in view of the finding that this compound readily undergoes rearrangement in alkaline solution to 16-oxo-oestradiol-17β there can be little doubt that the KC-5 previously isolated by Marrian *et al.* (1957) was indeed a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxo-oestradiol-17β as was suggested. The available evidence strongly suggests that extracts of late-pregnancy urine contain no 16-oxo-oestradiol-17β other than that which must be artifactually formed from 16α-hydroxyoestrone during the extraction of the phenolic fraction by aqueous alkali from ether. In view of this the recent claim by Levitz, Spitzer & Twombly (1956) to have detected the presence of radioactive 16-oxo-oestradiol-17β in the urine of human subjects after the administration of [16-^{14}C]oestradiol-17β must be treated with reserve.

The isolation of 16α-hydroxyoestrone from urine lends support to the previous suggestion of Marrian *et al.* (1957) that this compound may be formed from oestrone by 16α-hydroxylation and may be the metabolic intermediate in the 'hydration' of oestrone to oestriol.

SUMMARY

1. 16α-Hydroxyoestrone diacetate, prepared from 16α:17α-epoxyoestra-1:3:5-triene-3:17β-diol diacetate by the method of Leeds *et al.* (1954), yielded 16α-hydroxyoestrone on cold acid hydrolysis. 16α-Hydroxyoestrone was also prepared directly from the epoxyacetate by cold acid treatment.

2. 16α-Hydroxyoestrone readily undergoes rearrangement to 16-oxo-oestradiol-17β in aqueous alkali at room temperature. The same rearrangement occurs when it is heated *in vacuo* to 220–230°.

3. A preparation of the ketonic-phenolic Kober chromogen (KC-5) prepared from enzymically-hydrolysed late-pregnancy urine as described by Marrian *et al.* (1957) yielded a product after acetylation which has been conclusively identified as 16α-hydroxyoestrone diacetate.

4. In view of the rearrangement undergone by 16α-hydroxyoestrone in alkaline solution it is concluded that KC-5 as isolated from urine by the methods described consists of 16α-hydroxyoestrone contaminated with about 20% of artifactually produced 16-oxo-oestradiol-17β.

5. It is suggested that 16α-hydroxyoestrone may be the metabolic intermediate between oestrone and oestriol.

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Synthesis of Porphyrins by Cell Suspensions of *Tetrahymena vorax*: Effect of Members of the Vitamin B Group

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It is well established that the biosynthesis of the porphyrin nucleus in animal tissues proceeds by condensation of glycine with an unsymmetrical derivative of succinate (derived from the tricarboxylic acid cycle) with intermediate formation of δ-aminolaevulic acid (ALA) and porphobilinogen (Shemin, 1955; Dresel & Falk, 1956*a–c*; Falk, Dresel, Benson & Knight, 1956; Berlin, Neuberger & Scott, 1956*a*, *b*). There is evidence that a similar pathway occurs in photosynthetic bacteria of the Athiorhodaceae group (Lascelles, 1955; Cooper, 1956).

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Less is known of the individual enzymes catalysing these various steps. Gibson, Neuberger & Scott (1955) have studied the properties of purified ALA dehydrase, which converts ALA into porphobilinogen. This enzyme is widely distributed in higher animals and bacteria; it has also been found in preparations from spinach leaves, *Chlorella* and *Tetrahymena* (Granick, 1954). Enzyme fractions from spinach and from chick erythrocytes, which convert porphobilinogen into unidentified precursors of porphyrins, have also been briefly reported (Bogorad, 1955; Granick, 1955). The enzymes responsible for the initial reaction between glycine and the succinate derivative have not been isolated and the mechanism of the reaction is obscure.

Studies of animal nutrition have shown that many B group vitamins are concerned, directly or indirectly, in haemopoiesis (see Lemberg & Legge, 1949; Jukes, 1953). In particular, dietary deficiency of pyridoxine results in hypochromic anaemia in pigs, dogs and rats (Chick, Macrae, Martin & Martin, 1938; Fouts, Helmer & Lepkovsky, 1940; Kornberg, Tabor & Sebrell, 1945). The concentration of free protoporphyrin in the erythrocytes of pyridoxine-deficient pigs was found by Cartwright & Wintrobe (1948) to be markedly lower than that in the control animals, suggesting that this vitamin is directly concerned in the synthesis of protoporphyrin. Hypochromic anaemia has also been observed in pantothenate-deficient rats (Carter, Macfarlane, O'Brien & Robb-Smith, 1945).

