4. The coenzyme ratio

$$([\text{NAD}] + [\text{NADH}_2]) : ([\text{NADP}] + [\text{NADPH}_2])$$

fell from 6–10 in the livers of prenatal and newborn lambs to 2–3 in older lambs and adult sheep.

5. These findings are discussed with reference to the growth and intermediary metabolism of the liver in lambs and sheep.

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REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Burton, K. (1956). *Biochem. J.* **62**, 315.
 Caiger, P., Morton, R. K., Filsell, O. H. & Jarrett, I. G. (1962). *Biochem. J.* **85**, 351.
 Cloete, J. H. L. (1939). *Onderstepoort J. vet. Sci.* **13**, 417.
 Dawes, G. S. (1961). *Brit. med. Bull.* **17**, 148.
 Dawkins, M. J. R. (1959). *Proc. Roy. Soc. B*, **150**, 284.
 Dawkins, M. J. R. (1961). *Nature, Lond.*, **191**, 72.
 Dickens, F. (1961). In *Haematin Enzymes*, p. 625. Ed. by Falk, J. E., Lemberg, R. & Morton, R. K. Oxford: Pergamon Press Ltd.
 Dipietro, D. L., Sharma, C. & Weinhouse, S. (1962). *Biochemistry*, **1**, 455.
 Dische, Z. (1930). *Mikrochemie*, **2**, 4.
 Gallagher, C. H. & Buttery, S. H. (1959). *Biochem. J.* **72**, 575.
 Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
 Jarrett, I. G. & Filsell, O. H. (1958). *Aust. J. exp. Biol. med. Sci.* **36**, 433.
 Jarrett, I. G. & Filsell, O. H. (1960). *Nature, Lond.*, **188**, 418.
 Jarrett, I. G. & Potter, B. J. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 207.
 Leslie, I. (1955). In *The Nucleic Acids*, vol. 2, p. 1. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
 Lowenstein, J. M. (1961). *J. theoret. Biol.* **1**, 98.
 McLean, P. (1958). *Biochim. biophys. Acta*, **30**, 303.
 Morton, R. K. (1958). *Nature, Lond.*, **181**, 540.
 Morton, R. K. (1961). *Aust. J. Sci.* **24**, 260.
 Mott, J. C. (1961). *Brit. med. Bull.* **17**, 144.
 Nemeth, A. M. & Dickerman, H. (1960). *J. biol. Chem.* **235**, 1761.
 Raiha, N. C. R. (1961). *Amer. J. Physiol.* **201**, 961.
 Roux, J. F., Gordon, E. E., Dinnerstein, A. & Romney, S. L. (1962). *Fed. Proc.* **21**, 241.
 Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Shelley, H. J. (1960). *J. Physiol.* **153**, 527.
 Vendrely, R. & Vendrely, C. (1949). *Experientia*, **5**, 327.
 Villee, C. A. (1954). *Cold Spr. Harb. Symp. quant. Biol.* **19**, 186.
 Villee, C. A. (1961). *Ciba Found. Symp.: Somatic Stability in the Newly Born*, p. 246. Ed. by Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.
 Villee, C. A. (1962). *Biochem. J.* **83**, 191.
 Villee, C. A. & Loring, J. M. (1961). *Biochem. J.* **81**, 488.
 Webb, J. M. & Levy, H. B. (1955). *J. biol. Chem.* **213**, 107.
 Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 243. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
 Zamenhof, S. (1958). *Biochem. Prep.* **6**, 8.

Biochem. J. (1963) **89**, 100

The Biosynthesis of Coproporphyrinogen, Magnesium Protoporphyrin Monomethyl Ester and Bacteriochlorophyll by *Rhodospseudomonas capsulata*

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The biosynthesis of porphyrins and haem compounds in photosynthetic bacteria has been reviewed by Lascelles (1962). Conditions affecting the overall production of bacteriochlorophyll have also been studied, mainly by Lascelles (1956*a*, 1959, 1960, 1962), Cohen-Bazire, Sistrom & Stanier (1957), Tait & Gibson (1961), Gibson, Neuberger & Tait (1962*a*, *b*, *c*) and Sistrom (1962),

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but little is known of the later steps on the pathway leading to bacteriochlorophyll. The observation that mutant strains of *Chlorella*, which were unable to synthesize chlorophyll, produced protoporphyrin, protoporphyrin monomethyl ester, magnesium protoporphyrin, magnesium protoporphyrin monomethyl ester and magnesium vinyl phaeoporphyrin *a*₈ led Granick (1948*a*, *b*, 1950, 1961) to suggest that these compounds might be intermediates in the biosynthesis of chlorophyll

from protoporphyrin. The ability of chromatophores from *Rhodospseudomonas spheroides* to convert added magnesium protoporphyrin into its monomethyl ester (Tait & Gibson, 1961), and the detection of traces of the monomethyl ester in cultures of the same organism (Jones, 1963), suggest that bacteriochlorophyll and plant chlorophyll may be formed from protoporphyrin by similar pathways.

Porphyrinogens, the colourless fully reduced derivatives of porphyrins (Fischer, Bartholomäus & Röse, 1913; Fischer & Zerweck, 1924; Fischer & Stern, 1940; Mauzerall & Granick, 1958), are intermediates in the biosynthesis of protoporphyrin from porphobilinogen by enzyme systems isolated from plants, animals and bacteria (Bogorad, 1955*a, b*, 1958; Granick, 1955; Nevé, Labbe & Aldrich, 1956; Granick & Mauzerall, 1958; Hoare & Heath, 1959; Sano & Granick, 1961). The production of a similar colourless precursor of coproporphyrin by suspensions of *Rhodospseudomonas capsulata* and anaerobic cultures of *R. spheroides* has been briefly reported (Cooper, 1956; Cohen-Bazire *et al.* 1957).

