

Studies on the Biosynthesis of Porphyrin and Bacteriochlorophyll by *Rhodopseudomonas spheroides*

2. THE EFFECTS OF ETHIONINE AND THREONINE

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Gibson, Neuberger & Tait (1962) mentioned briefly that, when ethionine was added to suspensions of *Rhodopseudomonas spheroides* illuminated in 'mixture I' of Lascelles (1956), large amounts of porphyrin accumulated in the medium, although growth and bacteriochlorophyll synthesis were inhibited. This was rather surprising since other inhibitors of growth prevented the formation of porphyrin and bacteriochlorophyll. In the present paper the effect of ethionine is described in more detail. Of a large number of other compounds only threonine was found to have a similar effect. In the presence of ethionine or threonine the synthesis of bacteriochlorophyll can be restored and porphyrin excretion reduced by methionine or homocysteine. These results suggested that ethionine exerts its effect by interfering with the synthesis or utilization of methionine. This was confirmed when it was found that the methyl ester group of bacteriochlorophyll is formed from the methyl group of methionine. Some of these results have been briefly presented earlier (Neuberger, 1961; Gibson, Matthew, Neuberger & Tait, 1961).

MATERIALS AND METHODS

Chemicals

DL-Homocysteine thiolactone was obtained from Mann Research Laboratories Inc., New York; DL-ethionine from Roche Products Ltd., Welwyn Garden City, Herts; D-ethionine, L-ethionine and DL-homoserine from the California Corp. for Biochemical Research, Los Angeles; L-[Me-¹⁴C]methionine from The Radiochemical Centre, Amersham, Bucks. The sources of other chemicals were as given by Gibson *et al.* (1962).

Methods

The organisms used, the conditions of growth and illumination, and the methods used for colorimetric estimations and for assaying enzymes were as described by Gibson *et al.* (1962).

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RESULTS

Effect of ethionine on the synthesis of porphyrins and bacteriochlorophyll

During investigation of the role of biotin in porphyrin synthesis (Gibson *et al.* 1962), various inhibitors of growth were examined to see whether they would prevent the restoration of growth and the synthesis of porphyrins and bacteriochlorophyll when deficient organisms are illuminated in the presence of biotin. The compounds tested were DL-*p*-fluorophenylalanine, 8-azaguanine, 5-bromouracil and DL-ethionine. The first three are partly successful in preventing growth and porphyrin and bacteriochlorophyll synthesis when added with biotin to biotin-deficient organisms illuminated in mixture I. In the presence of DL-ethionine, although no growth or bacteriochlorophyll synthesis occurs, a large amount of porphyrin is excreted into the medium, even in the absence of added biotin. Table 1 shows the results with DL-ethionine and also with DL-*p*-fluorophenylalanine. Ethionine has the same effect on anaerobically grown organisms as it has on aerobically grown organisms (Table 2). Table 2 also shows that the effect of ethionine in promoting accumulation of porphyrins decreases as the degree of biotin deficiency of the organisms increases. Identical results are obtained when organisms deficient in nicotinic acid or thiamine are illuminated in mixture I with the addition of DL-ethionine.

The absorption spectrum over the range 355–610 m μ of the porphyrins accumulating in the presence of ethionine is identical with that of the porphyrins accumulating in its absence. The maximum of the Soret band in N-hydrochloric acid is 401 m μ . Paper chromatography of the porphyrins in the lutidine–water system of Nicholas & Rimington (1949) shows that no new porphyrins are formed in the presence of ethionine, and none of the porphyrins normally present is absent. The major porphyrin both in the presence and absence of ethionine is coproporphyrin.

Ethionine also stimulates porphyrin synthesis and inhibits formation of bacteriochlorophyll when

added to organisms illuminated in succinate and glycine ('mixture IS', see Table 3). As pointed out by Gibson *et al.* (1962); (see also Lascelles, 1959), when organisms are illuminated in aminolaevulate

('mixture II') they accumulate porphyrins although they do not grow or form bacteriochlorophyll. Ethionine also stimulates porphyrin synthesis in mixture II, which contains aminolaevulate

Table 1. *Effect of DL-p-fluorophenylalanine and DL-ethionine on growth and the synthesis of porphyrin and bacteriochlorophyll in mixture I*

Organisms grown aerobically in the dark in medium S containing 0.8 μM -biotin were harvested and illuminated under standard conditions for 26 hr. in mixture I. DL-p-Fluorophenylalanine (10 mM), DL-ethionine (10 mM) and biotin (40 μM) were added where stated. In this and subsequent Tables the concentration of bacteriochlorophyll and porphyrins is expressed as μM -moles/ml. of illumination mixture. Non-illuminated controls were kept at 0° in the dark; under these conditions the concentration of bacteriochlorophyll does not alter.

Additions	Dry wt. of organisms (mg./ml.)	Porphyrins (μM -moles/ml.)	Bacteriochlorophyll (μM -moles/ml.)
None	1.08	2.8	10.8
p-Fluorophenylalanine	0.60	3.9	7.8
Ethionine	1.26	30.5	7.0
Biotin	3.24	4.9	38.6
p-Fluorophenylalanine + biotin	0.54	4.4	10.5
Ethionine + biotin	1.26	29.0	6.6
None (non-illuminated control)	0.96	1.1	4.9

Table 2. *Effect of DL-ethionine on the synthesis of porphyrin and bacteriochlorophyll by normal and biotin-deficient organisms*

Organisms were grown anaerobically in the light in medium S containing the concentration of biotin stated. They were then illuminated under standard conditions for 20 hr. in mixture I. DL-Ethionine (5 mM) was added where stated.