The experiments with growing animals do not show the step or steps in the synthesis of porphyrin at which the vitamins act. This was the aim of the present work, with micro-organisms as experimental material, and an organism was sought which required for growth a number of B group vitamins and which also formed free porphyrins in easily detectable quantities. The photosynthetic bacteria were not suitable since they require only a limited number of B group vitamins for growth, and the free-living ciliate *Tetrahymena vorax* was finally chosen. The nutrition of this group of organisms has been studied in great detail and growth can be obtained in a completely defined medium (Kidder & Dewey, 1951; Kidder, 1953). Furthermore, free protoporphyrin is formed by cultures of some strains of *Tetrahymena* (Rudzinska & Granick, 1953); the strain used in the present work was particularly active in forming porphyrins during growth under certain conditions (personal communication from Dr V. Dewey). Porphyrin synthesis from glycine and from ALA was studied with washed organisms harvested from media containing growth-limiting concentrations of B group vitamins; respiratory activity was also examined, as a check of the possible indirect effects arising from the vitamin deficiency.

EXPERIMENTAL

Organism and media

Tetrahymena vorax, strain V 2, was obtained from Dr V. Dewey. Stock cultures were maintained in 2% (w/v) peptone (Evans Medical Supplies Ltd., Liverpool) adjusted to pH 7·0. These were subcultured fortnightly and incubated for 48 hr. at 30°; they were stored at room temperature (about 20°).

The organisms for experiments with suspensions were harvested from the semi-defined medium T, which contained per litre: acid-hydrolysed casein (vitamin-free), prepared by the method of Snell & Rannefeld (1945), equivalent to 2·5 g. of original casein; DL-tryptophan, 140 mg.; L-cysteine hydrochloride, 100 mg.; glucose, 3·6 g.; $Na_2HPO_4,12H_2O$, 1·4 g.; KH_2PO_4, 540 mg.; $MgSO_4,7H_2O$, 100 mg.; $MnSO_4,4H_2O$, 1 mg.; adenine, guanine and uracil, 10 mg. each; Tween 80 (Honeywill and Stein Ltd., London, S.W. 1), 0·4 ml. The complete medium contained the following concentrations of B group vitamins: nicotinic acid, 8 μM; thiamine hydrochloride, 3 μM; calcium pantothenate, 4·2 μM in terms of pantothenic acid; riboflavin, 1·3 μM; pyridoxal hydrochloride, 0·4 μM; pteroylglutamic acid, 0·1 μM; DL-6-thioctic acid, 25 μmM; biotin, 4 μmM. The pH was adjusted to 7·4 with N-NaOH. With the exception of pyridoxal, all additions were made before autoclaving for 10 min. at 10 lb./in.^{2} pressure.

Growth of organisms

Organisms were grown in Roux bottles containing 100 ml. of medium T modified as indicated. Each bottle was sown with 0·5 ml. (about 0·25 mg. dry wt. of cells) of a 48 hr. culture grown at 30° in 2% (w/v) Evans peptone; the bottles were incubated at 37° for 48 hr. The growth attained (about 0·5 mg. dry wt./ml. of culture) varied from 50 to 70% of that reached on more prolonged incubation (72 hr.) under the same conditions. It was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a neutral density filter.

Procedures for study of porphyrin synthesis

Harvesting of organisms. The cultures were centrifuged gently at 500 *g* for 4 min., the supernatant fluid was removed quickly by suction and the cells were washed as above in half the original culture volume of 0·85% NaCl. They were finally suspended in the salts solution described below. The dry weight was determined by heating samples to constant weight at 90–100° and correcting the values for the weight of added salts.