The present experiments show that high concentrations of magnesium protoporphyrin monomethyl ester and coproporphyrinogen are produced from simple substrates by cultures and suspensions of *R. capsulata*. Conditions for optimum production of these compounds are compared with those that favour bacteriochlorophyll synthesis.

EXPERIMENTAL

Isolation and culture of Rhodospseudomonas capsulata

A strain of *R. capsulata* was isolated from a local mud rich in decomposing organic matter. Light-grown enrichment cultures with lactate and glutamate as main carbon sources were followed by a series of three successive single-colony isolations; the colonies selected were those releasing most porphyrin into the surrounding agar. The bacterium was identified from morphological and nutritional characters by the system of van Niel (1944).

Techniques for culture and maintenance of the organism and for preparation of suspensions were similar to those used by Lascelles (1956*a*) with *R. spheroides*, except that the following medium was substituted for Lascelles's medium S: KH_2PO_4 , 500 mg.; K_2HPO_4 , 500 mg.; $(\text{NH}_4)_2\text{HPO}_4$, 800 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; CaCl_2 , 40 mg.; DL-malic acid, 2.7 g.; sodium L-glutamate monohydrate, 3.7 g.; acid-hydrolysed casein (Snell & Rannefeld, 1945), 20 ml.; thiamine hydrochloride, 1 mg.; nicotinic acid, 5 mg.; biotin, 10 μg .; water, 1 l.; the pH was adjusted to 6.8 with 10*N*-NaOH.

Conditions for the incubation of organisms

Unless stated otherwise, cultures and suspensions were incubated at 28–31° and illuminated at 100–150 foot-candles from one side by 15w and 25w incandescent lamps. Sus-

pensions (4–5 mg. dry wt. of organisms in 5 ml. volumes) were incubated for 22–26 hr. in air in test tubes (150 mm. $\times$ 12 mm.) plugged with cotton wool; these are the 'standard conditions' referred to below. Anaerobic incubations were in Thunberg tubes under hydrogen.

Three incubation mixtures were used for suspensions, depending on the desired main end-product of synthesis. All three mixtures contained (final concentrations): glycine, 10 mM; sodium succinate, 40 mM; nicotinic acid, 0.04 mM; $(\text{NH}_4)_2\text{HPO}_4$, 4 mM; MgSO_4 , 4 mM; MnSO_4 , 0.005 mM; CaCl_2 , 0.4 mM. The individual mixtures were completed as follows. Mixture A (for coproporphyrinogen): DL-methionine, 2 mM; potassium phosphate buffer, pH 7.0, 20 mM. Mixture B (for magnesium protoporphyrin monomethyl ester): DL-methionine, 0.5 mM; iron citrate, 0.02 mM; Tween 80 (polyoxyethylene sorbitan mono-oleate), 0.2% (v/v); potassium phosphate buffer, pH 6.6, 20 mM. Mixture C (for bacteriochlorophyll): iron citrate, 0.2 mM; potassium phosphate buffer, pH 6.6, 20 mM. The stock solution of iron citrate (4 mM) was made up as described by Lascelles (1956*a*).

After the suspensions had been centrifuged, bacteriochlorophyll in the organisms, and coproporphyrinogen and magnesium protoporphyrin monomethyl ester in the supernatant fluids, were determined as described below.

Identification and determination of products

Absorption spectra were assessed rapidly with a hand spectroscope or Hartridge reversion spectroscope, or in greater detail with Unicam SP. 500 or SP. 600 spectrophotometers.

Magnesium protoporphyrin monomethyl ester. Detection of this compound was facilitated by its orange-pink fluorescence in ultraviolet light and by its two strong absorption bands at 590 and 551 μm , properties characteristic of both magnesium protoporphyrin and its monomethyl ester (Hill, 1925; Granick & Gilder, 1947; Granick, 1948*b*, 1961). The band at 590 μm is well placed for detection in the presence of a large excess of coproporphyrin.

For large-scale preparation of magnesium protoporphyrin monomethyl ester, cultures inoculated and incubated in the same way as were those destined for experiments with suspensions were grown for 48 hr. in 8 oz. medical flat bottles containing 200 ml. of the medium supplemented with iron citrate (0.04 mM) and Tween 80 (0.2%, v/v). The Tween 80 was sterilized separately by autoclaving at 10 lb./in.² for 10 min., and was added to the sterile medium no more than 2 days before inoculation. Such cultures yielded 2–3 mg. dry wt. of organisms/ml. in 48 hr.

The culture fluid (300 ml.), containing 12 μmoles of magnesium protoporphyrin monomethyl ester and no spectroscopically detectable amount of other porphyrin, was brought to pH 5.5–6.0 with 0.8 ml. of acetic acid and shaken in near-darkness with a mixture of 200 ml. of peroxide-free ether and 200 ml. of ethanol: the ethanol prevented emulsification by the Tween 80. The top layer was then reduced in volume by shaking it with its own volume of 30% (v/v) ethanol in water, and the new top layer in turn shaken with its own volume of 30% ethanol. The process was repeated twice more, and the top layer (now 10 ml.) was further concentrated by bubbling a stream of nitrogen in the dark, to yield 1 ml. of a clear viscous cherry-red oil containing 10 μmoles of magnesium protoporphyrin monomethyl ester. Its absorption spectrum

indicated no other compound absorbing light between 300 and 1000 m μ , but it probably contained Tween 80. The absorption spectrum agreed in the positions and relative heights of the peaks with the spectrum reported by Granick (1948*b*, 1961) for synthetic magnesium protoporphyrin and its monoester (Table 1).

