THE BIOSYNTHESIS OF PORPHYRINS AND HAEMS BY *CORYNEBACTERIUM DIPHTHERIAE.*

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IT has been known for some time that when *C. diphtheriae* is cultured under conditions suitable for toxin production, porphyrin also appears in the culture fluid, and indeed a quantitative relationship between toxin and porphyrin was observed by Coulter and Stone (1931). The nature of the porphyrin has been studied by several workers (Campbell-Smith, 1930; Coulter and Stone, 1931; Dhéré, Glücksmann and Rapetti, 1933). Gray and Holt (1947, 1948) demonstrated, by chromatographic adsorption methods, that the major pigment is coproporphyrin III, but that there are also present small quantities of uroporphyrin I and of an unidentified porphyrin. Subsequent partition chromatographic analysis (Nicholas and Rimington, 1949) supported this conclusion, and indicated that two unidentified porphyrins are present which almost certainly contain 5 and 6 carboxyl groups respectively.

The quantitative relationship between iron content of the medium on the one hand and toxin and total porphyrin production on the other has been studied recently by Pappenheimer (1947*a*, *b*), and Pappenheimer and Hendee (1947), who state that for every four atoms of iron added over and above the optimal level for toxin production, one molecule of toxin and four molecules of porphyrin fail to appear in the culture medium. The conclusion was drawn that iron-porphyrin complex and toxin are related as prosthetic group and protein moiety in a haemoprotein fulfilling some enzymic function in the cell of *C. diphtheriae*. Although 95 per cent of the excreted porphyrin belongs to the series derived from aetioporphyrin III, there are present in the culture fluid other porphyrins, one of which is known to be related to aetioporphyrin I. This fact is not easily reconciled with Pappenheimer's theory, which postulates the building up of haemoprotein (Type III) when iron is freely available, and the excretion of the porphyrin prosthetic group and protein moiety when the iron content of the medium is restricted. Implicit in the hypothesis is the idea that the porphyrin which fails to appear in the culture medium is retained in the cell in the form of an iron complex of a Type III porphyrin, together with toxin protein. Thus it would appear possible that at higher iron levels an accumulation of coprohaem might occur within the cells. Rawlinson and Hale (1948, 1949) have investigated the nature of the haems present in *C. diphtheriae* cells and find no evidence for the presence of coprohaem; the major haemoprotein yields protohaem No. 9 (isomeric series III).

As far as pigment metabolism is concerned, the situation would thus appear to be that in the presence of abundant iron, the growing cells synthesize haemoproteins mainly derived from protoporphyrin No. 9 and pass little porphyrin into the culture fluid. When iron supply is restricted, however, less intracellular protohaem is produced and porphyrins appear in the culture fluid. Coproporphyrin III, which makes up by far the larger part of this pigment, could conceivably be either a precursor of protoporphyrin or be derived from it by metabolic transformation, but the isomerically different uroporphyrin I of the culture fluid could not possibly bear such relationships to the protoporphyrin. Alternatively it is possible that each pigment is synthesized independently, and that the appearance, according to the available supply of iron, of intracellular protohaem or of extracellular coproporphyrin and uroporphyrin, etc., is merely the expression of some redirection of the biosynthetic mechanism at a comparatively early stage in tetrapyrrol synthesis.

We considered that this system offered an opportunity to study, by the aid of isotopes, some of the questions raised. The results of our investigations are set forth below.

METHODS.

Organism.

Corynebacterium diphtheriae, Park Williams No. 8 Toronto strain, has been used throughout.

Culture media.

In Experiments 1 to 4 a synthetic medium, found by trial to be optimal for growth and porphyrin production, was used; the composition of this medium is recorded in Table I. In Experiments 5 and 6 a semi-synthetic medium was employed as described by Holt (1948), modified only by using 0·2 per cent (w/v) instead of 0·15 per cent (w/v) of glycine. The requisite quantities of iron were added as $FeSO_4.7H_2O$. The medium was distributed in quantities of 100 ml. in Roux bottles, sterilized, and after inoculation cultivated at 32° for 7 days. In Experiments 1 to 4 known amounts of glycine were added, the glycine containing an excess of ^{15}N in Experiments 2 and 4. In Experiments 5 and 6 it was necessary to determine the glycine content (method of Alexander, Landwehr and Seligman, 1945) of the casein digest prior to the addition of the isotopic amino-acid in order to calculate the final isotope dilution.