Synthesis from glycine. The washed organisms were suspended in a mixture of salts similar to that used by Ryley (1952); it contained: NaCl, 0·047 M; KCl, 0·002 M; $MgSO_4$, 0·001 M; $MnSO_4$, 5×10^{-6} M; phosphate buffer, pH 7·3, 0·025 M (prepared from 0·2 M-Na_2HPO_4 and -KH_2PO_4). The other components of the reaction mixture (I) were: glycine, 0·02 M; sodium fumarate, 0·02 M; pyridoxal hydrochloride, 10^{-6} M; Tween 80, 0·1%; penicillin G and streptomycin sulphate, each 20 μg./ml. The final volume was 2·5 ml. and the concentration of organisms 3–4 mg. dry wt./ml. Incubation was in 18 mm. × 150 mm. tubes for 48 hr. at 37°; the tubes were held in a sloped position to increase aeration. The antibiotics were added to prevent growth of bacteria during incubation of the suspensions; they had no observed effect on the metabolism of *T. vorax*.

Synthesis from δ-aminolaevulic acid. The organisms were incubated in a mixture of inorganic salts similar to that described above, with addition of penicillin G and streptomycin sulphate, each 20 μg./ml., and mM-δ-aminolaevulic acid hydrochloride (mixture II). The conditions of incubation were as for studying synthesis from glycine.

Estimation and identification of porphyrins

Estimation. Most of the porphyrin formed by the organisms passed out into the suspending fluid, but a small proportion (5–10%) was retained by the cells. Samples of the whole reaction mixture (cells and suspending fluid) were diluted in 1·4 N-HCl, thereby removing the free porphyrin present in, or absorbed on to, the cells. The samples were centrifuged and the optical density of the supernatant fluid was measured at the Soret peak (405–407 mμ.); the Unicam spectrophotometer SP. 600 (Unicam Instruments Ltd., Cambridge) was used. Since uroporphyrin was the major component of the mixtures of porphyrins formed, the concentration of porphyrin was calculated in terms of this compound, the extinction coefficient of Rimington & Sveinsson (1950) being used.

The proportion of uro-, copro- and proto-porphyrin was determined by the fractionation procedure described previously (Lascelles, 1956*a*).

Identification. The porphyrins formed were identified by the paper-chromatographic methods of Eriksen (1953), Chu, Green & Chu (1951) and Falk & Benson (1953); samples were prepared for chromatography by the methods described before (Lascelles, 1956*a*).

Manometric experiments

The conventional Warburg technique was used. Each vessel contained cells (3–4 mg. dry wt./ml.) suspended in the salts mixture described above, together with glucose (0·02 M); the final volume was 2·5 ml. The centre well of each vessel contained 20% NaOH and a pleated filter paper. Incubation was in air at 37°. The results are expressed as Q_{O_2} values (μl. of oxygen consumed/mg. dry wt. of organisms/hr.). The Q_{O_2} values for cells grown on the complete medium T varied from 20 to 30 with different batches of cells.

Chemicals

Details concerning the solutions of ALA, iron citrate and of the porphyrins used as reference standards for chromatography have been given previously (Lascelles, 1956*a*).

RESULTS

Porphyrin formation during growth

Porphyrins were formed by growing cultures of *T. vorax* in medium T; addition of glycine (0·01 M) to the medium, which contained acid-hydrolysed casein, increased the formation of porphyrin. Glucose was essential for production of porphyrin though the organism could grow on medium T without it.

The organism grows well at 30°, but appreciable formation of porphyrin was observed only in cultures incubated at 37°; Dr V. Dewey (private communication) made similar observations with cultures grown in a peptone medium. All further experiments were carried out with suspensions of organisms grown in medium T at 37°.

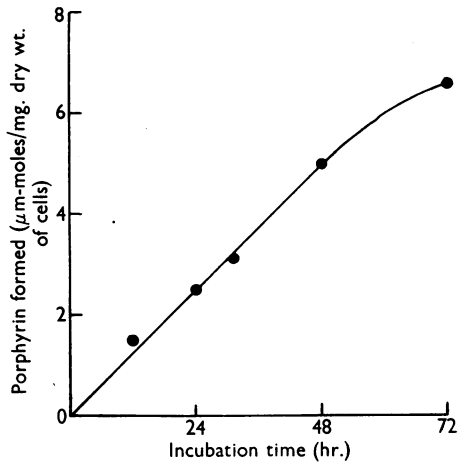

Fig. 1. Rate of porphyrin synthesis by cell suspensions. The washed organisms (3·3 mg. dry wt./ml.) were harvested from medium T and were incubated for varying periods in mixture I at 37°.