For identification of the methyl ester group, a portion of the concentrated ethereal extract (extract I) was shaken with 1.4*N*-HCl to split the magnesium complex. The HCl was neutralized with a saturated solution of sodium acetate, and the ether layer (extract II) was washed twice with water. A portion of this extract was shaken with *N*-KOH and left standing at room temperature for 30 min. to hydrolyse the ester linkage. The KOH was neutralized with saturated sodium acetate, and the ether layer (extract III) was washed twice with water. Ethanol was added when necessary to disperse emulsions.

The extracts were analysed by ascending chromatography on Whatman no. 1 paper with a solvent mixture of 2,6-lutidine and 0.05*N*-NH₃ (10:7, by vol.) (Granick, 1961). Each extract and a protoporphyrin marker produced single spots fluorescing pink in ultraviolet light, with *R_F* values as follows: extract I, 0.84; extract II, 0.79; extract III, 0.68; protoporphyrin marker, 0.69. Such changes in *R_F* would be expected when the magnesium is removed (to form extract II), and the ester subsequently hydrolysed (to form extract III) (Granick, 1961). The conclusion that the magnesium protoporphyrin produced by *R. capsulata* was the monoester and not the diester was confirmed by hydrolysing three separate portions of extract II with *N*-KOH for 1 min., 2 min. and 5 min. respectively, followed by neutralization and extraction with ether. Paper chromatography of the three extracts produced only two spots (*R_F* 0.78 and 0.69) of different relative intensities, and not the three spots that Granick observed with partially hydrolysed protoporphyrin dimethyl ester.

Hydrolysis of the ester group caused the expected change in acid-solubility: 0.5*N*-HCl extracted porphyrin from extract III but not from extract II, whereas 2*N*-HCl extracted porphyrin from both extract II and extract III. When extract III was shaken with 2*N*-HCl and part of the

aqueous layer made alkaline with 10*N*-NaOH, the wavelengths of the visible-absorption bands of both acid and alkaline solutions were identical in the Hartridge reversion spectroscopy with those of protoporphyrin similarly treated.

To identify the ester group, 2 ml. samples (containing about 2 μ moles of porphyrin) of extracts II and III were freeze-dried in the bulbs of Thunberg tubes, and then incubated with 6*N*-H₂SO₄ and distilled over into the tubes; the distillates were tested for methanol (Granick, 1961). Extract II but not extract III gave a positive reaction with the chromotropic acid reagent, and therefore contained a methyl ester group.

Finally, the metal of the complex was checked by extracting extract I (0.5 ml.) with a drop of 2*N*-HCl, and spot-testing the neutralized extract by the quinalizarin method (Feigl, 1954; Granick, 1948*b*). The positive result could, according to Feigl, be due either to magnesium, lanthanum, beryllium, or to some of the rare-earth elements; it was assumed that magnesium was responsible.

A method was devised for determining both magnesium protoporphyrin monomethyl ester and coproprophyrinogen in the mixture of the two which was found in some experiments. Samples of supernatant fluid (2 ml.) were diluted with 50% (v/v) ethanol and 0.02*N*-KOH (final concentrations), and centrifuged to remove precipitated salts; the extinctions were measured at 418 m μ for magnesium protoporphyrin monomethyl ester and 394 m μ for coproprophyrin (the coproprophyrinogen having been rapidly oxidized to coproprophyrin in this solvent mixture). The extinctions at each wavelength were corrected for absorption from the compound with its peak at the other wavelength:

$$\text{Corrected } E_{418 \text{ m}\mu} = \frac{E_{418 \text{ m}\mu} - 0.17 E_{394 \text{ m}\mu}}{0.96}$$

$$\text{Corrected } E_{394 \text{ m}\mu} = \frac{E_{394 \text{ m}\mu} - 0.26 E_{418 \text{ m}\mu}}{0.96}$$

These correction factors were derived from the spectra of purified magnesium protoporphyrin monomethyl ester and of coproprophyrin, each dissolved in 50% (v/v) ethanol containing KOH (0.02*N*). The molar extinction coefficients at these peaks in this solvent mixture are 2.7×10^5 for magnesium protoporphyrin and its monomethyl ester (Granick, 1948*b*, 1961), and 2.5×10^5 for coproprophyrin. The latter value was derived by comparison with a similar sample of coproprophyrin dissolved in 1.4*N*-HCl, for which the extinctions are known (Jope & O'Brien, 1945). The accuracy of the method was checked on known mixtures of the authentic samples of magnesium protoporphyrin monomethyl ester and coproprophyrin, with or without added supernatant fluid from suspension experiments. Such determinations were reliable (mean deviation < 5%) except for a component which comprised less than 5% of the total porphyrin.

Coproprophyrinogen. The supernatant fluid from suspensions of organisms incubated with mixture A in the light, under either the anaerobic or the standard semi-anaerobic conditions, contained high concentrations of coproprophyrinogen, a colourless compound that was oxidized to coproprophyrin III on standing in air (Cooper, 1956). Coproprophyrinogen was determined as coproprophyrin (see below) after acidified samples had been left in open tubes in the dark for 24 hr.

