test a hypothesis put forward by DeKock (1954–55) that in iron-deficient plants there is a deficiency of ferrous iron and that consequently the aconitase activity is diminished. A possible connexion between the effects upon aconitase and upon the concentrations of citric acid and malic acid and hence upon the phosphorus/iron and potassium/calcium ratios is further discussed by DeKock *et al.* (1960*b*). According to these arguments the concentration of citric acid should be raised and that of malic acid depressed, or at least the ratio of citric acid to malic acid should be greater in the leaves of iron-deficient plants. Experiments in progress (see also DeKock & Morrison, 1958) show this to be the case, but proof is still lacking that the lower level of aconitase is directly responsible. As we have already remarked (Bacon *et al.* 1961), it is difficult to decide, simply from a knowledge of its level of activity in extracts, whether an enzyme is a limiting factor in a sequence of metabolic reactions. What is required is a direct examination of the interconversion of organic acids in normal and deficient leaves.

SUMMARY

1. Aconitase activity has been measured in extracts of the leaves of mustard plants growing in water culture and receiving three concentrations of iron (0·2, 1 and 5 p.p.m.).

2. The aconitase activity in plants receiving 0·2 p.p.m., which always show signs of iron-deficiency, was about half that in those receiving 1 p.p.m. At 5 p.p.m. the activity was variable, but usually greater than at 1 p.p.m.

3. Simultaneous measurements of malic-dehydrogenase activity showed that it was only slightly depressed by iron-deficiency; fumarase activity was slightly raised.

4. When iron-deficient plants or leaves were fed with adequate iron the aconitase activity was restored to the normal level.

5. The aconitase activity of extracts from deficient plants could not be increased by treatment with ferrous iron and cysteine, nor by any other means tested.

6. The significance of these results is discussed in relation to the possible connexion of iron with aconitase action, and to possible effects of aconitase deficiency on the metabolism of the leaf.

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Some Enzymic Reactions Concerned in the Metabolism of Acetoacetyl-Coenzyme A in Athiorhodaceae

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Photosynthetic bacteria of the Athiorhodaceae family grow either aerobically in the dark or anaerobically in the light. Carotenoids and bacteriochlorophyll are formed only during growth in the light or under low conditions of aeration in the dark (van Niel, 1944; Cohen-Bazire, Sistrom & Stanier, 1957; Lascelles, 1959).

Organisms grown anaerobically in the light (pigmented) are considerably more active in catalysing the enzymic reactions concerned in the early stages of bacteriochlorophyll synthesis than are those cultivated aerobically in the dark (non-pigmented). The increase in activity, which is due to enhanced ability to synthesize the enzymes,

accompanies pigment formation in organisms adapting from the non-pigmented (aerobic) to the pigmented (photosynthetic) state (Lascelles, 1959, 1960). Similar changes in the activity of enzymes concerned in the early stages of carotenoid synthesis might also occur in Athiorhodaceae adapting from aerobic to photosynthetic conditions, and the present work was originally undertaken with this possibility in view.

There is considerable evidence that mevalonic acid is a precursor not only of cholesterol (Tavormina, Gibbs & Huff, 1957) but also of carotenoids (Braithwaite & Goodwin, 1957; Anderson, Nargard & Mangiarotti, 1959; Yokoyama, Chichester & Mackinney, 1960).

The early stages of carotenoid synthesis up to the formation of mevalonic acid probably proceed by the following pathway (in which CoA is coenzyme A, HMG β-hydroxy-β-methylglutaryl-, TPN triphosphopyridine nucleotide, and TPNH reduced triphosphopyridine nucleotide) (Ferguson, Durr & Rudney, 1959; Knappe, Ringlemann & Lynen, 1959):

$$2\ \text{Acetyl-CoA} \xrightleftharpoons{\beta\text{-ketothiolase}} \text{acetoacetyl-CoA} + \text{CoA} \quad (1)$$

$$\text{Acetoacetyl-CoA} + \text{acetyl-CoA} \xrightarrow[\text{enzyme}]{\text{HMG-CoA condensing}} \text{HMG-CoA} + \text{CoA} \quad (2)$$

$$\text{HMG-CoA} + \text{TPNH} \xrightarrow[\text{reductase}]{\text{HMG-CoA}} \text{mevalonic acid} + \text{TPN} \quad (3)$$