Concn. of biotin in growth medium (μM)	Additions to illumination mixture	Porphyrins (μM -moles/ml.)	Bacteriochlorophyll (μM -moles/ml.)
40	None	13.2	17.6
40	DL-Ethionine	57.6	6.4
40	None (non-illuminated control)	0.0	12.2
2	None	4.8	8.0
2	DL-Ethionine	53.3	6.7
2	None (non-illuminated control)	0.0	11.7
0.8	None	3.6	4.7
0.8	DL-Ethionine	32.7	4.0
0.8	None (non-illuminated control)	0.0	8.0
0.4	None	1.8	3.6
0.4	DL-Ethionine	6.3	2.1
0.4	None (non-illuminated control)	0.0	3.3

Table 3. *Effect of DL-ethionine on the synthesis of porphyrin and bacteriochlorophyll by organisms illuminated in different media*

Organisms grown aerobically in the dark in medium S containing 0.8 μM -biotin were harvested, and illuminated under standard conditions for 26 hr. in the media stated. DL-Ethionine (10 mM) and biotin (40 μM) were added where stated.

Medium	Additions	Dry wt. of organisms (mg./ml.)	Porphyrins (μM -moles/ml.)	Bacteriochlorophyll (μM -moles/ml.)
Mixture IS	None	0.87	3.6	3.7
Mixture IS	Biotin	1.86	28.2	8.1
Mixture IS	DL-Ethionine	1.17	37.2	3.6
Mixture II	None	0.87	11.8	2.3
Mixture II	Biotin	0.85	10.7	1.2
Mixture II	DL-Ethionine	0.93	38.1	2.9
Non-incubated control		0.93	0.0	1.8

(Table 3), but this effect appears to differ in nature from that in mixture I or IS (see below).

As shown in Fig. 1, DL-ethionine (final concentration 5 mM), when added to non-deficient organisms illuminated in mixture I, completely inhibits the synthesis of bacteriochlorophyll and carotenoids and only partly inhibits growth. However, the molar amount of porphyrin accumulating in the presence of ethionine is twice as great as the sum of the porphyrin and bacteriochlorophyll formed in its absence. In a number of experiments this ratio was 3-4 (Table 4). Table 4 shows that even 0.1 mM-ethionine stimulates porphyrin production, i.e. at a concentration at which it reduces bacteriochlorophyll synthesis only slightly. At equal concentrations of each the ratios of porphyrin accumulating were 1.5:1.2:1.0 for the L-, DL-, and D-isomers respectively.

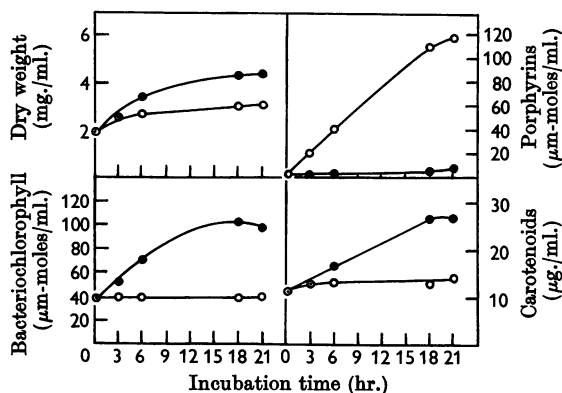


Fig. 1. Effect of DL-ethionine on growth and the synthesis of porphyrin, bacteriochlorophyll and carotenoid. Organisms, grown anaerobically in the light in medium S, were illuminated at 2 mg. dry wt./ml. in mixture I. ●, Without additions; ○, in the presence of DL-ethionine (5 mM).

Table 4. Effect of different concentrations of DL-ethionine on the synthesis of porphyrin and bacteriochlorophyll in mixture I

Organisms grown anaerobically in the light in medium S were illuminated under standard conditions for 20 hr. in mixture I. DL-Ethionine was added at the concentration stated.

Concn. of DL-ethionine (mM)	Porphyrin ($\mu\text{m-moles/ml.}$)	Bacteriochlorophyll ($\mu\text{m-moles/ml.}$)
5	67.8	12.3
1	95.0	15.8
0.5	80.0	16.3
0.1	19.0	23.8
0	5.7	26.7
0*	0.0	13.0

* Non-illuminated control.

Ethionine does not stimulate porphyrin excretion when added to organisms incubated aerobically in the dark in mixture I. No porphyrin or bacteriochlorophyll is normally formed under these conditions. For the synthesis of porphyrins and bacteriochlorophyll by organisms illuminated in mixture I, Lascelles (1956, 1959) found that all the components were necessary. In the presence of ethionine, ammonium phosphate can be omitted. Of the metal ions in mixture I (Mg^{2+} , Ca^{2+} , Mn^{2+}), only Mg^{2+} ions are required for ethionine to show a large effect on porphyrin excretion.

