'intermediate' spots are stated possibly to correspond to hepta-, hexa-, penta- or tri-carboxylic porphyrins, these are to be taken as tentative, rather than definitive, terms. Photographs of the various types of chromatogram described below have been published elsewhere (Falk, 1955).

'Coproporphyrin' fractions from incubation mixtures. In a sample of the total 'coproporphyrin' fraction from incubation of PBG or ALA with the chicken-erythrocyte haemolysate (Dresel & Falk, 1956*a*), only the component moving to the same position as the coproporphyrin III marker was present in sufficient quantity to show up in a fluorescence photograph. Spots relatively much less intense were visible to the eye under ultra-violet light. These trace spots corresponded to uroporphyrin-like materials and to 'hepta-', 'hexa-' and 'penta-carboxylic' porphyrins; the last was the most prominent among these minor components. A small 'tricarboxylic' spot was sometimes found in this fraction as well as in the protoporphyrin fraction (see below). This mixture was fractionated several times on columns of Al_2O_3 (grade V, cf. Nicholas, 1951). Paper chromatography of the main fraction from the columns revealed coproporphyrin III (or IV) only. In some fractions from the columns there were indications of contamination of the coproporphyrin III with very small amounts of coproporphyrin I, but we were not able

to isolate this material, or even to confirm its presence satisfactorily. Paper chromatography of the main fraction by the method of Chu, Green & Chu (1951) also revealed only coproporphyrin III; their method is, however, less sensitive than the new lutidine method (cf. Falk, 1955). From our experience with the latter it is certain that, if coproporphyrin I were present, it amounted to no more than 5% of the total.

Separation and identification of uroporphyrin isomers

Synthetic uroporphyrins. The behaviour of synthetic uroporphyrins II and IV has been examined on the paper chromatograms previously found suitable for separation of the isomers I and III (the 'dioxan' method of Falk & Benson, 1953). Tested as octamethyl esters (Table 1) uroporphyrin II behaved like uroporphyrin I, and uroporphyrin IV like uroporphyrin III; thus, this method can distinguish only between the two pairs of isomers. This same separation into pairs of isomers was found, as reported by Falk (1954*a*) when all four coproporphyrin tetramethyl esters were run by the method of Chu *et al.* (1951).

Since uroporphyrins are easily decarboxylated in the laboratory to coproporphyrins, the 'lutidine' method can be used to check the proportions of uroporphyrins I and III (or IV) found by the 'dioxan' method, and also to distinguish any uroporphyrin II, if present. Comparisons of analyses of 'uroporphyrins' from a number of sources, and the coproporphyrins obtained by their decarboxylation, are shown in Table 1. There is complete agreement between the two methods. The results of Table 1 also indicate identical behaviour of the coproporphyrins from decarboxylation of MacDonald's synthetic uroporphyrins II and IV, and of the synthetic coproporphyrins II and IV from Fischer's collection, which were used as markers.

Table 1. Behaviour of some 'uroporphyrins' (Uro) on paper chromatograms before and after decarboxylation

Sample	Before decarboxylation			After decarboxylation		
	By 'dioxan' chromatography			By 'lutidine' chromatography		
	Uro I or II	Uro III or IV	<i>pseudo</i> Uro	Copro I	Copro III or IV	Copro II
Uro I marker	+++	.	Trace	+++	Trace	.
Uro III marker	.	+++	.	.	+++	.
Uro I:Uro III						
1:9	+	+++	Trace	+	+++	.
1:1	+++	+++	Trace	+++	+++	.
9:1	+++	+	Trace	+++	+	.
'Uro' fraction, from incubation of PBG with haemolysate	.	+++	+++	? Trace	+++	.
MacDonald samples <i>a</i> and <i>b</i> *	+++	Trace	? Trace	.	.	+++
MacDonald sample <i>c</i> *	.	+++	Trace	.	+++	.

* Samples *a* and *b* were synthetic uroporphyrin II and sample *c* was synthetic uroporphyrin IV (see materials).