TABLE I.—*Composition of Synthetic Medium.*

		g.			*g.*
DL	Tryptophane . .	0·1	DL	Aspartic acid . .	0·5
	Glycine . . .	1·0	L	Proline . . .	0·6
DL	Valine . . .	0·5	DL	Phenylalanine . .	0·4
DL	Alanine . . .	0·5	L	Cystine . . .	0·1
DL	Leucine . . .	1·0		NaCl	2·0
L-	Glutamic acid . .	2·5		KH_2PO_4 . . .	0·1
DL	Methionine . . .	0·4		$MgSO_4.7H_2O$ . .	0·3
DL	Tyrosine . . .	0·4			*mg.*
L	Arginine . . .	0·3		Pimelic acid . .	5·0
L	Histidine . . .	0·2		β Alanine . . .	5·0
DL	Lysine . . .	0·2		Nicotinic acid . .	10·0

Materials dissolved in 500 ml. distilled water with the exception of cystine, which was dissolved in minimum of *ca.* 5N HCl and then added. pH adjusted to 7·6, volume made up to 900 ml., and after the addition of 3 ml. of 3·3 per cent (w/v) $CaCl_2$ boiled 15 min. and filtered. $MgSO_4$ and tryptophane added, made up to 1000 ml. sterilized by autoclaving. 3·5 ml. 45 per cent (w/v) maltose solution and 4 ml. of 0·001 per cent (w/v) $FeSO_4.7H_2O$ added per 90 ml. medium.

Harvesting.

The cultures were centrifuged and the cells washed three times with 10 vol. of 0·9 per cent (w/v) NaCl. They were again resuspended in saline and portions removed for determination of total N and ^{15}N excess. The remainder was used for total haem extraction. The supernatant fluid, after determination of Lf and total coproporphyrin content, was treated with formaldehyde, to convert the toxin to toxoid.

Isolation of the porphyrins from the toxoid solution.

This was achieved by methods described by Gray and Holt (1948). In the case of experiments with high iron levels it was not possible to effect isoelectric precipitation. The coproporphyrin was then extracted with ether, and the material that separated at the interface was worked up for uroporphyrin. The porphyrins were subjected to chromatographic purification after esterification.

Isolation of protoporphyrin from intracellular haems.

The haems extracted from the washed cell residue by the method described by Rawlinson and Hale (1949) were adsorbed from the ethereal solution upon an alumina column, eluted by glacial acetic acid and converted to porphyrins by the method of Warburg and Negelein (1932). The porphyrins were taken into ether and the protoporphyrin removed by shaking with 5 per cent HCl. The porphyrin of the cytochrome *a* complex remained in the ether, but was present in too small a quantity to make isolation practicable. The protoporphyrin was purified by repeated transfers between acid and ether.

TABLE II.—*C. diphtheriae. Large*

Experiment.	Total Glycine N in culture fluid (mg.).	^{15}N in Glycine (atom % excess).	Total Bact. N (mg.).	^{15}N in Bact. Protein (atom % excess).	Lf.
(1) Low Fe* normal glycine	858·4	*Nil*	475	*Nil*	14
(2) Low Fe* ^{15}N glycine	678·5	5·584	487·5	0·903	20
(3) High Fe* normal glycine	522·5	..	560	0·008	4–5
(4) High Fe* ^{15}N glycine	678·5	5·584	632·5	0·863	12
					Large Experiment 2 (20 litre/batch).
	(g.)		(g.)		
(5) Low Fe* ^{15}N glycine	7·40	4·92	9·25	0·790	55
(6) High Fe* ^{15}N	7·14	4·92	8·57	0·729	5

* Low Fe, opt. iron for toxin production 0·147 μg./ml. High Fe = 5 × opt. iron.

Isotope analysis.