Effect of threonine on the synthesis of porphyrins and bacteriochlorophyll

In an attempt to elucidate the mechanism of action of ethionine, a number of compounds were tested to see whether they had a similar effect when added to organisms illuminated in mixture I. The compounds, which were all tested at concentrations between 1 and 10 mM, were DL-methionine, DL-homocystine, DL-homocysteine thiolactone, L-cysteine, reduced glutathione and L- and D-penicillamine, all of which contain sulphur; choline, betaine, formaldehyde, DL-serine and methanol, all of which are capable of yielding biologically active C_1 -fragments; DL-norleucine, which like ethionine is an antagonist of methionine for protein synthesis (Vaughan & Steinberg, 1959); acetic acid, acetaldehyde, ethanol, L-aspartic acid, L-asparagine, DL-tyrosine, L-glutamic acid, L-glutamine, DL-alanine, DL-tryptophan, DL-threonine, L-arginine, L-lysine, pyridoxine, pyridoxamine, DL- β -hydroxybutyric acid and aminoacetone. Some of these compounds slightly stimulate porphyrin production, some slightly depress it, and others have no effect at all. The only compound which was found to have a marked effect is DL-threonine, which stimulates porphyrin accumulation to about the same extent as does ethionine and also inhibits the synthesis of bacteriochlorophyll (Table 5). At higher concentrations threonine also inhibits growth. Threonine also resembles ethionine in stimulating porphyrin production by biotin-deficient organisms in the absence of added biotin. Whereas 0.1 mM-ethionine shows an appreciable effect, the lowest concentration of threonine which is active is 1 mM. L-Threonine and DL-threonine at a concentration of 5 mM stimulate porphyrin production to about the same extent. The porphyrins accumulating in the presence of threonine were found to be the same as those formed in its absence, coproporphyrin again being the major component.

Ethionine has no effect on aminolaevulate-synthetase or aminolaevulate-dehydratase activity in cell-free systems. When organisms are illuminated in mixture I in the presence of ethionine there is no increase in the activity of either of these

enzymes. Neither ethionine nor threonine can replace L-alanine in the L-alanine- γ - δ -dioxovalerate-aminotransferase (transaminase) reaction, nor do they inhibit that reaction.

Reversal of the effects of ethionine and threonine

As shown in Table 5 added L-methionine or DL-homocysteine thiolactone (final concentration 1 mM) by themselves have no significant effect on the synthesis of porphyrins or bacteriochlorophyll by organisms illuminated in mixture I. However, MM-methionine completely reverses the effect of MM-ethionine or 5 MM-threonine; 0.1 mM-methionine abolished the effect of ethionine completely in some experiments but only partly in others. Added homocysteine thiolactone (final concentration 1 mM) reverses the effect of ethionine partly and of threonine completely. In an illumination mixture from which organisms were omitted, the concentration of sulphhydryl groups at the end of 8 hr. was 25% of the theoretical maximum. It is therefore probable that homocysteine itself is the active compound in these experiments. Homoserine (final concentration 1 mM) completely reverses the action of 5 mM-threonine, but does not

antagonize the effect of MM-ethionine, either by itself or in the presence of MM-cysteine (Table 6). DL-Cysteine, DL-homocystine, DL-norleucine and pyruvate, at a final concentration of 1 mM, have no effect. The following compounds which were tested only in the presence of ethionine are also inactive: choline, betaine, glutathione, DL-penicillamine and L-penicillamine. Formaldehyde (final concentration 2.5 mM) inhibits porphyrin synthesis in the presence of MM-ethionine and 5 mM-threonine, but no synthesis of bacteriochlorophyll was observed; 0.5 mM-formaldehyde has no effect.

In contrast with the findings discussed above, which were obtained in mixture I, methionine stimulates the production of porphyrins to about the same extent as ethionine when organisms are illuminated in the presence of aminolaevulate (mixture II), as shown in Table 7. The effect of homocystine is qualitatively similar. It is possible that these substances are converted in the cell into sulphhydryl compounds, which are known to activate the enzymes involved in the conversion of aminolaevulate into coproporphyrinogen (Gibson, Neuberger & Scott, 1955; Granick & Mauzerall, 1958; Mauzerall & Granick, 1958).

Table 5. *Reversal of the effect of ethionine and threonine by methionine and homocysteine*

Organisms grown anaerobically in the light were illuminated under standard conditions for 21 hr. in mixture I. DL-Threonine (5 mM), L-ethionine, L-methionine and DL-homocysteine thiolactone (all at a concentration of 1 mM) were added where stated.

Additions	Porphyrins (μ m-moles/ml.)	Bacterio- chlorophyll (μ m-moles/ml.)
None	16.2	45.1
L-Methionine	12.8	53.8
DL-Homocysteine thiolactone	15.8	49.8
L-Ethionine	77.0	26.4
L-Ethionine + L-methionine	17.2	51.3
L-Ethionine + DL-homocysteine thiolactone	34.4	49.5
DL-Threonine	77.0	32.3
DL-Threonine + L-methionine	19.0	53.0
DL-Threonine + DL-homocysteine thiolactone	18.1	55.0
None (non-illuminated control)	0.0	26.5

Table 6. *Effect of DL-homoserine on the stimulation of porphyrin production and inhibition of bacteriochlorophyll synthesis caused by ethionine and threonine*

Organisms grown anaerobically in the light were illuminated under standard conditions for 21 hr. in mixture I. DL-Threonine (5 mM), DL-homoserine (2 mM), L-ethionine (mM) and DL-cysteine (mM) were added where stated.