'Uroporphyrin' fractions from incubation mixtures. It is characteristic of 'uroporphyrins' that they often give a double spot on paper chromatograms developed with lutidine-water mixtures; this double spot has been thought to be a 'paper artifact' (cf. Nicholas & Comfort, 1949). The faster-running part of the double spot is, in most uroporphyrin samples, very much fainter than the slower-running component, but in the uroporphyrin fraction from incubation of ALA or PBG with the chicken-erythrocyte haemolysate (cf. Dresel & Falk, 1956b), or from incubation of ALA with intact chicken erythrocytes (Dresel & Falk, 1956a), the amount of the faster component was approximately the same as that of the slower one. We have called the slower and faster spots L8 and L8a respectively, to suggest their approximation to the 'octacarboxylic porphyrin' position on 'lutidine' chromatograms. Those samples that, according to 'lutidine' chromatograms, had relatively large proportions of L8a, also separated on the 'dioxan' chromatograms into approximately equal amounts of uroporphyrin III and a faster-running porphyrin. It was found that such mixtures could be resolved on CaCO₃ columns into two components: one component ran now on 'lutidine' chromatograms as spot L8 only, and on 'dioxan' chromatograms as uroporphyrin III only; the other component on 'lutidine' chromatograms was L8a only, and 'dioxan' chromatograms revealed only the spot faster than uroporphyrin III. The faster-running material had visual-absorption characteristics very like those of a uroporphyrin: maxima in CHCl₃ (Beck-Hartridge reversion spectroscopy) were: I, 625; II, 570; III, 534; IV, 497.5.

In a preliminary publication (Falk, 1955) we called this material, which runs faster than uroporphyrin III on both types of chromatogram, 'above uro III'. Because it is now known that it is not a uroporphyrin, and because of its similarity in solubility properties during extraction procedures, its spectroscopic similarity and its chromato-

graphic proximity to a uroporphyrin, we now call it *pseudouroporphyrin*. The relatively smaller amounts of spot L8a seen in uroporphyrin samples from some other sources can also be separated from the main uroporphyrin present by CaCO₃ columns. As shown in Table 2, the slower-running component (L8) may be either uroporphyrin I or III.

Countercurrent distribution of 'uroporphyrins'. *cycloHexanone* is a useful solvent for the extraction of 'uroporphyrins' from aqueous media (Dresel & Falk, 1956a). *cycloHexanone*-aqueous HCl systems can therefore conveniently be used for the countercurrent distribution of such porphyrins, *K* being of the order of 1 when the aqueous phase consists of 0.628N-HCl. In our 20-tube apparatus, uroporphyrins I and III, and *pseudouroporphyrin* were, however, not satisfactorily separated from each other.

'Protoporphyrin' fraction from incubation mixtures

On 'lutidine' chromatograms, these fractions always showed a main spot moving like the protoporphyrin marker, but they also sometimes contained traces of material moving to about the position expected of a 'tricarboxylic' porphyrin. This method is not specific for protoporphyrin, other dicarboxylic porphyrins moving similarly; confirmatory evidence was therefore sought from other methods.

'Protoporphyrin' fractions obtained after incubation of glycine with whole chicken blood (Dresel & Falk, 1956a) and of PBG with the haemolysate (Dresel & Falk, 1956b) and of uroporphyrin III with the haemolysate (Dresel, 1955), have been examined by countercurrent distribution. For these studies the free porphyrin was transferred to ether, and the ether solution was taken to dryness as described; the residue was used without further treatment. On countercurrent distribution, a small proportion of the material remained in the first few tubes (cf. Fig. 1). This material of lower

Table 2. Separation of uroporphyrins I and III from pseudouroporphyrin on CaCO₃ columns

UI=Uroporphyrin I; UIII=uroporphyrin III; Ψuro=*pseudouroporphyrin*; L8 and L8a are described in text; ≈=approximately equal to and ≫=much greater than.