All materials were digested for 24 hours with H_2SO_4 containing 2 per cent (w/v) SeO_2, copper and potassium sulphates being added as additional catalysts. Where a specimen contained insufficient nitrogen (*ca.* 0·25 ml.) for the mass spectrograph dilution was effected with some of the normal substance, the relative proportions being determined spectrophotometrically. All ^{15}N analyses were kindly carried out by Dr. R. Bentley at the National Institute for Medical Research.

RESULTS AND DISCUSSION.

The results of the experiments are presented in Table II. The isotope enrichment of both protein fractions and haems or porphyrins is seen to be little affected by the nature of the basal medium, i.e. either casein digest or mixture of synthetic amino acids respectively. In each case the labelling of the intracellular haem is higher than that of the bacterial protein, whilst that of the coproporphyrin III is distinctly lower. In the Experiments 5 and 6, where isolation of uroporphyrin I was also possible, the labelling of this porphyrin was approximately the same as that of the coproporphyrin.

The ways in which the coproporphyrin of the culture fluid might be expected to be formed are :

(1) By independent synthesis from a nitrogenous precursor or precursors different from that used for synthesis of the protoporphyrin of intracellular haem.

(2) By an independent synthesis from the precursor of the intracellular haem.

(3) By a route in which coproporphyrin III is a precursor of protoporphyrin or vice versa (Fig. 1).

(4) By metabolic transformation from the protoporphyrin of the intracellular haem.

The last of these is unlikely, because the coproporphyrin excreted into the culture medium cannot have been derived directly by some metabolic transformation from the protoporphyrin of the intracellular haems or vice versa, otherwise a comparable degree of labelling would have been found.

Experiment 1. (*Synthetic Medium.*)

^{15}N in Toxoid Protein (atom % excess).	^{15}N in Non-toxoid Protein (atom % (excess).	^{15}N in Intracellular Haem (atom % excess).	Copro-porphyrin (mg.).	^{15}N in Copro-porphyrin (atom %) excess).	Uropor-phyrin (mg.).	^{15}N in Uropor-phyrin (atom % excess).
Nil	*Nil*	..	7·0	0·023	—	—
0·88	0·88	..	9·9	0·489	..	..
..	..	..	2·2	..	..	..
..	..	1·247	8·2	0·511	..	..

(*Casein digest Medium.*)