Table 1. *Effect of glucose during growth on porphyrin synthesis by harvested organisms*

Organisms were grown for 48 hr. in medium T with various amounts of glucose. Harvested cells were incubated for 48 hr. in mixture I or II.

Concn. of glucose in growth medium (M)	Porphyrin formed (μm-moles/mg. dry wt. of organisms)	
	Mixture I	Mixture II
0	0	5·2
0·005	1·3	5·2
0·01	4·2	5·6
0·02*	5·0	5·8

* Normal concn. in medium T.

Conditions for synthesis by suspensions of organisms

When the washed organisms were incubated aerobically in mixture I at 37° porphyrin synthesis occurred at a rate which was linear for 48 hr. or more (Fig. 1). The rate of synthesis was directly proportional to the concentration of organisms within the range 1·5–4 mg. dry wt./ml. When the suspensions were incubated at 30° the amount of porphyrin formed was less than 20% of that formed at 37°. No porphyrins were formed when the suspensions were incubated anaerobically in mixture I.

Organisms harvested after growth in medium T without glucose or with low concentrations of the sugar formed little or no porphyrin from glycine (Table 1); addition of glucose to such suspensions

incubated in mixture I did not restore activity. Omission of glycine from mixture I reduced synthesis to about 40 % of that in the complete mixture (Table 2). Omission of fumarate had a less-marked effect, and other carboxylic acids tested were less active or inactive (Table 2). Acetate inhibited porphyrin synthesis both in the presence and absence of fumarate. Tween 80 was also included in mixture I, as it increased synthesis slightly; it is necessary for growth of this organism in medium T.

δ-Aminolaevulic acid promoted porphyrin synthesis in the absence of both glycine and fumarate (Table 2). Further experiments with this substance were in mixture II, which contained neither Tween 80 nor pyridoxal; these compounds did not affect porphyrin synthesis from ALA. Organisms grown without glucose formed porphyrin from ALA though not from glycine (Table 1).

Effect of B group vitamins

T. vorax requires for growth nicotinic acid, thiamine, riboflavin, pantothenic acid, pteroylglutamic acid, thioctic acid and pyridoxal or pyridoxamine; its requirements for B group vitamins are therefore similar to those of *Tetrahymena geleii* (Kidder & Dewey, 1951; Kidder, 1953). With the exception of pyridoxal, addition of these vitamins, either singly or as mixtures, did not increase porphyrin synthesis by suspensions of organisms harvested from medium T; pyridoxal sometimes increased porphyrin formation from glycine (see later) and it was therefore normally included in mixture I.

The effect of the B group vitamins was examined in organisms rendered deficient in the vitamin in question by growing them with suboptimum concentrations. Usually the concentration was sufficient to give between 60 and 80 % of the growth attained on the complete medium T.

Pyridoxal. Organisms grown with suboptimum concentrations of pyridoxal formed little porphyrin from glycine. The Q_{O_2} value of the deficient cells was similar to that of the control cells, whereas the amount of porphyrin formed was less than 20 % of that synthesized by the normal organisms (Table 3).

Table 2. *Substrates required for porphyrin synthesis by suspensions of organisms*

Organisms harvested from medium T were incubated for 48 hr. in mixture I without glycine and fumarate, with the additions shown. The carboxylic acids were added as their sodium salts.