Sample	Fractions	Paper chromatograms	
		'Lutidine'	'Dioxan'
Uroporphyrin I marker	Before CaCO ₃	L8 ≫ L8a	UI ≫ Ψuro
	After CaCO ₃ , fraction (1)	L8a	Ψuro
	After CaCO ₃ , fraction (2)	L8, trace L8a	UI, trace Ψuro
Uroporphyrin III marker	Before CaCO ₃	L8, trace L8a	UIII
	After CaCO ₃ , fraction (1)	L8a	Ψuro
	After CaCO ₃ , fraction (2)	L8, trace L8a	UIII
'Uroporphyrin' from incubation of PBG with haemolysate	Before CaCO ₃	L8 ≈ L8a	UIII ≈ Ψuro
	After CaCO ₃ , fraction (1)	L8a	Ψuro
	After CaCO ₃ , fraction (2)	L8	UIII

K probably arose from protoporphyrin during the extraction. As mentioned above (Methods) material of low *K* arises during storage of free protoporphyrin in the dry state. Similar material was found to arise also when pure, free protoporphyrin was subjected to the procedure used for extraction and determination of this porphyrin after incubation experiments (Dresel & Falk, 1956*a*).

The centre of the main concentration band varied between tubes 11 and 12 in different preparations. It was sometimes possible to detect asymmetry in the band, the tendency being for the left-hand side of the curve (earlier tubes) to be raised (Fig. 1). This was particularly noticeable in

Fig. 1. Countercurrent distribution of various 'protoporphyrins'. 'Protoporphyrin' from incubation of whole blood with glycine (Δ); of incubation of haemolysate with PBG ($\bullet$); and of incubation of haemolysate with uroporphyrin III ($\circ$); reference material ($\times$).

the samples obtained after incubation of whole blood with glycine. This might possibly have been due to small amounts of 'tricarboxylic' porphyrin with two vinyl groups; in the absence of such groups, the compound should have a much lower *K* value than protoporphyrin. It is unlikely that the hump was due to monohydroxyethylmonovinyl deuteroporphyrin (cf. Granick, Bogorad & Jaffe, 1953). From the data of Granick *et al.* (1953) it may be calculated that for this porphyrin *K* (0.3*N*-HCl) is of the order of 0.015, so that it should certainly lie in the first few tubes together with the dihydroxyethyl compound (haematoporphyrin), for which *K* (0.3*N*-HCl) is of the order of 0.06. Whatever the cause of this slight distortion of the concentration curve, in all cases the majority of the material, at least, was behaving similarly to the reference protoporphyrin. No micro-method is yet available for isomer-analysis of protoporphyrin, and we cannot therefore state definitely that the biosynthetic samples were protoporphyrin IX.

Spectrophotometric measurements were made on the aqueous layer of the tube representing the centre of the main porphyrin band (tube 12); in this solvent (0.3*N*-HCl saturated with ether) the Soret maximum was at 406 $m\mu$. in the experimental samples referred to above as well as in the reference material, and the shapes of the curves around this maximum were identical. With one sample, solutions were made of the porphyrin in 1.37*N*-, 2.74*N*- and 4.1*N*-HCl; the positions of the Soret maxima in these solutions were 408, 409 and 410 $m\mu$. respectively; identical positions were found with the reference material under the same conditions (cf. also Grinstein & Wintrobe, 1948).

In Table 3, the positions and ratios of intensity of the visual absorption bands of the experimental and reference 'protoporphyrins' are compared in dioxan solution.

Action of aqueous acids on protoporphyrin ester

Under conditions commonly used for the hydrolysis of protoporphyrin ester, some hydration of the vinyl side chains might be expected to occur. By

Table 3. *Visual absorption spectra of protoporphyrin samples from various sources*

Positions of maxima ($m\mu$.) and ratio of intensities (in parentheses) of the visual absorption bands of various samples of protoporphyrin in dioxan, the intensity of band IV being taken as 1.0.