Table 1. *Absorption maxima of magnesium protoporphyrin monomethyl ester*

Magnesium protoporphyrin monomethyl ester was extracted from culture fluid (see the Experimental section) and dissolved in 0.02*N*-KOH in 50% (v/v) ethanol containing 5% (v/v) of ether. The values for synthetic magnesium protoporphyrin and its monomethyl ester dissolved in 0.02*N*-KOH in 50% (v/v) ethanol are those of Granick (1948*b*, 1961).

Extract from culture fluid		Synthetic magnesium protoporphyrin	
Wavelength of peak (m μ)	Ratio of extinction to that at 589 m μ	Wavelength of peak (m μ)	Ratio of extinction to that at 590 m μ
589	1.00	590	1.00
551	1.07	551	1.08
510	0.15	510	0.20
418	15.5	418	15.6
340	1.21	342	1.66

For large-scale production of coproporphyrinogen, 2 oz. medical flat bottles were filled to the brim (about 65 ml.) with suspension (0.8–1.0 mg. dry wt. of organisms/ml.) in mixture A, loosely screw-capped, and incubated for 26 hr. with illumination at 150–200 foot-candles from 40 w lamps. Before centrifuging, 2 ml. of 20% (w/v) ascorbic acid was added to each 100 ml. of combined suspension. When coproporphyrin was determined by diluting a sample of the supernatant fluid with 1.4N-HCl (final concentration) and immediately measuring the extinction at 403 m μ , the amount present was less than 5% of that eventually appearing, which was usually about 30 μ moles/100 ml. of supernatant fluid (see Table 3). Attempts to purify coproporphyrinogen from the supernatant fluid, by adding HCl (final concentration 0.25N), extracting it into peroxide-free ether and re-extracting it into a small volume of aqueous alkali (all operations in the dark as much as possible), were only partially successful. Such preparations (Fig. 1) contained much coproporphyrin and an intermediate with an absorption peak at 500 m μ in acid solution (see below). Coproporphyrinogen was oxidized by air more rapidly in ethyl acetate or butan-1-ol than in ether.

To confirm the identity of the compound produced by *R. capsulata*, coproporphyrinogen III was chemically prepared by a method similar to that used for mesoporphyrinogen by Fischer *et al.* (1913). Coproporphyrin III (3 mg.), dissolved in 6 ml. of 0.1N-NaOH and then diluted with 18 ml. of water, was added to 10 g. of 3% (w/w) sodium amalgam in a thick-walled test tube and shaken in darkness for 3 hr. The colourless mixture, which rapidly became orange in the light, was decanted from the mercury and neutralized with HCl. This preparation was identical with that from *R. capsulata* in the following properties. (1) Acidic solutions allowed to oxidize in air or by treatment with iodine or hydrogen peroxide had a sharp strong absorption peak at 500 m μ (Fig. 1), which increased and then decreased in intensity while the peak at 548 m μ , from coproporphyrin, was increasing steadily (Fig. 2). After the peak at 500 m μ had reached its maximum, the ratio of fall in extinction at 500 m μ to rise in extinction at 548 m μ , over any given period of time, was constant at 2.1 under various redox conditions (air, iodine, hydrogen peroxide or cysteine). (2) Freshly prepared ethereal extracts from 0.25N-HCl solutions had sharp absorption peaks at 372, 437 and 481 m μ superimposed on the spectrum of coproporphyrin; when the extracts were exposed to air all peaks rapidly increased and eventually the coproporphyrin spectrum obscured any other. Alkaline solutions of preparations rich in the intermediate absorbing at 500 m μ in acid also had peaks at about 370 and 480 m μ . (3) Oxidation by air was greatly accelerated by light, alkali and organic solvents, and was slowest at neutral pH in water. (4) Freeze-dried preparations were insoluble in light petroleum, sparingly soluble in chloroform, moderately soluble in ether, ethyl acetate and dilute acid, and very soluble in lower alcohols, acetone and aqueous alkali. Ether extracted coproporphyrinogen from 0.1–0.3N-HCl, but little from N-HCl and none from alkaline solutions or 2N-acetic acid. The compound absorbing at 500 m μ was extracted from 0.1N-HCl but not from 0.25N-HCl. (5) The pH of minimum solubility of the compound absorbing at 500 m μ , as judged by the discharge of colour from the red solution, was 3.6–3.8. (6) Two of the tests commonly used for bile pigments (Gray, 1953) were applied. Coproporphyrinogen solution

(2 ml.) was treated with 2 ml. of 2% (w/v) *p*-dimethylaminobenzaldehyde in 5N-HCl (Ehrlich's reagent) and then 4 ml. of saturated aqueous sodium acetate. There were no changes in colour or spectrum other than those due to oxidation. Another sample of coproporphyrinogen was treated with an equal volume of ethanolic 10% (w/v) zinc

Fig. 1. Absorption spectrum of partially oxidized coproporphyrinogen extracted from bacterial suspensions (—) or from a solution of coproporphyrin reduced by sodium amalgam (---) (see the Experimental section). Each preparation was immediately diluted with 1.4N-HCl and 0.5% (w/v) ascorbic acid (final concentrations) and the spectra were surveyed rapidly. The spectrum of pure coproporphyrin in 1.4N-HCl, in the region of 500 m μ , is also shown (.....).