In addition to the enzymes concerned in this synthesis other reactions have been demonstrated in preparations from animal tissues and yeast which could compete for key intermediates. These include β-hydroxybutyryl-coenzyme A dehydrogenase (reaction 4) (Wakil, 1955); coenzyme A transferase (reaction 5) described by Stern, Coon, del Campillo & Schneider (1956*b*) and β-hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme (reaction 6) (Bachhawat, Robinson & Coon, 1955).

$$\beta\text{-Hydroxybutyryl-CoA} + \text{diphosphopyridine nucleotide} \rightleftharpoons \text{acetoacetyl-CoA} + \text{reduced diphosphopyridine nucleotide} \quad (4)$$

$$\text{Acetoacetyl-CoA} + \text{succinate} \rightleftharpoons \text{succinyl-CoA} + \text{acetoacetate} \quad (5)$$

$$\text{HMG-CoA} \longrightarrow \text{acetoacetate} + \text{acetyl-CoA} \quad (6)$$

Recent work has shown that acetoacetate formation by liver is due, at least in part, to cleavage of β-hydroxy-β-methylglutaryl-coenzyme A (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958).

In the present work formation of free acetoacetate from acetyl-coenzyme A has been observed in extracts of Athiorhodaceae, suggesting the presence of some of the enzymes mentioned above. Their level of activity has been examined in extracts of pigmented and unpigmented organisms to see if there is a correlation between enzymic activity and ability to form carotenoids.

EXPERIMENTAL

Materials

CoA (70–75% pure), diphosphopyridine nucleotide (DPN) and TPNH were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; glutathione was obtained from British Drug Houses, Ltd., Poole, Dorset; β-hydroxy-β-methylglutaric acid and acetyl phosphate (dilithium salt) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Diketene was a gift from Dr D. Bigley, Dyson Perrins Laboratory, Oxford. Other chemicals were of the purest commercial grade available.

A partially purified preparation of phosphotransacetylase was made from an ultrasonic extract of *Escherichia coli* by a method similar to that of Goldman (1958). The preparation used in this work had an activity of 940 units/ml. (containing 47 mg. of protein) measured by arsenolysis of acetyl phosphate (Stadtman, 1955).

Acetyl-CoA was prepared by reaction of CoA and acetic anhydride (Simon & Shemin, 1953).

Acetoacetyl-CoA was prepared from diketene and CoA by the method described by Lynen *et al.* (1958).

β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) was prepared from β-hydroxy-β-methylglutaric anhydride and CoA (Hilz, Knappe, Ringelmann & Lynen, 1958). The anhydride was made from the acid by the method of Hilz *et al.* (1958); its authenticity was established by melting-point determination and by chromatography of the hydroxamate derivative (Hilz *et al.* 1958). Acetoacetate was prepared from freshly distilled ethyl acetoacetate by hydrolysis of the ester with 2N-NaOH at 37° for 1 hr. (Krebs & Eggleston, 1945).

Tris buffers were prepared from 2-amino-2-hydroxymethylpropane-1:3-diol and HCl according to Gomori (1955).

Growth and harvesting of organisms

Rhodopseudomonas spheroides N.C.I.B. no. 8253, *R. capsulata* van Niel, 2.3.11, and *R. palustris* van Niel, 2.1.7 were used. Stock cultures were maintained as described by Lascelles (1959).

R. spheroides was grown on malate plus glutamate medium (Lascelles, 1959), and *R. palustris* on the same medium supplemented with μM-*p*-aminobenzoate. For growth of *R. capsulata* sodium succinate (0·01 M) was used in place of malate.

Cultures were inoculated, incubated aerobically in the dark or anaerobically in the light, and harvested as described by Lascelles (1959). The washed organisms were finally suspended in 0·02M-tris buffer, pH 8·5, to a concentration of about 20 mg. dry wt./ml.