	IV	III	II	I
Protoporphyrin*	504 (1.0)	538 (0.728)	576 (0.436)	631 (0.352)
Protoporphyrin methyl ester*	503 (1.0)	537 (0.790)	575 (0.463)	630 (0.381)
'Reference sample' (3 $\times$ purified) $\dagger$	504 (1.0)	537 (0.798)	575 (0.483)	629.5 (0.394)
Product of incubation of glycine with whole chicken blood (2 $\times$ purified) $\dagger$	504 (1.0)	538 (0.840)	575 (0.544)	630 (0.389)
Protoporphyrin from incubation of PBG with haemolysate (2 $\times$ purified) $\dagger$	504 (1.0)	538 (0.829)	576 (0.553)	630.5 (0.383)

* Stern & Wenderlein (1934).

$\dagger$ Sample purified by countercurrent distribution.

choosing a suitable concentration of HCl, analysis could be carried out by countercurrent distribution for unchanged ester, free porphyrin and 'hydration products' simultaneously.

After purification by countercurrent distribution as described above (Methods), protoporphyrin ester was transferred to ether and after washing with water the ether solution was evaporated to dryness. Portions of the ester were then dissolved in 2.5 ml. of aqueous HCl of different concentrations and, after standing at room temperature for 42 hr., the solutions were evaporated to dryness *in vacuo*, over solid NaOH, at room temperature. The evaporation required about 2 hr. These conditions were chosen because they are those conventionally used in this laboratory for the hydrolysis of protoporphyrin ester. In a control experiment, instead of evaporating to dryness directly after standing for 4 hr. in 2.74N-HCl, the porphyrin was transferred to ether and the ether solution was evaporated to dryness *in vacuo*; analysis of the porphyrin showed the same proportions of each component (see below) as when the evaporation technique was used. The results of the analysis, by countercurrent distribution, of a series of samples treated with increasing concentrations of HCl are shown in Fig. 2.

In the sample (Fig. 2) treated with 0.274N-HCl for 42 hr. most of the ester remained unchanged; at the next higher concentration (1.37N-HCl) tested, and at every concentration higher than this, hydrolysis was complete, but significant 'hydration' also occurred as shown by the appearance of porphyrins of low *K* value. For routine hydrolysis, the lowest concentration of HCl in which dry protoporphyrin ester can conveniently be dissolved at

room temperature is about 3N. It seemed possible that treatment with acid of this concentration for shorter times might allow hydrolysis of the ester with less formation of 'hydration products'. When, however, the ester was treated with 2.74N-HCl for only 2 hr., some 20% of the ester remained unchanged, but about as much (16% of the total) hydration product was present as was found after 42 hr. (15%, Fig. 2). Since it was clear that there would be no advantage in hydrolysing for short times, the critical time for hydrolysis as such was not studied. Complete hydrolysis without the formation of by-products was thus not possible. It appeared that the best compromise might be to treat the ester for 42 hr. with 2.74N-HCl, and then to remove porphyrins of low HCl number by ether-HCl fractionation in bulk. It was found that 0.1N-HCl was too weak to remove all such products, but that after exhaustive extraction with 0.274N-HCl all the material remaining in the ether layer behaved like pure protoporphyrin on countercurrent distribution; by-products were, however, formed on storage of such material (see Methods).

It was also of some interest to test whether any reversal of the hydration process occurred under these conditions, since Schumm (1927) has claimed that on refluxing in 6.5N-HCl both haematoporphyrin and protoporphyrin are converted into an equilibrium mixture of the two porphyrins. Haematoporphyrin was allowed to stand for 42 hr. in 4.8N- and in 8.2N-HCl. Analysis of the products revealed no component other than haematoporphyrin in either case, though (Fig. 2) protoporphyrin under these two conditions yielded 20 and 57% respectively of 'hydration product'. As shown in Fig. 2, the formation of 'hydration products' increased steadily with concentration of acid up to about 3N-HCl, remained steady to about 6N-HCl, and increased steadily again at higher concentrations.

Treatment with perchloric acid resulted in complete hydrolysis of the ester at every concentration tested; 'hydration products' were formed (Fig. 2), but no plateau appeared in the concentration curve; these findings are discussed below.