Additions	Porphyrins (μ m-moles/ml.)	Bacterio- chlorophyll (μ m-moles/ml.)
None	23.4	28.4
L-Ethionine	77.5	21.4
DL-Homoserine	25.7	26.0
DL-Cysteine	29.7	26.7
L-Ethionine + DL-homoserine	82.0	21.8
L-Ethionine + DL-homoserine + DL-cysteine	101.9	21.8
DL-Threonine	70.8	23.0
DL-Threonine + DL-homoserine	25.2	27.2
DL-Threonine + DL-homoserine + DL-cysteine	19.3	28.9
None (non-illuminated control)	0.0	20.0

Reversal of the effects of ethionine and threonine by ferrous ions

Lascelles (1956) showed that addition of Fe^{2+} ions to mixture I decreased the amount of porphyrin excreted and increased the amount of bacteriochlorophyll formed. The increase of bacteriochlorophyll in the presence of Fe^{2+} ions was much less than the change in the porphyrin excreted.

The effect on porphyrin production of the addition of different amounts of ferrous ammonium sulphate to organisms illuminated in mixture IS in the presence of different amounts of ethionine and threonine is shown in Table 8. The effect of ethionine at concentrations below about 1 mM can be reversed by addition of ferrous ammonium sulphate, but at a concentration of 5 mM the addition of ferrous ammonium sulphate even at a concentration of 0.1 mM does not alter the amount of porphyrin excreted. The results with 5 mM-threonine are very similar to those with mM-ethionine.

Origin of the methyl ester group of bacteriochlorophyll

The results reported above suggest that ethionine and threonine inhibit the biosynthesis of bacteriochlorophyll by interfering with some reaction in-

volving methionine. One possibility was that the methyl ester group of bacteriochlorophyll might be derived from methionine. The only previous report on the origin of this methyl ester group is that of Green, Altman, Richmond & Salomon (1957), who showed that chlorophyll isolated from cultures of *Chlorella vulgaris* grown in the presence of [^{14}C]-formate was labelled in the methyl ester group but not in the rest of the molecule. It was therefore decided to test this directly with [$Me\text{-}^{14}\text{C}$]methionine.

Anaerobically grown *Rps. spheroides* (2 mg. dry wt./ml.) were illuminated for 18 hr. at 34° in mixture I containing L- [$Me\text{-}^{14}\text{C}$]methionine of various concentrations and specific radioactivities in a total volume of 100 ml. During the incubation the dry weight of the organisms doubled, as did the amount of bacteriochlorophyll and carotenoid. A control of identical composition was kept in the dark at 0° . After illumination, portions of the experimental and control mixtures were removed for estimation of bacteriochlorophyll and carotenoid. The remainder was harvested by centrifuging, washed with water and made into a thick suspension which was crushed in the Hughes (1951) press.

The pigments were isolated by a method modified from that described by Komen (1956). The crushed organisms were extracted with acetone, a small volume of light petroleum (b.p. $60\text{--}80^\circ$) was added, and the acetone was removed by passing the solution three times through a large volume of water. The pigments remained in the light-petroleum layer, which was removed, dried over MgSO_4 and evaporated under reduced pressure. The residue was dissolved in the minimum volume of light petroleum containing a trace of CCl_4 to facilitate dissolution of the bacteriochlorophyll.

Sucrose (commercial sugar) was powdered in a mortar and sieved (40 mesh). A thick sludge was made in light petroleum and a column prepared and allowed to settle. The pigment solution was applied to the column. Elution with light petroleum brought off first the yellow then the red carotenoid which were collected together. The bacteriochlorophyll, which remained as a blue band at the top of the column during elution with light petroleum, was removed with light petroleum-acetone (9:1, v/v). This preparation was pure as judged by the spectrum between 400 and $830\text{ m}\mu$, which was identical with that given by Komen (1956).

Table 7. *Effect of DL-ethionine, DL-methionine and DL-homocystine on the production of porphyrins by organisms illuminated in mixture II*

Organisms were grown anaerobically in the light in medium S, then illuminated under standard conditions for 20 hr. in mixture II. DL-Ethionine (mM), DL-methionine (5 mM) and DL-homocystine (5 mM) were added where stated.

Expt.	Additions	Porphyrins ($\mu\text{m-moles/ml.}$)
1	None	21.6
	DL-Ethionine	57.8
	DL-Methionine	63.2
2	None	9.4
	DL-Homocystine	21.4

Table 8. *Effect of ferrous ions on porphyrin production in mixture IS in the presence and absence of ethionine and threonine*

Organisms grown anaerobically in the light were illuminated under standard conditions for 20 hr. in mixture IS. DL-Ethionine, DL-threonine and ferrous ammonium sulphate were added where stated.