Fig. 2. Changes in extinctions at 500 m μ and 548 m μ during the oxidation of coproporphyrinogen. Supernatant fluids from bacterial suspensions incubated under the standard conditions in mixture A (see the Experimental section) were diluted 1:5 at zero time with mixtures of 1.4N-HCl and either 0.1 mM-iodine (○) or 18 mM-hydrogen peroxide (●) (final concentrations).

acetate. No green fluorescence was seen, but only a diffuse spectrum that changed immediately on acidification to that of coproporphyrin. It is probable that an acid-labile zinc complex was formed. (7) No porphyrin other than coproporphyrin III was detected by paper chromatography (see below) of the fully oxidized preparations.

Other porphyrins. Porphyrins, other than magnesium protoporphyrin monomethyl ester, in the oxidized supernatant fluids were prepared for chromatography by the methods of Lascelles (1956*a*), and identified by the paper chromatography of Eriksen (1953) for porphyrins with different numbers of carboxyl groups/molecule and that of Chu, Green & Chu (1951) for the isomers of coproporphyrin. Determinations by the method of Lascelles (1956*a*) showed amounts of uroporphyrin which varied but which were not more than 6% of the total porphyrin in the oxidized supernatant fluids; there were only traces of porphyrins other than uroporphyrin, coproporphyrin and magnesium protoporphyrin monomethyl ester. Thus, except with mixtures containing both coproporphyrin and magnesium protoporphyrin monomethyl ester, which were determined as described above, total porphyrins in supernatant fluids were determined as coproporphyrin by measuring the extinction at 403 m μ of dilutions in 1.4*N*-HCl (Jope & O'Brien, 1945).

Bacteriochlorophyll. Bacteriochlorophyll in the organisms was determined by Lascelles's (1956*a*) modification of the method of van Niel & Arnold (1938).

Chemicals

Tween 80 was obtained from Honeywell and Stein Ltd., London, and protoporphyrin from L. Light and Co. Ltd., Colnbrook, Bucks. Coproporphyrin methyl esters were kindly given by Professor C. Rimington.

RESULTS

Rates of synthesis of coproporphyrinogen, magnesium protoporphyrin monomethyl ester and bacteriochlorophyll

The time-courses of synthesis of the three compounds, each in its standard incubation mixture, are shown in Fig. 3. The concentrations of bacteriochlorophyll or magnesium protoporphyrin monomethyl ester produced never exceeded 50 μ m-moles/ml. of suspension, whereas in the iron-deficient mixture A up to 310 μ m-moles of coproporphyrinogen/ml. (0.02%) were produced.

R. capsulata always grew during incubation of the suspensions; the dry weight of organisms doubled in the iron-deficient mixture A and increased fivefold in mixtures B and C. Bacteriochlorophyll per unit dry weight increased by 30–50% during incubation in mixture C.

Effects of omitting components from the incubation mixtures

Decreased rates of synthesis due to the omission of components are listed in Table 2. The omission of a given component, except for iron citrate,

methionine or Tween 80, decreased the synthesis of all three products by the same proportion.

Iron. The effect of different concentrations of iron (Fig. 4) was studied with mixture B, which could be modified to support near-maximal rates of synthesis of any of the three products by altering its content of iron citrate. Optimum concentrations of added iron for the synthesis of coproporphyrinogen, magnesium protoporphyrin monomethyl ester and bacteriochlorophyll were respectively: none, 0.02 and 0.2 mM. The increase in coproporphyrinogen due to iron deficiency was about five times as great as the decrease in bacteriochlorophyll. At the lower iron concentrations, synthesis of magnesium protoporphyrin monomethyl ester was similar to that of bacteriochlorophyll, and at the higher concentrations to that of coproporphyrinogen.

Methionine. Increasing concentrations (up to 2 mM) of DL-methionine decreased bacteriochlorophyll synthesis and increased coproporphyrinogen synthesis (Fig. 5). Synthesis of magnesium protoporphyrin monomethyl ester was greatest at an intermediate methionine concentration. Additions of iron had little effect on the percentage changes in synthesis due to additions of methionine. Methionine was completely replaced in its effects on the three products in mixtures A and B by glutathione (1 mM), and partially by L-cysteine (1 mM), but not by DL-serine, DL-threonine, DL-alanine, sodium L-glutamate, sodium sulphide, sodium thiosulphate (0.2 mM and 1 mM), cobalamin (1 μ M) or *p*-aminobenzoic acid (1 μ M).

Tween 80 (*polyoxyethylene sorbitan mono-oleate*). This non-ionic detergent had only small and variable effects on the growth of cultures or on the production of bacteriochlorophyll or copropor-

Fig. 3. Course of synthesis of coproporphyrinogen (O) in mixture A, magnesium protoporphyrin monomethyl ester (●) in mixture B, and bacteriochlorophyll (▲) in mixture C, by bacterial suspensions incubated under the standard conditions (see the Experimental section).

Table 2. *Effect of omitting components of incubation mixtures*

Bacterial suspensions were incubated under the standard conditions in mixture A (for coproporphyrinogen), mixture B (for magnesium protoporphyrin monomethyl ester), or mixture C (for bacteriochlorophyll); details are given in the Experimental section.