Preparation of cell-free extracts

Extract of organisms were prepared by ultrasonic vibration (Lascelles, 1959); remaining whole organisms and debris were removed by centrifuging at 25 000*g* for 10 min.

at 0°. The supernatant fluid was used in all the work reported. Treatment of bacterial extract by Dowex 1 was effected by passing a portion of extract (equivalent to 15 mg. of protein) down a column (7·5 cm. × 1·5 cm.) of Dowex 1X8 (100–200 mesh; chloride form), the column having been cooled to 0°.

Estimations

Dry weight of organisms. The extinctions of suspensions of the bacteria were measured at 680 mμ, and the dry weight was calculated from the calibration curve for the appropriate organism grown under the same conditions (Cohen-Bazire *et al.* 1957).

Protein. The concentration of protein in extracts was determined colorimetrically by a method based on the biuret reaction (Layne, 1957) after removal of photosynthetic pigments by treatment with hot acid–ethanol (Vernon & Kamen, 1953). Crystalline bovine plasma albumin (Armour Laboratories, Hampden Park, Eastbourne, Sussex) was the standard.

Photosynthetic pigments. Carotenoids and bacteriochlorophyll were estimated by the method of Cohen-Bazire *et al.* (1957).

Acetoacetate. This was estimated colorimetrically as the *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan, formed after reaction with an excess of *p*-nitrobenzenediazonium hydroxide (Walker, 1954).

Spectrophotometry

All spectrophotometric measurements were with the Unicam spectrophotometer model SP. 500 (Unicam Instruments Ltd., Cambridge).

Assay of enzymic activities

β-Ketothiolase. The assay of this enzyme was based upon measurement of the decline in absorption at 310 mμ due to acetoacetyl-CoA in the presence of Mg^{2+} ions, which enhances the absorption of the enolate ion of the β-keto thio ester (Stern, 1955, 1956). The assay was conducted in silica microcuvettes (light-path of 1 cm.) containing (in μmoles): tris buffer, pH 8·5, 75; $MgCl_2$, 5; CoA, 0·01; glutathione, 3; acetoacetyl-CoA, 0·02; bacterial extract, 1–5μg. of protein; water to a final volume of 0·4 ml. The reaction was started by the addition of the enzyme and the absorption at 310 mμ measured at half-minute intervals against a control which lacked acetoacetyl-CoA. The reaction was carried out at room temperature (about 22°).

The enzyme activity was expressed in arbitrary units, one unit causing an initial rate of decline in $E_{310\ m\mu}$ of 0·01/min.

Coenzyme A transferase (succinyl-β-oxoacyl-S-coenzyme A transferase.) The procedure for this assay is based on that for the enzyme in pig heart (Stern *et al.* 1956*b*), except that the breakdown, rather than formation, of acetoacetyl-CoA was determined by measuring the decline in $E_{310\ m\mu}$. The assay was carried out in silica microcuvettes (light-path 1 cm.) containing (in μmoles): tris buffer, pH 8·5, 75; $MgCl_2$, 5; acetoacetyl-CoA, 0·02; sodium succinate, 5; bacterial extract, 5–30μg. of protein; water to a final volume of 0·4 ml. The reaction was started by the addition of succinate and the decline in absorption measured against a control lacking acetoacetyl-CoA. The assay was conducted at room temperature (about 22°).

One unit of enzyme activity was the amount causing a rate of decline in $E_{310\ m\mu}$ of 0·01/min.

β-Hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme. The cleaving of HMG-CoA to form equimolar amounts of acetoacetate and acetyl-CoA was assayed by estimation of the acetoacetate formed. Tubes (1 cm. diameter) contained (in μmoles): tris buffer, pH 8·5, 200; HMG-CoA, 0·20; bacterial extract, 1 mg. of protein; water to a final volume of 0·9 ml. The reaction was started by the addition of the bacterial extract, and after incubation for 30 min. at 34° 0·1 ml. of 30% trichloroacetic acid was added. After removal of the precipitate by centrifuging, acetoacetate was estimated by the method of Walker (1954).