Concn. of $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4$ (μM) ...	Porphyrins ($\mu\text{m-moles/ml.}$)			
	0	1	10	100
Additions (concn.)				
None	11.6	4.4	1.7	0.9
DL-Ethionine (0.25 mM)	32.2	18.7	7.9	4.2
DL-Ethionine (mM)	51.2	37.5	20.7	17.6
DL-Ethionine (5 mM)	44.6	47.5	48.9	48.9
DL-Threonine (5 mM)	50.0	—*	24.5	—

* Not determined.

Table 9. *Radioactivity of the pigments on illumination of organisms in the presence of L-[Me-¹⁴C]methionine*

Organisms were grown anaerobically in the light then illuminated in mixture I plus L-[Me-¹⁴C]methionine as described in the text. The extraction and purification of pigments is described in the text. Samples of the purified pigments were plated out on 2 cm.² polythene planchets and their radioactivity was determined at infinite thinness in a Geiger end-window counter. A linear increase in counts with increasing amounts of bacteriochlorophyll was obtained up to 300 µg./2 cm.² and all samples were counted at concentrations lower than this.

Expt.	Concn. of L-methionine (mM)	Radioactivity (counts/min./µmole)		
		Methionine	Bacteriochlorophyll	Carotenoids
1	0.1	28 970	3 022	5 586
2	0.1	28 970	3 600	—*
2	0.5	5 800	1 883	—

* Not determined.

The incorporation of radioactivity from L-[Me-¹⁴C]methionine into bacteriochlorophyll on illumination of organisms in mixture I is shown in Table 9. The specific radioactivities of the pigments are expressed/unit synthesized during illumination assuming that there is no metabolic turnover. It is clear that the methyl group of methionine is an effective precursor of some carbon atom of bacteriochlorophyll. In Expt. 2 of Table 9 the effect of varying the concentration of methionine on the molar radioactivity of the bacteriochlorophyll was studied. With 0.5 mM-L-methionine the radioactivity was transferred with a threefold dilution of molar radioactivity. In Expt. 1 of Table 9 the mixed carotenoids were also highly labelled. This is not surprising since both the carotenoids contain one methoxy group/molecule (Stanier, 1960) which, by analogy with other methoxy groups which have been investigated, is probably formed from *S*-adenosylmethionine (cf. Axelrod & Tomchick, 1958).

Degradation of the bacteriochlorophyll and isolation of fragments

The bacteriochlorophyll isolated in Expt. 1 of Table 9 was degraded by a method similar to that described by Fischer & Stern (1940), who showed that alkaline hydrolysis splits bacteriochlorophyll into bacteriochlorin *e*₈, phytol, methanol and Mg²⁺ ions.

Labelled bacteriochlorophyll (1.78 mg.) was dissolved in 24% (w/v) methanolic potassium hydroxide (1.5 ml.) and the solution was refluxed under nitrogen for 3 hr. The methanol, containing the methanol derived from the methyl ester of bacteriochlorophyll, was distilled off, collected in a receiver cooled in solid CO₂, and kept at -20° until required. The residue was redissolved in methanol and shaken with ether and water. The ether phase was removed and the aqueous phase was re-extracted with ether. The combined ether extracts were dried to give a residue which contained phytol. The aqueous phase was acidified to pH 1.5 with HCl and extracted with ether until all the

Table 10. *Radioactivity in fragments of bacteriochlorophyll*

The purified bacteriochlorophyll of Expt. 1 of Table 9 was degraded and the fragments isolated as described in the text. The radioactivity of the samples was determined by scintillation counting as described in the text. The values are expressed/unit of bacteriochlorophyll assuming that 100% of the fragments of the bacteriochlorophyll was recovered.

Fraction	Radioactivity (counts/min./µmole)
Bacteriochlorophyll	9800
Methanol	9920
Phytol	64
Bacteriochlorin	6

green pigments were removed. This ether extract contains the bacteriochlorin. In paper chromatography in 2,6-lutidine-water [5:3, v/v (Falk, Dresel, Benson & Knight, 1956)] more than one porphyrin spot was obtained, showing that some of the bacteriochlorin had been degraded. However, the complete fraction was estimated by liquid scintillation counting. The samples, where necessary, were dissolved in 0.5 ml. of methanol and mixed with 5 ml. of the dioxan-based scintillator of Bray (1960). The samples were counted in a Panax scintillation counter with the discriminator bias set at 12 v and the external high tension at 1480 v. The solutions of bacteriochlorophyll and bacteriochlorin quenched the scintillation. The specific radioactivity of the bacteriochlorophyll was determined by extrapolation to zero concentration. The radioactivity of the bacteriochlorin was found to be extremely small: when the bacteriochlorin was plated on a 2 cm.² polythene planchet and counted with a Geiger end-window counter, only 6 counts/min./µmole were observed.

Table 10 shows that virtually all the radioactivity originally present in the bacteriochlorophyll is confined to the methyl ester group, the counts of the phytol and bacteriochlorin being negligible. The specific radioactivity of the methanol derived from the methyl ester group of bacteriochlorophyll was calculated from the fact that a known amount of bacteriochlorophyll was dissolved in a known

volume of methanolic potassium hydroxide, and therefore a given volume of the methanol distillate contained the methanol derived from a known weight of bacteriochlorophyll.