Omissions	Products formed ($\mu\text{m-moles/ml.}$)		
	Copro-porphyrinogen	Magnesium protoporphyrin monomethyl ester	Bacterio-chlorophyll
None	245	31	39
Glycine	64	8	14
Succinate	0	0	2
Nicotinic acid	132	28	31
$(\text{NH}_4)_2\text{HPO}_4$	53	10	9
MgSO_4	4	0	1
MnSO_4	24	5	5
CaCl_2	207	30	32
Potassium phosphate buffer	116	15	10
Iron citrate	—	0	4
Methionine	140	19	—
Tween 80	—	2	—

Fig. 4. Effect of iron on the synthesis of coproporphyrinogen (○), magnesium protoporphyrin monomethyl ester (●) and bacteriochlorophyll (▲). Bacterial suspensions were incubated under the standard conditions in mixture B (see the Experimental section) with its content of iron citrate varied as shown.

Fig. 5. Effect of methionine on the synthesis of coproporphyrinogen (○), magnesium protoporphyrin monomethyl ester (●) and bacteriochlorophyll (▲). Bacterial suspensions were incubated under the standard conditions in mixture B (see the Experimental section) with its content of DL-methionine varied as shown.

pyrroline by suspensions, but stimulated 10- to 20-fold the production of magnesium protoporphyrin monomethyl ester by suspensions and cultures. The optimum concentration of Tween 80

was 0.2 %; 1 % or more inhibited both growth of cultures and the synthesis of all three products. Because of the low solubility of magnesium protoporphyrin monomethyl ester in aqueous solutions, it was possible that Tween 80 promoted not the synthesis of this product but merely its solution in the supernatant fluid during centrifuging. To resolve this question, cultures were grown by the same methods and in the same medium as for large-scale preparations (see the Experimental section), except that Tween 80 was omitted from the medium but added (0.2 %, v/v) to the cultures at the end of their 48 hr. period of growth in the light. Cultures were then reincubated in the dark at 30° for 24 hr. and centrifuged. Such culture fluids, as well as those from controls without Tween 80, contained less than 1 $\mu\text{m-mole}$ of magnesium protoporphyrin

Table 3. *Production of coproporphyrinogen and coproporphyrin under different incubation conditions*

Bacterial suspensions in mixture A, with iron citrate added as shown, were incubated anaerobically in Thunberg tubes under hydrogen or under the standard semi-anaerobic conditions; details are given in the Experimental section. Coproporphyrin was estimated (a) immediately after centrifuging the suspensions and diluting the supernatant fluids with 1.4 N-HCl (final concn.), and (b) after keeping the dilutions 24 hr. in air in the dark, which gives total porphyrin synthesized. Coproporphyrinogen was obtained by difference.

Conditions of incubation	Iron citrate added (μM)	Porphyrin present ($\mu\text{m-moles/ml.}$)		
		As coproporphyrinogen	As coproporphyrin	Total
Anaerobic	0	276	14	290
	10	78	14	92
	100	8	10	18
Semi-anaerobic	0	162	143	305
	10	44	30	74
	100	0	16	16

monomethyl ester/ml. Additional controls grown in the light with Tween 80 and then similarly incubated in the dark for 24 hr. produced 30 $\mu\text{m-moles/ml.}$ Thus probably little magnesium protoporphyrin monomethyl ester had accumulated during growth without Tween 80.

Types of porphyrin produced

Paper chromatography and spectrum analysis revealed no porphyrin with less than three carboxyl groups/molecule, other than magnesium protoporphyrin monomethyl ester, in either cultures or suspensions, except occasional faint traces of a dicarboxylic porphyrin, probably unesterified magnesium protoporphyrin. Traces of free protoporphyrin or protoporphyrin monomethyl ester could have escaped detection. The effect of anaerobic and the standard semi-anaerobic incubations on the proportion of coproporphyrin to coproporphyrinogen in suspensions analysed immediately after centrifuging is shown in Table 3. Additions of iron citrate, which greatly affected the total amount of coproporphyrin finally appearing after full oxidation, little affected the amount of coproporphyrin in fresh supernatant fluids from anaerobic incubations. No isomer of coproporphyrin other than type III was detected in the fully oxidized supernatant fluids. Other porphyrins detected were uroporphyrin and traces of porphyrins with three, five, six and seven carboxyl groups/molecule. The proportion of uroporphyrin from the standard 24 hr. incubations varied in different experiments from 2 to 6% of the total porphyrin, increasing up to 10–15% after 40 hr. of incubation. After 40 hr. of anaerobic incubation, similar proportions of uroporphyrin were found in supernatant fluids where the total porphyrin had increased 20-fold during oxidation. Therefore suspensions probably produced both coproporphyrinogen and uroporphyrinogen.

Growing cultures were used to produce magnesium protoporphyrin monomethyl ester relatively

free from other porphyrins, because it was always accompanied by coproporphyrinogen in experiments with suspensions. The standard cultures from large-scale preparations (see the Experimental section) produced 30–50 $\mu\text{m-moles}$ of magnesium protoporphyrin monomethyl ester/ml. in 30–48 hr. and only traces of other porphyrins. After a further 24 hr. of growth, such cultures contained the same amount of magnesium protoporphyrin monomethyl ester but it was now accompanied by 40–60 $\mu\text{m-moles}$ of coproporphyrinogen/ml.

DISCUSSION

The locally isolated strain of *R. capsulata* is similar to *R. spheroides* (Lascelles, 1956a, 1962) in many aspects of its synthesis of porphyrins and bacteriochlorophyll. There were only minor changes in composition of the incubation mixtures for bacterial suspensions: methionine was added (see below), succinate was an efficient carbon source and thus replaced the mixture of fumarate and α -oxoglutarate used for *R. spheroides*, and nicotinic acid was added because it increased porphyrin synthesis despite its high concentration in the growth medium. However, *R. capsulata* is noteworthy mainly in its vigorous and readily elicited production of two probable intermediates in bacteriochlorophyll synthesis, magnesium protoporphyrin monomethyl ester and coproporphyrinogen.