Additions			Porphyrin formed (μm-moles/mg. dry wt. of organisms)
Glycine (M)	Fumarate (M)	Other compounds	
0	0	—	0·8
0	0·02	—	1·9
0·005	0·02	—	4·1
0·01	0·02	—	4·4
0·02	0·02	—	5·0
0·04	0·02	—	4·9
0·02	0	—	3·9
0·02	0·005	—	4·4
0·02	0·01	—	5·0
0·02	0	Succinate (0·02 M)	4·6
0·02	0	Acetate (0·02 M)	2·8
0·02	0	α-Oxoglutarate (0·02 M)	3·5
0	0	ALA (0·001 M)	4·2

Table 3. *Effect of deficiency of pyridoxal on porphyrin synthesis*

Organisms were harvested from medium T containing various concentrations of pyridoxal. They were incubated for 48 hr. in mixture I with or without pyridoxal (10^{-6} M), or in mixture II.

Expt. no.	Concn. of pyridoxal in growth medium (M)	Growth* (%)	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)		
				Mixture I		
				Without pyridoxal	With pyridoxal	Mixture II
1	10^{-8}	71	20	0·7	2·5	4·3
	2×10^{-8}	81	24	0·6	2·1	4·3
	4×10^{-7} (normal concn.)	—	25	4·8	5·0	4·4
2	$2·5 \times 10^{-8}$	85	23	1·0	5·6	—
	4×10^{-7}	—	24	4·2	5·8	—

* Growth attained by the deficient culture as % of that reached in the control culture.

Table 4. *Effect of deficiency of pantothenate on porphyrin synthesis*

Deficient organisms were grown on medium T containing 8×10^{-8}M-pantothenate, and normal organisms on the same medium with 4×10^{-6}M-pantothenate; the growth of the deficient cultures was 68–75% of that in the controls. The harvested organisms were incubated for 48 hr. either in mixture I supplemented with pantothenate (4×10^{-5}M) where shown, or in mixture II.

Expt. no.	Type of organisms	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)		
			Mixture I		
			Unsupplemented	Supplemented	Mixture II
1	Deficient	26	2·0	1·9	3·4
	Normal	22	4·3	4·2	2·7
2	Deficient	22	1·5	1·5	5·1
	Normal	24	4·4	—	4·9
3	Deficient	22	1·2	1·1	4·0
	Normal	23	3·8	—	3·8

Table 5. *Effect of deficiency of riboflavin on porphyrin synthesis*

Organisms were harvested from medium T containing varying concentrations of riboflavin. The suspensions were incubated for 48 hr. in mixture I or in mixture II, supplemented with riboflavin (2×10^{-6}M) where indicated.

Expt. no.	Concn. of riboflavin in growth medium (M)	Growth* (%)	Q_{O_2}	Porphyrin formed (μm-moles/mg. dry wt. of organisms)			
				Mixture I		Mixture II	
				Unsupplemented	Supplemented	Unsupplemented	Supplemented
1	2×10^{-8}	60	28	1·5	2·9	2·1	2·6
	4×10^{-8}	70	29	2·9	4·1	2·7	3·4
	10^{-6} (normal concn.)	—	29	5·0	4·8	6·5	6·5
2	2×10^{-8}	70	29	2·3	5·1	2·0	3·2
	10^{-6}	—	25	5·8	5·8	4·3	4·4

* Growth attained by the deficient culture as percentage of that reached in the control culture.

Addition of pyridoxal to suspensions of the deficient organisms increased their activity; in some experiments pyridoxal also increased porphyrin formation by cells harvested from media containing optimum concentrations of the vitamin (Table 3). Pyridoxal phosphate also increased synthesis by the deficient cells but it had only about one-half of the activity of pyridoxal on a molar basis. The phosphorylated vitamin is also less active for growth of *Tetrahymena* (Kidder & Dewey, 1951).

Porphyrin synthesis from ALA was not affected by deficiency of pyridoxal (Table 3, last column).

Pantothenate. Deficiency of pantothenate during growth reduced the ability of the organisms to synthesize porphyrin from glycine but did not affect synthesis from ALA (Table 4). The effect was observed only with organisms grown with concentrations of pantothenate which limited growth to 75% or less of the normal value. The Q_{O_2} values for such cells did not differ significantly from those of normal cells, whereas the amount of porphyrin formed from glycine varied from 30–50% of that synthesized by the controls. Addition of pantothenate to suspensions of the deficient cells did not increase porphyrin synthesis (Table 4).