DISCUSSION

Origin of the methyl ester group of bacteriochlorophyll

Green *et al.* (1957) found considerable radioactivity in the methyl ester group of chlorophyll, but not in the rest of the molecule, when *Chlorella vulgaris* was grown in the presence of [¹⁴C]formate. This showed that C₁-compounds are involved in the synthesis of this methyl group, but the appreciable dilution of radioactivity indicated that formate was not a direct or highly specific precursor. The relatively small dilution observed in the present work with the methyl group of methionine (Table 9) shows that the latter is a more immediate precursor than formate. No other carbon atom of bacteriochlorophyll was significantly labelled. In one experiment radioactivity was found both in bacteriochlorophyll and in the carotenoids. The two major carotenoids of *Rps. spheroides* both contain one methoxy group/molecule. The molar radioactivity of the mixed carotenoids was about 80% higher than that of bacteriochlorophyll. However, as the carotenoids were not degraded, it is impossible to say whether only the methoxy carbon atoms were labelled. Moreover, the assumption made in the calculations that no metabolic turnover of bacteriochlorophyll or carotenoids occurs during illumination may not be correct, and indeed the two types of pigment may turn over at different rates. However, the low dilution (in one experiment only 3·1) observed in the conversion of the methyl group of methionine into the methyl ester group of bacteriochlorophyll, and the fact that molar radioactivities of the latter compound and of the mixed carotenoids are of the same order of magnitude, indicate strongly that methionine is a specific and fairly direct precursor of the methyl ester and methyl ether groups respectively in both types of pigment. Tait & Gibson (1961) have shown that the immediate precursor of the methyl ester group of bacteriochlorophyll is *S*-adenosylmethionine, since chromatophores isolated from *Rps. spheroides* catalysed the formation of magnesium protoporphyrin monomethyl ester from magnesium protoporphyrin and *S*-adenosylmethionine.

Ethionine, threonine and the synthesis of bacteriochlorophyll

When *Rps. spheroides* is illuminated in the presence of ethionine, the synthesis of bacteriochlorophyll is strongly inhibited. This inhibition can be overcome by the addition of methionine or

its precursor homocysteine, indicating that ethionine exerts its effect by acting as an antagonist of methionine. Two mechanisms for such an antagonism can be considered. First, the synthesis of bacteriochlorophyll is coupled to growth, so that if growth is inhibited the pigment is not formed (Gibson *et al.* 1962). This is probably the mechanism by which *p*-fluorophenylalanine, 8-azaguanine and 5-bromouracil prevent the synthesis of bacteriochlorophyll. Since the production of porphyrins from α -oxoglutarate and glycine is also coupled to growth (Gibson *et al.* 1962), this process would be expected to be decreased as well, and this is so when these three inhibitors are employed. By contrast, ethionine markedly stimulates the production of porphyrins. Further evidence which suggests that the action of ethionine is dissimilar to that of the other inhibitors of growth is the fact that norleucine, which also antagonizes methionine in its role as a constituent of proteins, has no effect on the synthesis of bacteriochlorophyll or porphyrins.

Secondly, ethionine might interfere with the utilization of methionine in a specific reaction involved in the synthesis of bacteriochlorophyll. It is now clear that this is the true explanation of the action of ethionine, and that the specific reaction which is inhibited is that leading to the formation of the methyl ester group of bacteriochlorophyll. At an enzymic level it has been found that *S*-adenosylethionine inhibits the methylation of magnesium protoporphyrin (K. D. Gibson, A. Neuberger & G. H. Tait, unpublished work). The inhibition of carotenoid synthesis observed in organisms illuminated in the presence of ethionine may be brought about by a similar mechanism, but this has not been established.

The action of threonine on the synthesis of bacteriochlorophyll is more complicated. The striking similarity between the results obtained with threonine and those with ethionine strongly suggests that threonine also exerts its effect by inhibiting the formation of the methyl ester group of bacteriochlorophyll. As shown by Gibson *et al.* (1962), cultures of *Rps. spheroides* grow when they are illuminated under the conditions employed in this work. The media used for illumination, namely mixtures I and IS, contain no methionine, and it must be presumed that the organisms synthesize this compound during incubation. The pathways for the synthesis of threonine and methionine have several steps in common, i.e. those in which aspartic acid is converted, via β -aspartyl phosphate and aspartic β -semialdehyde, into homoserine (Black & Wright, 1955*a, b, c*). Homoserine can be converted either into threonine (Watanabe & Shimura, 1955) or into homocysteine and methionine in succession (Teas, Horowitz & Fling, 1948;

Berg, 1951). A probable explanation for the effect of threonine on the synthesis of bacteriochlorophyll in *Rps. spheroides* is that it inhibits the synthesis of homoserine and thus also of methionine. If this is correct, the action of threonine should be reversed by homoserine as well as by methionine and homocysteine, and this was found to be so (Table 6). In *Escherichia coli* and *Saccharomyces cerevisiae* threonine inhibits the phosphorylation of aspartic acid (Stadtman, Cohen, Lebras & de Robichon-Szulmajster, 1961), whereas in *Micrococcus glutamicus* threonine inhibits the conversion of aspartic β -semialdehyde into homoserine (Nara *et al.* 1961). In extracts of *Rps. spheroides* also threonine competitively inhibits homoserine dehydrogenase (Gibson *et al.* 1961).