The production of magnesium protoporphyrin monomethyl ester from simple substrates by cultures and suspensions provides further evidence for its participation in the biosynthesis of chlorophylls. No identifiable amounts of either its probable precursors, protoporphyrin and magnesium protoporphyrin (Tait & Gibson, 1961), or the free protoporphyrin monomethyl ester accompanying it in cultures of a *Chlorella* mutant (Granick, 1961), were found in cultures or suspensions of *R. capsulata*.

An explanation for the striking stimulation by Tween 80 of the production of magnesium protoporphyrin monomethyl ester may lie in the known solubilization of porphyrins, including dimethyl protoporphyrin ester, by aqueous solutions of detergents (see Dempsey, Lowe & Phillips, 1961). Leakage of magnesium protoporphyrin monomethyl ester from the sites of bacteriochlorophyll synthesis may thus be promoted by Tween 80, which, however, did not lessen bacteriochlorophyll production.

The effects of iron on the synthesis of porphyrin and bacteriochlorophyll were broadly similar to those with *R. sphaeroides* and other photosynthetic bacteria (Lascelles, 1962); more bacteriochlorophyll and much less coproporphyrinogen were produced when the concentration of iron was increased. With *R. sphaeroides*, the production of both protoporphyrin and haem from δ -aminolaevulinic acid is increased at the expense of coproporphyrin by adding iron, though five times as much iron is needed for maximum haem production as for maximum protoporphyrin production. Lascelles (1956*a, b*) concluded that, apart from its incorporation into the haem molecule, iron has complex functions, one of which is at a stage in the formation of the vinyl side chains of protoporphyrin. That this function is also important in *R. capsulata* may be concluded from the increased production of both magnesium protoporphyrin monomethyl ester and bacteriochlorophyll at concentrations of iron (0.02 mM) that greatly inhibited coproporphyrinogen production. However, further additions of iron up to very high concentrations (0.2 mM) produced more bacteriochlorophyll but less magnesium protoporphyrin monomethyl ester; thus iron probably regulates stages in bacteriochlorophyll synthesis beyond magnesium protoporphyrin monomethyl ester. The effects of iron are so complex that any detailed conclusions should be deferred until more is known of the reaction sequence leading to bacteriochlorophyll and the overall regulation of pigment production in photosynthetic bacteria.

The effects of methionine on the synthesis of the three products were also complex, and do not relate in any simple way to the known function of methionine in bacteriochlorophyll synthesis, i.e. the provision of the methyl ester group of magnesium protoporphyrin monomethyl ester (Tait & Gibson, 1961; Gibson *et al.* 1962*a, b*); with added methionine, suspensions produced more magnesium protoporphyrin monomethyl ester, but also more coproporphyrinogen and less bacteriochlorophyll. Any simple relationship between methionine and iron was precluded by the similar effects of methionine at widely different iron concentrations. The replacement of methionine by glutathione or cysteine suggests either that these sulphur compounds can

spare the methionine which the organism can synthesize for itself, or that the effects on porphyrin metabolism come from sulphur compounds other than methionine. It should be emphasized that suspensions of bacteria grew during the 24 hr. incubation in the complex mixtures. Synthesis of porphyrin and of bacteriochlorophyll from glycine is always associated with growth (Lascelles, 1956*a*; Gibson *et al.* 1962*a, b*), and compounds added to the incubation mixtures would be expected to have complex effects.

Coproporphyrinogen III was produced efficiently by iron-deficient suspensions of *R. capsulata* incubated anaerobically with glycine and succinate; its final concentration accounted for up to 25 % of the added glycine. The proportion of coproporphyrinogen to coproporphyrin appearing was greater when the access of air during incubation was decreased (Table 3); probably no coproporphyrin was formed in anaerobic suspensions. It remains undecided whether in aerobic suspensions coproporphyrinogen is oxidized to coproporphyrin before or after it passes from the bacteria.

The coproporphyrinogen synthesized from simple substrates by the organism is apparently identical with the product of chemical reduction of coproporphyrin III, and homologous with uroporphyrinogen III described by Mauzerall & Granick (1958). The compound with a strong absorption peak at 500 m μ in acid solution is intermediate in the oxidation of coproporphyrinogen to coproporphyrin (Fig. 2). It is probably homologous with a product, also showing a peak at 500 m μ , which Mauzerall & Granick observed during the oxidation of uroporphyrinogen, and which they identified as a dipyrromethene tetrapyrrole (an open chain of four pyrrole groups, in which one of the linking methylene groups has been oxidized to a methine group).

After coproporphyrin formation in acid solution in air had ceased, there was a persistent small peak at 500 m μ , suggesting the formation of a minor component closely related to the intermediate but unable to form porphyrin by oxidation.

SUMMARY

1. Suspensions of a strain of *Rhodospseudomonas capsulata*, incubated in the light in a mixture containing glycine and succinate, synthesized high concentrations of bacteriochlorophyll and two probable intermediates in its biosynthesis: coproporphyrinogen III and magnesium protoporphyrin monomethyl ester.

2. More bacteriochlorophyll and much less coproporphyrinogen were produced with increasing concentrations of iron. The production of magnesium protoporphyrin monomethyl ester was greatest at an intermediate iron concentration.