This method, which depends upon the reaction of acetoacetic acid with excess of *p*-nitrobenzenediazonium hydroxide, is, under the conditions employed, essentially specific for this compound (Walker, 1954; Drummond & Stern, 1960). Acetoacetyl-CoA does not form the coloured compound, *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan, produced by acetoacetate (Lynen *et al.* 1958; Drummond & Stern, 1960).

Activity is expressed as μmoles of acetoacetate formed/hr./mg. of protein, under the standard conditions.

β-Hydroxybutyrate dehydrogenase. The assay of D(−)-β-hydroxybutyrate dehydrogenase was based upon measurement of the amount of reduced diphosphopyridine nucleotide (DPNH) formed from DPN in the presence of β-hydroxybutyrate.

Silica microcuvettes (light-path 1 cm.) contained (in μmoles): tris buffer, pH 8·5, 75; DPN, 0·35; sodium β-hydroxybutyrate, 1·0; bacterial extract, 50–200μg. of protein; water to a final volume of 0·4 ml. The addition of the bacterial extract initiated the reaction and the increase in $E_{340\ m\mu}$ was recorded. The formation of DPNH, taking readings at 30 sec. intervals, was linear over the range 50–250μg. of protein for the first 4 min.

One unit of activity was the amount causing an increase of 0·01/min. in $E_{340\ m\mu}$.

RESULTS

Synthesis of photosynthetic pigments by Athiorhodaceae under different growth conditions

The concentration of bacteriochlorophyll in *R. spheroides* and *R. palustris* grown aerobically in the dark under the conditions used in this work was about 1% of that in photosynthetically grown organisms (Table 1). Aerobically grown *R. capsulata* was more pigmented than the other organisms when grown under comparable conditions, containing about 4% of the amount of bacteriochlorophyll found in photosynthetically grown organisms. The levels of carotenoid were also considerably reduced by growth under aerobic conditions (Table 1).

Formation of acetoacetate

Extracts of *R. spheroides*, *R. capsulata* and *R. palustris* formed acetoacetate when incubated with acetyl-CoA (generated from acetyl phosphate by phosphotransacetylase) and succinate (Table 2).

Table 1. *Bacteriochlorophyll and carotenoid content of Athiorhodaceae grown anaerobically in the light and aerobically in the dark*

Organism	Growth conditions	Bacteriochlorophyll (μm-moles/mg. dry wt.)	Carotenoid (μm-moles/mg. dry wt.)
R. spheroides	Anaerobic, light	23·0	6·0
	Aerobic, dark	0·3	0·2
R. capsulata	Anaerobic, light	34·0	9·0
	Aerobic, dark	1·4	1·4
R. palustris	Anaerobic, light	24·0	—*
	Aerobic, dark	0·2	—

* Not examined.

Table 2. *Acetoacetate formation by extracts of Athiorhodaceae*

Each tube (1 cm. diam.) contained: bacterial extract, 1 mg. of protein; tris buffer, pH 8·5, 100 μmoles; acetyl phosphate, 25 μmoles; sodium succinate, 10 μmoles; CoA, 0·05 μmole; Na_2S, 4 μmoles; phosphotransacetylase, 5 units; water to 0·9 ml. Tubes were incubated at 34° for 30 min. and the reaction was stopped by addition of 0·1 ml. of 30% trichloroacetic acid. The precipitate was removed by centrifuging and the acetoacetate determined as described in the Experimental section. The activity is expressed as μmole of acetoacetate formed/hr./mg. of protein. The results are the average of three determinations with each type of extract; the range is shown in parentheses.

Organism	Acetoacetate synthesis by extracts from organisms grown: Aerobic, dark	Anaerobic, light
R. capsulata	0·32 (0·20–0·40)	0·15 (0·14–0·16)
R. spheroides	0·20 (0·16–0·26)	0·10 (0·08–0·12)
R. palustris	0·23 (0·18–0·28)	0·09 (0·09)

The amount formed/mg. of protein under the standard conditions was two to three times higher with extracts from aerobically grown bacteria than with those from organisms grown anaerobically in the light (Table 2).