DISCUSSION

pseudoUroporphyrin

Material behaving on paper chromatograms similarly to pseudouroporphyrin has been observed in this Laboratory in porphyrin samples derived from the urine of patients with various forms of porphyria. It was present in relatively small proportions in most samples studied, including specimens from typical 'acute' and 'congenital' porphyric patients; most of these samples had been crystallized as methyl esters, and it is not certain what

Fig. 2. Action of HCl and HClO₄ on protoporphyrin ester. O, 'Hydration product' after treatment of pure protoporphyrin with different concentrations of HCl for 42 hr. at about 20°; ●, protoporphyrin ester remaining after this treatment; x, and + (a separate experiment at slightly lower temperature), 'hydration product' found after similar treatment with HClO₄.

proportion of the *pseudouroporphyrin* may have been lost in the mother liquors. Relatively large amounts of a porphyrin (methyl ester m.p. 211–216°) were found in the urines of a series of 'cutaneous' porphyrias by Canivet & Rimington (1953). We have examined some of this material kindly sent to us by Dr Canivet, and have found it to behave, on both types of paper chromatogram, like the *pseudouroporphyrin* from our tissue incubations. After decarboxylation of this material and of *pseudouroporphyrin*, only coproporphyrin III (or IV) was found by 'lutidine' chromatography.

Watson and collaborators have isolated a material (methyl ester m.p. 208°) from the urine of certain cases of 'acute' porphyria, and have found this to be a heptacarboxylic porphyrin (Grinstein *et al.* 1945; Watson, Schwartz & Hawkinson, 1945). The behaviour of this porphyrin on paper chromatograms is quite different from that of *pseudouroporphyrin*, and thus also from Canivet's material (cf. Falk & Benson, 1954), although we find that this material also yields only coproporphyrin III (or IV) on decarboxylation.

pseudoUroporphyrin cannot be one of the uroporphyrin isomers I, II, III or IV, none of which runs faster than uroporphyrin III on the 'dioxan' chromatograms. This porphyrin appears to arise in significant quantity *in vitro* only during the enzymic conversion of porphyrin precursors into porphyrins (cf. Dresel & Falk, 1956*a*). The traces of material behaving similarly in some of the synthetic uroporphyrins of MacDonald & Michl (1955) (cf. Table 1) may or may not be the same substance.

Action of aqueous acids on protoporphyrin ester

The analysis for the hydration products formed in acid includes all materials of *K* value very low compared with that of protoporphyrin. The method does not distinguish between the mono- and dihydrated products of free protoporphyrin and the *K* values of hydration products of the ester are unknown. It is possible that the latter might lie close to free protoporphyrin, but, unless they are in fact almost the same, the symmetry of the free protoporphyrin concentration band in our countercurrent distributions makes their presence unlikely.

The plateau in the HCl-concentration curve, between about 3*N*- and 6*N*-HCl, suggests that the hydration of the second vinyl group of protoporphyrin requires a much higher HCl concentration than that of the first, though this is difficult to explain. It is also possible that hydrochlorination of the vinyl groups occurs at the higher HCl concentrations; the resulting dichloroethylporphyrin might be expected to have, like the dihydroxyethyl product (haematoporphyrin), a *K* value much lower than that of protoporphyrin. The absence of

any plateau in the concentration curve with perchloric acid suggests that there was, in fact, chloride-ion attack on the molecule with the higher concentrations of HCl. There could perhaps have been either direct addition of hydrogen chloride to the vinyl groups or esterification by chloride of secondary alcoholic groups arising from their hydration. With perchloric acid only the hydration should occur and apparently did so.

No method for the hydrolysis of protoporphyrin ester without the formation of by-products is yet available, but it is clear that the conventional hydrolysis with 6.85*N*-HCl leads to some 40% of 'hydration products'. As a compromise, we have suggested a method in which 2.74*N*-HCl is used for the hydrolysis, and the impurities are removed by ether-HCl fractionation in bulk. Alkaline hydrolysis was not considered because protoporphyrin ester is insoluble in cold aqueous alkali and unstable to heating in alkali. It has been shown above that material of low *K* value arises from free protoporphyrin both during extraction of this from tissue preparations and during storage in the dry state. We have not identified this material as haematoporphyrin with certainty, but the presence of the artifact made it impossible to determine whether any haematoporphyrin was formed enzymically in our experiments. If any were formed, it could only have been present in very small amounts.