Table 6. *Effect of deficiency of nicotinic acid, thiamine, thioctic acid and pteroylglutamic acid on porphyrin synthesis*

In each experiment organisms were grown in medium T containing the modified concentrations of the vitamins shown in parentheses. The washed organisms were incubated for 48 hr. in mixture I. Results are expressed as % of the values obtained with normal organisms harvested from medium T.

Cells deficient in	Growth of culture*	Activity compared with normal organisms (%)	
		Q_{O_2}	Porphyrin synthesis
Nicotinic acid (2×10^{-7}M)	68	100	52
Thiamine (3×10^{-9}M)	95	83	40
Thioctic acid (5×10^{-10}M)	60	95	66
Pteroylglutamic acid ($2{\cdot}5 \times 10^{-9}$M)	70	100	95

* Growth of culture from which the deficient organisms were harvested as percentage of that reached by the control culture.

Riboflavin. Of the growth factors concerned in the hydrogen-transport mechanisms of the cell, riboflavin had the most marked influence on porphyrin synthesis (Table 5). Organisms grown with limiting concentrations of this vitamin synthesized only 30–50 % of the amount of porphyrin formed by normal cells, though the Q_{O_2} values did not differ. Synthesis from both glycine and from ALA was decreased in organisms deficient in riboflavin; in both cases addition of the vitamin partially restored synthesis (Table 5).

Other vitamins. Organisms deficient in nicotinic acid, thiamine and thioctic acid also had reduced activity with glycine (Table 6), but synthesis from ALA was not impaired. Deficiency in pteroylglutamic acid did not significantly affect formation of porphyrin (Table 6).

Effect of iron

Previous work with the photosynthetic organism *Rhodopseudomonas spheroides* showed that iron salts markedly influenced porphyrin formation (Lascelles, 1956*a*). Addition of iron to suspensions of this organism almost completely prevented porphyrin synthesis from glycine plus α-oxoglutarate. Synthesis from ALA was not prevented by iron but in the presence of iron both protoporphyrin and haematin were formed (Lascelles, 1956*a*, *b*). These substances were not formed in the absence of iron.

Addition of iron citrate to suspensions of *T. vorax* decreased, but did not abolish, porphyrin synthesis from glycine and from ALA (Table 7). However, cells harvested from medium T containing added iron formed no detectable porphyrin from glycine; porphyrin was formed from ALA by the iron-rich cells but the yield was decreased (Table 7). The proportion of protoporphyrin formed by such cells was increased (see later).

Types of porphyrin formed

The porphyrins formed by the suspensions were examined by paper chromatography and by extraction with organic solvents followed by fractionation with HCl (see Methods). Cells harvested from medium T formed a mixture of porphyrins of identical composition when incubated in either mixture I or II. Uroporphyrin predominated, accounting for 50–60 % of the total porphyrins (Fig. 2, Expt. *A*); it behaved mainly as the type III isomer when subjected to paper chromatography by the method of Falk & Benson (1953). Protoporphyrin was also formed (20–30 % of the total) together with coproporphyrin III (15–25 % of the total). These three porphyrins are also formed predominantly by chick-erythrocyte preparations when incubated with ALA or with porphobilinogen (Falk *et al.* 1956).

Effect of iron and pantothenate. Organisms harvested from medium T supplemented with iron citrate also formed a mixture of uro-, proto- and copro-porphyrins from ALA, but the proportion of protoporphyrin was increased at the expense of uroporphyrin; there was little change in the relative amount of coproporphyrin (Fig. 2, Expt. *B*).

Deficiency of pantothenate did not affect the ability of the cells to form uro- and copro-porphyrin from ALA (see above), but synthesis of protoporphyrin was decreased (Fig. 2, Expt. *C*). The effect of deficiency of pantothenate on the formation of protoporphyrin from ALA was more pronounced in organisms grown in the presence of iron

Table 7. *Effect of iron on porphyrin synthesis*

Organisms were grown on medium T without added iron ('normal organisms') or on the same medium supplemented with 4×10^{-6}M-iron citrate ('iron-rich organisms'). The harvested organisms were incubated for 48 hr. in mixture I or II with addition of iron citrate as shown.