Ethionine, threonine and the formation of porphyrins

The production of porphyrins by organisms illuminated in the presence of α -oxoglutarate and glycine is 'coupled' to the synthesis of bacteriochlorophyll, in that if the latter process does not take place neither does the former (Gibson *et al.* 1962). This has been observed under various experimental conditions, including the presence of *p*-fluorophenylalanine, 8-azaguanine and 5-bromouracil as shown in the present paper. In contrast, ethionine and threonine appear to uncouple porphyrin formation from the synthesis of bacteriochlorophyll. Though they decrease the amount of bacteriochlorophyll formed, they markedly stimulate the accumulation of porphyrin. There are several striking features of this stimulation. First, the amount of porphyrin produced in the presence of ethionine or threonine is much greater than the sum of the porphyrin and bacteriochlorophyll formed in their absence. Secondly, as with bacteriochlorophyll, the effect of ethionine and threonine on porphyrin formation is reversed by methionine and homocysteine. This indicates that their action on the synthesis of porphyrin is connected with the fact that in these experiments they both interfere with some specific role of methionine. A third point is illustrated in Fig. 2 [incorporating the data of Table 7 of Gibson *et al.* (1962) and Table 2 of the present paper], which shows the concentrations of aminolaevulate synthetase in extracts of organisms grown with different concentrations of biotin, together with the amounts of porphyrin which these cultures accumulate when illuminated in the presence of α -oxoglutarate and glycine with and without ethionine and in the absence of biotin. As discussed above only organisms grown with an excess of biotin can form appreciable amounts of porphyrin when illuminated in the absence of ethionine. There is no relation between the amount of porphyrin produced under these conditions and

the concentration of aminolaevulate synthetase. On the other hand there is a marked correlation between the concentration of the enzyme and the amount of porphyrin produced when ethionine is present.

These observations strongly suggest that ethionine and threonine stimulate the formation of porphyrins by interfering with mechanisms which regulate the synthesis of aminolaevulate. Gibson *et al.* (1962) pointed out that the coupling of porphyrin formation to the synthesis of bacteriochlorophyll almost certainly includes a means for regulating the synthesis of aminolaevulate. Three types of regulation other than enzyme repression were discussed, involving respectively variations in the concentrations of the substrates, alterations in amount and availability of pyridoxal phosphate and specific inhibition of aminolaevulate synthetase. It is difficult to see how two substances as dissimilar in their structure and metabolism as ethionine and threonine could influence either the concentrations of succinyl-CoA or glycine or the activation of aminolaevulate synthetase by pyridoxal phosphate. Thus the most likely explanation of the action of ethionine and threonine is that they reverse some specific inhibition of the synthesis of aminolaevulate.

The nature of the postulated inhibition of aminolaevulate synthetase was discussed by Gibson *et al.* (1962), who pointed out that no physiological inhibitor has yet been definitely identified. The results with ethionine and threonine encourage

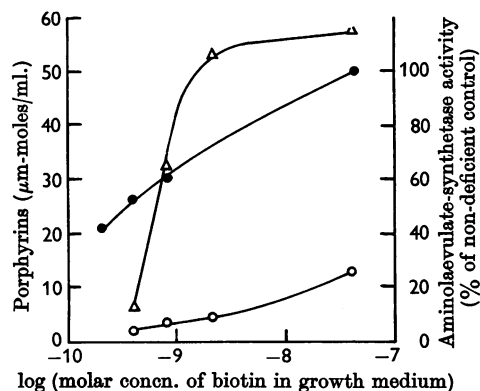


Fig. 2. Aminolaevulate-synthetase activity and porphyrin formation. Organisms were grown anaerobically in the light in medium S containing the amounts of biotin shown. At the end of growth portions of the organisms were crushed and aminolaevulate synthetase activity was estimated in extracts (●). Portions of the organisms were also illuminated under standard conditions in mixture I, without additions (○) or in the presence of DL-ethionine (5 mM) (Δ), for 20 hr. and the amount of porphyrin formed was estimated.

certain speculations on this point. Inhibition of the synthesis of bacteriochlorophyll at the step in which magnesium protoporphyrin is methylated would decrease the concentrations of later intermediates. It is suggested that accumulation of one of these later intermediates in bacteriochlorophyll synthesis is responsible for the inhibition of the formation of aminolaevulate. The proposition was made more specific by Gibson *et al.* (1961), who put forward the hypothesis that one of the intermediates was a feed-back inhibitor of aminolaevulate synthetase. This hypothesis has one drawback: the enzyme which methylates magnesium protoporphyrin is confined to the chromatophores (Tait & Gibson, 1961), and it seems reasonable to suppose that the reactions leading from magnesium protoporphyrin monomethyl ester to bacteriochlorophyll are also carried out there; however, aminolaevulate synthetase is in the cytoplasm (Kikuchi, Kumar, Talmage & Shemin, 1958; Gibson, 1958). A substance formed in the chromatophores would have to diffuse into the cytoplasm to inhibit aminolaevulate synthetase, and no example of feed-back inhibition yet established involves any barrier to the free diffusion of the inhibitor; indeed any such barrier would seem likely to impede the proper functioning of this type of metabolic control. Hence the mechanism by which an intermediate in the synthesis of bacteriochlorophyll might inhibit the formation of aminolaevulate is probably more complicated than direct feed-back inhibition. However, at present too little experimental evidence can be adduced in favour of any alternative hypothesis to make further discussion profitable.