SUMMARY

1. The constitution of a number of porphyrin fractions obtained in *in vitro* experiments with chicken red-cell preparations has been studied.

2. An improved paper-chromatographic method, which allows distinction between coproporphyrin isomers I, II and (III and IV) is described; use of this method reveals that, in the 'coproporphyrin' fraction, coproporphyrin III (or IV) preponderates, but is accompanied by relatively very small amounts of materials which may be hepta-, hexa-, penta- and tri-carboxylic porphyrins; possibly a trace of coproporphyrin I is also present.

3. By paper chromatography, it was also found that the 'uroporphyrin' fraction consisted, in some instances, of about equal amounts of uroporphyrin III (or IV) and a material which has been called *pseudouroporphyrin*. This porphyrin resembles in some of its properties a uroporphyrin, but it is not one of the isomers I, II, III or IV; it is suggested that it may be of significance in the biosynthesis of protoporphyrin.

4. The 'protoporphyrin' fraction sometimes contained traces of a 'tricarboxylic' porphyrin, but by all available criteria, including countercurrent distribution, it appears to consist mainly of protoporphyrin.

5. The countercurrent-distribution technique has also been used for studies of the action of aqueous HCl on protoporphyrin and its methyl ester.

We thank Professor C. Rimington, F.R.S., for his continuous encouragement and support and Miss B. E. Tooth for technical assistance. We are grateful to B. Lewis and Co. for generous supplies of chicken blood. During part of the time that this work was carried out, one of us (J.E.F.) held a Foulerton Research Fellowship of the Royal Society. The Nuffield Unit is supported by a grant from the Nuffield Foundation to Professor C. Rimington.

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Carbohydrate Metabolism in Citric Acid Fermentation

4. PURIFICATION AND PROPERTIES OF ALDOLASE FROM *ASPERGILLUS NIGER**

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(Received 31 August 1955)

Meyerhof & Lohmann (1934) discovered and investigated in detail the enzyme aldolase which catalyses the reaction

Aldolase from rabbit muscle was purified by Herbert, Gordon, Subrahmanyam & Green (1940). The enzyme from rat muscle was later crystallized by Warburg & Christian (1943), and from rabbit muscle by Taylor, Green & Cori (1948). Warburg & Christian (1943) partially purified yeast aldolase and discovered that it required heavy metals for its activity. The enzyme was found to be inhibited by metal-binding agents, such as cysteine, pyrophosphate and *o*-phenanthroline, and reactivated by Zn^{2+} , Fe^{2+} or Co^{2+} . Muscle aldolase did not require or contain these metals and was not inhibited by any of the metal-binding agents. Subsequent studies of the enzyme with preparations of

low purity from different tissues have shown the existence of both types of aldolase. The activation of aldolase from *Escherichia coli* by Mn^{2+} (Knox, Stumpf, Green & Auerbach, 1948) and the requirement of *Clostridium perfringens* aldolase for Fe^{2+} or Co^{2+} (Bard & Gunsalus, 1950) have been reported. Partially purified pea aldolase was, however, unaffected by heavy metals or metal-binding agents (Stumpf, 1948).

During a study of the glycolytic enzymes of *Aspergillus niger* it was observed that the aldolase in crude extracts of the mould mycelium was completely inhibited by the metal-chelating agent ethylenediaminetetraacetic acid, and was reactivated by Zn^{2+} (Jagannathan & Singh, 1954). The aldolases of other moulds which were investigated (*Aspergillus oryzae*, *Aspergillus wentii*) also resembled yeast aldolase and differed from muscle aldolase in their metal requirement. Since the metal-requiring aldolases had been studied mostly in crude form, the isolation of the enzyme from

* Part 3: Damodaran, Jagannathan & Singh (1956).