..	..	..	38·0	0·510	approx. 0·8	0·463
..	..	1·261	3·2	0·284	approx. 0·4	0·342

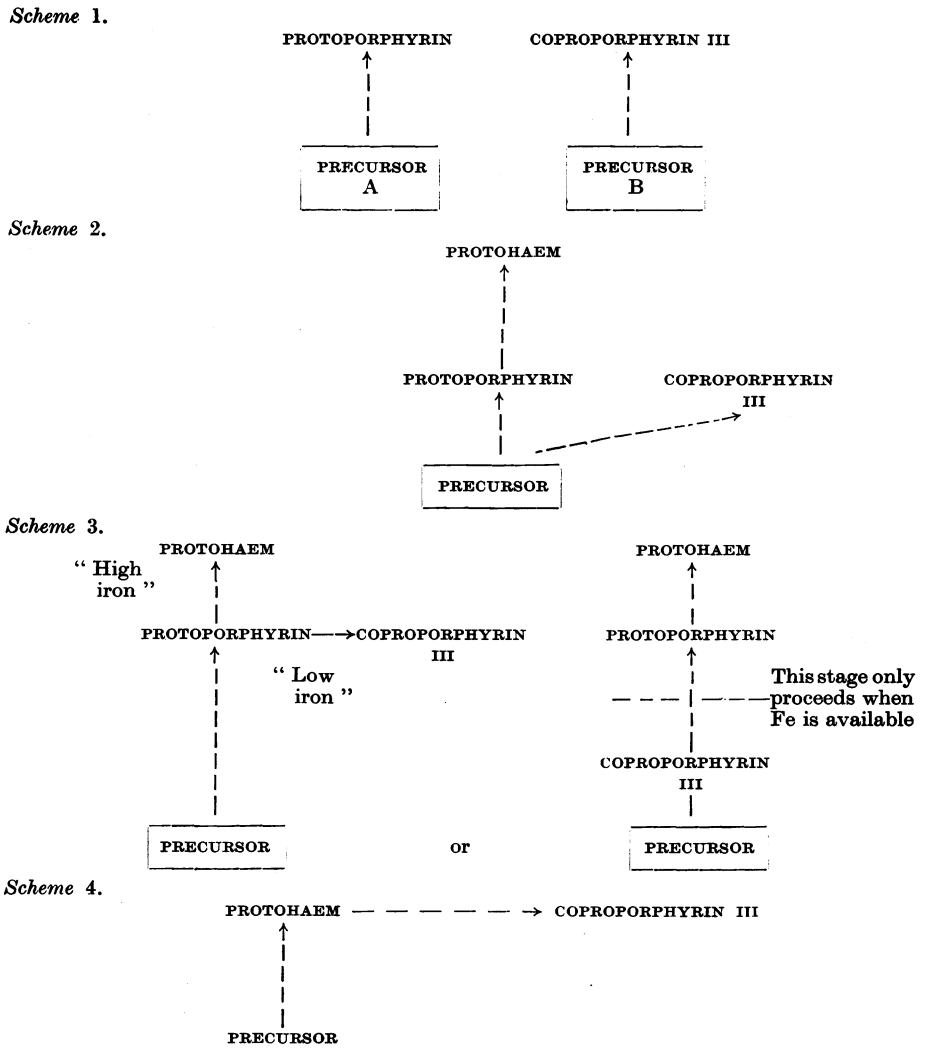

FIG. 1.—Possible pathways of coproporphyrin III synthesis.

At present there exists no conclusive experimental evidence concerning the specific nitrogenous precursors of coproporphyrin or uroporphyrin, although coproporphyrin I and uroporphyrin I excreted by a case of congenital porphyria have been shown by Grinstein, Aldrich, Hawkinson and Watson (1949) and Gray and Neuberger (1949) to be labelled to a high degree after the feeding of isotopic glycine to the subject. In the case of *C. diphtheriae* a different coproporphyrin isomer is concerned. In our experiment uroporphyrin belonging to the series I could not have been derived directly from the coproporphyrin III or vice versa, but the fact that the isotope concentration in these two substances was practically equal suggests that they may have been derived from the same nitrogenous precursor.

With regard to the possibilities (2) and (3), synthesis of coproporphyrin may occur at a later time during cultivation than that of protohaem. Protohaem

would most probably be formed while the isotope content was maximal, coproporphyrin possibly only after protohaem synthesis had decreased through reduction of the iron content of the medium. The isotope content of the glycine in the medium might by then have been lowered by randomization. In this respect separate experiments have shown that the growth of *C. diphtheriae* is independent of glycine, the organism being capable of synthesizing this amino acid. On the other hand, porphyrin production per mg. of bacterial nitrogen is slightly higher in cultures to which an external source of glycine has been added. Consistent with the hypothesis that coproporphyrin III is formed at a later stage than protohaem is the fact that the isotope content of the coproporphyrin in Experiment 5 with " low iron " is much higher than that in Experiment 6 with " high iron." In the latter experiment one may assume that protohaem production continued for a longer period than when less iron was available, so that the coproporphyrin production in this case was delayed until randomization had lowered the isotope concentration of the glycine. Unfortunately isotope analyses of the glycine in the medium were not carried out at intervals during the growth of the organism.

Our observations are therefore equally compatible with the possibilities (1), (2) and (3) listed above. The first hypothesis although a possibility is, we feel, less likely in view of the abundant reported evidence for the participation of glycine in the biosynthesis of the porphyrin ring.

SUMMARY.

The incorporation of ^{15}N glycine into the proteins, porphyrins and haems elaborated by *C. diphtheriae* has been studied.

Glycine is incorporated into all these compounds, but the degree of labelling differs. This is discussed and hypotheses are advanced to account for these findings.

The isotopes supplied were kindly made available by the Tracer-elements Sub-Committee of the Medical Research Council, and we are greatly indebted to Dr. R. Bentley for performing the isotopic analyses.

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