SUMMARY

1. Cultures of *Rhodospseudomonas spheroides* grown with suboptimum amounts of biotin grow and form porphyrins and bacteriochlorophyll on subsequent illumination in a medium containing α -oxoglutarate and glycine with the addition of biotin. Growth and formation of both pigments is prevented by *p*-fluorophenylalanine, 8-azaguanine and 5-bromouracil.

2. Growth and formation of bacteriochlorophyll are also inhibited by both D- and L-ethionine. In contrast, the production of porphyrin is stimulated, even when biotin is not added to the illumination medium. Ethionine has the same effect on organisms grown in a complete medium. The amount of porphyrin formed in the presence of ethionine is at least twice as great as the sum of the porphyrin and bacteriochlorophyll formed in its absence.

3. Of a large number of compounds tested only DL-threonine resembles ethionine in stimulating porphyrin production and decreasing the synthesis of bacteriochlorophyll.

4. The effects of both ethionine and threonine are reversed by addition of methionine or homocysteine thiolactone. The effect of threonine is also reversed by the addition of homoserine.

5. These results suggested that methionine might be specifically concerned in the biosynthesis of bacteriochlorophyll and particularly of the methyl ester group. Organisms were illuminated in the presence of [Me - ^{14}C]methionine and bacteriochlorophyll was isolated and degraded to yield methanol, phytol and bacteriochlorin. The bacteriochlorophyll possessed high radioactivity which was almost exclusively in the methyl ester group. In one experiment total carotenoids were isolated and found to have a molar radioactivity of the same order of magnitude as the bacteriochlorophyll.

6. The significance of these results is discussed. In particular it is concluded that ethionine inhibits the synthesis of bacteriochlorophyll by interfering with the formation of the methyl ester group, and that threonine, by virtue of the fact that it shares a common biosynthetic pathway with methionine, inhibits the synthesis of the latter compound.

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Physicochemical Studies on Ovalbumin

3. THE SULPHYDRYL AND DISULPHIDE CONTENTS OF OVALBUMIN AND AN IODINE-MODIFIED DERIVATIVE*

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The well-known instability of aqueous solutions of ovalbumin at room temperature often causes difficulty in the interpretation of physicochemical measurements on the substance. We attempted to prepare more stable derivatives by modifying the sulphhydryl groups of the cysteine residues, since these groups are probably implicated in the aggregation process (Frensdorff, Watson & Kauzmann, 1953; Halwer, 1954; cf. McKenzie, Smith & Wake, 1955). Of those reagents for which specificity for sulphhydryl groups has been claimed (cf. reviews by Olcott & Fraenkel-Conrat, 1947; Herriott, 1947; Putnam, 1953), iodine was selected because the available information indicated that the reaction was irreversible and the product stable. However, there is conflicting evidence concerning the specificity of the reaction. Anson & Stanley (1941) found conditions under which about 5 atoms of iodine reacted per molecule of ovalbumin, and concluded that the reaction was specific, under rather restricted conditions, for the oxidation of sulphhydryl groups to disulphide linkages because the modified material no longer gave a nitroprusside test after denaturation, and earlier studies (Greenstein, 1938; Brand & Kassel, 1940; Anson, 1941) had all indicated the presence of 5 sulphhydryl groups/molecule. However, since the sedimentation coefficients of the native and modified materials were found to be essentially identical (Anson, 1942), it appeared that the reaction was entirely intramolecular. Since at least 1 intermolecular

disulphide bridge must be formed if 5 sulphhydryl groups are converted into disulphide linkages, the reported stoichiometry is incompatible with specificity. We therefore investigated the stoichiometry of the reaction, and the sulphhydryl and disulphide content of starting material and product. In addition, the product has been analysed for iodine by a radiochemical procedure.

Part of this work has been described by Winzor (1960); a preliminary report was presented at the Symposium on Proteins held at Parkville, Victoria, Australia, on 10–11 September 1959, the proceedings of which have been summarized by Thompson (1959).

While this investigation was in progress, Cunningham & Nuenke (1959, 1960) reported studies of the reaction between iodine and ovalbumin, carried out under slightly different conditions, and yielding a product which, as discussed below, probably differs from the one we obtained.

EXPERIMENTAL

Ovalbumin. Four samples of crystalline ovalbumin were used. The first two, denoted ACS2 and ACS3 (Creeth, Nichol & Winzor, 1958), were prepared by the method of La Rosa (1927), and consisted of a paste of protein crystals and ammonium sulphate. The third, denoted ACK, was prepared by Dr R. A. Kekwick according to the procedure of Kekwick & Cannan (1936), and consisted of a dry powder containing much sodium sulphate. The fourth, denoted AC Ar, was a commercial crystallized material (Armour and Co. Ltd., Eastbourne, Sussex, Lot no. DM1490). The last two samples contained a small proportion of material insoluble at pH 7, but the

* Part 2: Nichol, Winzor & Creeth (1960).

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