3. Less bacteriochlorophyll and more coproporphyrinogen were produced with increasing concentrations of methionine. The production of magnesium protoporphyrin monomethyl ester was greatest at an intermediate methionine concentration.

4. The synthesis of magnesium protoporphyrin monomethyl ester by cultures or suspensions was increased 10- to 20-fold by adding Tween 80.

5. Coproporphyrinogen synthesized by *R. capsulata* was identical with the product of chemical reduction of coproporphyrin III in all properties examined. A compound with an absorption peak in acid solution at 500 m μ , probably the dipyrromethene tetrapyrrole, was intermediate in the chemical oxidation of coproporphyrinogen to coproporphyrin.

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REFERENCES

- Bogorad, L. (1955a). *Fed. Proc.* **14**, 184.
 Bogorad, L. (1955b). *Science*, **121**, 878.
 Bogorad, L. (1958). *J. biol. Chem.* **233**, 501.
 Chu, T. C., Green, A. A. & Chu, E. J. (1951). *J. biol. Chem.* **190**, 643.
 Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957). *J. cell. comp. Physiol.* **49**, 25.
 Cooper, R. (1956). *Biochem. J.* **63**, 25p.
 Dempsey, B., Lowe, M. B. & Phillips, J. N. (1961). In *Haematin Enzymes*, p. 29. Ed. by Falk, J. E., Lemberg, R. & Morton, R. K. Oxford: Pergamon Press Ltd.
 Eriksen, L. (1953). *Scand. J. clin. Lab. Invest.* **5**, 155.
 Feigl, F. (1954). *Spot Tests*, vol. 1, p. 210. Amsterdam: Elsevier Publishing Co.
 Fischer, H., Bartholomäus, E. & Röse, H. (1913). *Hoppe-Seyl. Z.* **84**, 262.
 Fischer, H. & Stern, A. (1940). *Die Chemie des Pyrrols*, Band II, Hälfte 2, p. 420. Leipzig: Akademische Verlagsgesellschaft.
 Fischer, H. & Zerweck, W. (1924). *Hoppe-Seyl. Z.* **137**, 242.
 Gibson, K. D., Neuberger, A. & Tait, G. H. (1962a). *Biochem. J.* **83**, 539.
 Gibson, K. D., Neuberger, A. & Tait, G. H. (1962b). *Biochem. J.* **83**, 550.
 Gibson, K. D., Neuberger, A. & Tait, G. H. (1962c). *Biochem. J.* **84**, 483.
 Granick, S. (1948a). *J. biol. Chem.* **172**, 717.
 Granick, S. (1948b). *J. biol. Chem.* **175**, 333.
 Granick, S. (1950). *J. biol. Chem.* **183**, 713.
 Granick, S. (1955). *Abstr. Pap. Amer. chem. Soc.* **128**, 69.
 Granick, S. (1961). *J. biol. Chem.* **236**, 1168.
 Granick, S. & Gilder, H. (1947). *Advanc. Enzymol.* **7**, 305.
 Granick, S. & Mauzerall, D. (1958). *J. biol. Chem.* **232**, 1119.
 Gray, C. H. (1953). *The Bile Pigments*, p. 22. London: Methuen and Co.
 Hill, R. (1925). *Biochem. J.* **19**, 341.
 Hoare, D. S. & Heath, H. (1959). *Biochem. J.* **73**, 679.
 Jones, O. T. G. (1963). *Biochem. J.* **86**, 429.
 Jope, E. M. & O'Brien, J. R. P. (1945). *Biochem. J.* **50**, 239.
 Lascelles, J. (1956a). *Biochem. J.* **62**, 78.
 Lascelles, J. (1956b). *J. gen. Microbiol.* **15**, 404.
 Lascelles, J. (1959). *Biochem. J.* **72**, 508.
 Lascelles, J. (1960). *J. gen. Microbiol.* **23**, 487.
 Lascelles, J. (1962). In *The Bacteria*, vol. 3, p. 335. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.
 Mauzerall, D. & Granick, S. (1958). *J. biol. Chem.* **232**, 1141.
 Nevé, R. A., Labbe, R. F. & Aldrich, R. A. (1956). *J. Amer. chem. Soc.* **78**, 691.
 Sano, S. & Granick, S. (1961). *J. biol. Chem.* **236**, 1173.
 Sistrom, W. R. (1962). *J. gen. Microbiol.* **28**, 599, 607.
 Snell, E. E. & Rannefeld, A. N. (1945). *J. biol. Chem.* **157**, 475.
 Tait, G. H. & Gibson, K. D. (1961). *Biochim. biophys. Acta*, **52**, 614.
 van Niel, C. B. (1944). *Bact. Rev.* **8**, 1.
 van Niel, C. B. & Arnold, W. (1938). *Enzymologia*, **5**, 244.

Biochem. J. (1963) **89**, 108

Metabolism of D-Glucuronolactone in Mammalian Systems

3. FURTHER STUDIES OF D-GLUCURONOLACTONE DEHYDROGENASE OF RAT LIVER*

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Marsh (1963b) described the identification in certain mammalian tissues of an enzyme system capable of converting D-glucuronolactone into

D-glucaric acid, and it was suggested that this could explain the presence of D-glucaric acid in normal human urine and the greatly increased excretion of this compound immediately after oral dosage of D-glucuronolactone (Marsh, 1963a). The

* Part 2 (Marsh 1963b).