Type of organisms	Iron citrate concn. (M)	Porphyrin formed (μm-moles/mg. dry wt. of organisms) Mixture I	Mixture II
Normal	0	4·7	4·9
	5×10^{-6}	4·9	4·0
	10^{-5}	2·7	3·7
'Iron-rich'	0	0	3·0

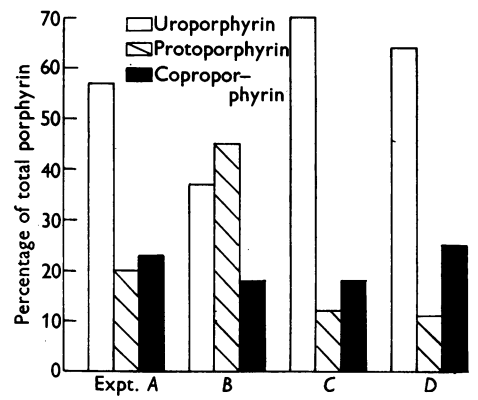

Fig. 2. Proportion of uro-, proto- and copro-porphyrins formed from ALA by organisms grown under various conditions. The cells were harvested from medium T (Expt. *A*), medium T supplemented with 4×10^{-6}M-iron citrate (Expt. *B*), from medium T containing a decreased concentration (8×10^{-8}M) of pantothenate (Expt. *C*), and from medium T containing 8×10^{-8}M-pantothenate and supplemented with 4 μM-iron citrate (Expt. *D*). In each experiment the washed cells were suspended in mixture II and distributed in ten replicate tubes (2·5 ml./tube); incubation was for 48 hr. The replicates were pooled and the proportion of the various porphyrins was determined (see Experimental).

salts (Fig. 2, Expt. *D*). Of the total porphyrin formed by such organisms, protoporphyrin accounted for about 11 % and uroporphyrin for about 64 %; the protoporphyrin formed by organisms grown with optimum concentrations of pantothenate, together with added iron, accounted for 45 % of the total porphyrin (Fig. 2, Expt. B).

DISCUSSION

The accumulation of free porphyrins by this strain of *T. vorax* probably occurs as a result of some derangement at one or more stages in the synthesis of intracellular haem compounds such as cytochromes and haemoglobin; these pigments have been detected in the cells of *T. geleii* (Ryley, 1952; Keilin & Ryley, 1953). The free porphyrins may not be true intermediates in the synthesis of the haem compounds but may be stabilized by-products arising from such intermediates. Dresel & Falk (1956*c*), using isotopic techniques, have provided evidence that free uro-, copro- and proto-porphyrins are not intermediates in the conversion of glycine, ALA or porphobilinogen into haem in erythrocyte preparations from chickens, though they are undoubtedly closely related to the genuine intermediates.

The results suggest that the path of porphyrin synthesis in *T. vorax* is similar to that occurring in higher animals. Glycine increased synthesis by the washed organisms, and ALA was converted into porphyrins. Addition of possible sources of the 4-C intermediate required for condensation with glycine had little effect on synthesis; the intermediate may have been provided by oxidation of endogenous material. The amount of oxidizable substances within the organisms was apparently large, since addition of substrates such as glucose did not increase the Q_{O_2} values; similar findings have been made with suspensions of *T. geleii* (Ryley, 1952). The 4-C intermediate may have arisen from the tricarboxylic acid cycle, individual reactions of which have been demonstrated in *T. geleii* (Seaman, 1955).

The experiments with cells deficient in vitamin B_6 indicate that this factor (presumably in coenzyme form) is necessary for conversion of glycine into porphyrins; synthesis from ALA was not affected. The effect of pyridoxal could be shown even with cells harvested from media containing concentrations of the vitamin sufficient to support full growth. Schulman & Richert (1955) have briefly reported that deficiency of vitamin B_6 in the diet reduces the incorporation of ^{14}C-labelled glycine and succinate into haem by preparations of duck erythrocytes; activity is restored by pyridoxal phosphate. The condensation between glycine and the 4-C intermediate in porphyrin synthesis may be similar to that between glycine and a 1-C derivative to give serine; this latter reaction is mediated by pyridoxal phosphate in both bacteria and in animal tissues (Lascelles & Woods, 1954; Blakley, 1955).

The participation of pantothenate in the initial condensation reaction is also suggested by the experiments with deficient organisms. A similar finding has been made with haemolysates from pantothenate-deficient ducks, which incorporate labelled glycine and succinate into haem less actively than do preparations from normal birds (Schulman & Richert, 1956). The vitamin may be needed for the formation of succinyl-coenzyme A, possibly the 4-C derivative required for condensation with glycine. This compound is a product of the oxidative decarboxylation of α-oxoglutarate in both animal tissues and in some bacteria (Gunsalus, 1953; Sanadi & Littlefield, 1953). There is also evidence that it is formed by the same reaction in cell-free extracts of *T. geleii* (Seaman, 1953). Pantothenate may also be concerned in the formation of protoporphyrin from ALA. Cells deficient in pantothenate showed decreased synthesis of protoporphyrin from ALA with a corresponding increase in the amount of uroporphyrin, suggesting that coenzyme A may participate in the oxidative decarboxylation necessary to produce the vinyl side chains of protoporphyrin.

The effects of nicotinic acid, thiamine and thioctic acid on porphyrin synthesis from glycine could be atrributed to their participation in the tricarboxylic acid cycle, in particular at the stage of oxidative decarboxylation of α-oxoglutarate (Gunsalus, 1953). The need for thioctic acid in the oxidation of α-oxoglutarate by extracts of *T. geleii* has been shown by Seaman (1953); the keto acid also accumulates in cultures of this organism grown with suboptimum concentrations of thioctic acid (Dewey & Kidder, 1952). Deficiency of riboflavin decreased porphyrin synthesis both from glycine and from ALA, suggesting that the vitamin may act in the oxidation of porphobilinogen (formed from ALA) to porphyrin; the overall reaction requires the loss of 6 atoms of hydrogen and 4 molecules of ammonia.

The inability of cells grown with added iron salts to form porphyrins from glycine is consistent with observations made with photosynthetic bacteria (Lascelles, 1955). Another effect of iron, observed with both photosynthetic bacteria and *Tetrahymena*, is in the conversion of ALA into protoporphyrin; with both types of organism iron salts increase the formation of this porphyrin, whereas the proportion of uroporphyrin or coproporphyrin is decreased. Thus this metal has similar effects on porphyrin synthesis by organisms containing bacteriochlorophyll as their main pyrrole component (together with smaller quantities of haematin

compounds) and by those containing only haematin compounds. The mode and locus of action of iron as well as of the B group vitamins cannot be finally ascertained until the enzymes involved in the formation of porphyrins from glycine have been separated and resolved.

SUMMARY

1. Suspensions of *Tetrahymena vorax* synthesize porphyrins when incubated aerobically at 37° with glycine and fumarate; they also convert δ-aminolaevulic acid into porphyrins.

2. Synthesis from glycine by organisms harvested from media containing growth-limiting concentrations of pyridoxal or pantothenate is reduced to 30% or less of that achieved by cells grown with optimum concentrations of these vitamins.

3. Cells deficient in riboflavin, nicotinic acid, thiamine or thioctic acid also form less porphyrin from glycine than do normal cells.

4. Synthesis from δ-aminolaevulic acid by cells deficient in riboflavin is reduced; deficiency in the other vitamins does not decrease the amount of porphyrins formed from this substrate.

5. The porphyrins formed from either glycine or δ-aminolaevulic acid consist of a mixture of uroporphyrin III, protoporphyrin and coproporphyrin III. Cells deficient in pantothenate form less protoporphyrin than do normal cells.

6. Organisms harvested from media containing added iron salts do not form porphyrins from glycine, but do so from δ-aminolaevulic acid. Such cells form more protoporphyrin from this substrate than do organisms grown without added iron.

7. It is concluded that pyridoxal and pantothenate may be concerned directly in the primary stage of porphyrin biosynthesis, which involves condensation between glycine and a 4-C intermediate.

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