Synthesis of acetoacetate was examined in more detail with extracts of aerobically grown *R. capsulata*.

Requirements for synthesis. When the extracts were incubated in the complete reaction mixture (containing tris buffer, acetyl phosphate, succinate, CoA, sodium sulphide and phosphotransacetylase) acetoacetate was formed at a linear rate over a period of 30 min. provided that the final protein concentration was from 0·5 to 3 mg./ml. (Fig. 1). The optimum pH for synthesis was about 8·5.

No acetoacetate was formed when acetyl phosphate was omitted from the reaction mixture and synthesis was reduced to about 20% in the absence of succinate (Table 3). Little synthesis

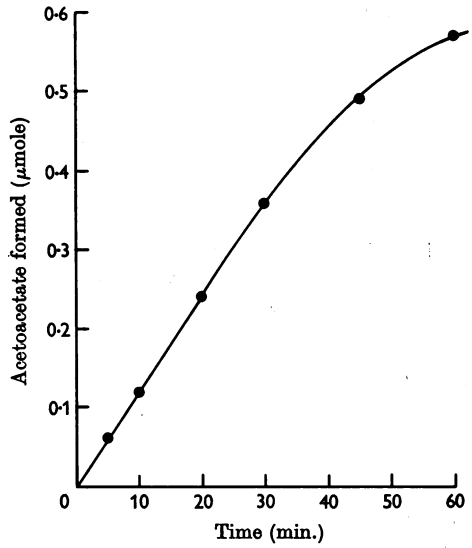

Fig. 1. Production of acetoacetate by *Rhodopseudomonas capsulata* extract (1·6 mg. of protein). Incubation conditions as in Table 2.

Table 3. *Requirement for acetoacetate formation by extracts of* Rhodopseudomonas capsulata

The complete system contained: extract of aerobically grown *R. capsulata*, 0·1 ml. (1·5 mg. of protein); tris buffer, pH 8·5, 100 μmoles; acetyl phosphate, 25 μmoles; sodium succinate, 10 μmoles; CoA, 0·05 μmole; Na_2S, 4 μmoles; phosphotransacetylase, 5 units; water to 0·9 ml. Incubation was for 30 min. at 34° before estimation of the acetoacetate as described (Table 2).

Component omitted	Acetoacetate formed (μmole)
None	0·44
Acetyl phosphate	0
Succinate	0·07
CoA	0·03
Phosphotransacetylase	0·01

occurred in the absence of CoA, and added phosphotransacetylase was also necessary (Table 3); the activity of this enzyme is only slight in these extracts. In the crude extracts stimulation by sulphide or glutathione was observed only occasionally. Glutathione, however, was needed for maximum synthesis by extracts treated with Dowex 1. It is also required for acetoacetate formation by liver preparations (Lynen *et al.* 1958).

Addition of adenosine di- or tri-phosphate or of inorganic phosphate (each at a concentration of 5 μmoles/ml.) did not increase acetoacetate formation.

Identification of acetoacetate. The method used to estimate acetoacetate involves its coupling with *p*-nitrobenzenediazonium hydroxide to give *NN'*-bis(nitrophenyl)-*C*-acetylformazan, which has an absorption maximum at 450 mμ (Walker, 1954). After reaction with *p*-nitrobenzenediazonium hydroxide the compound formed in the enzymic reaction mixture had the same absorption spectrum (over the range 300–600 mμ) as that given by authentic acetoacetate treated under the same conditions.

Identity of the product with acetoacetate was confirmed also by chromatography of the 2:4-dinitrophenylhydrazones (Dagley, Fewster & Happold, 1952).

Enzymes concerned in the formation of acetoacetate

The stimulation by succinate of acetoacetate formation from acetyl-CoA suggested that the CoA-transferase enzyme might participate; this would catalyse acetoacetate formation from acetoacetyl-CoA, arising from acetyl-CoA by the action of β-ketothiolase. The activity of these enzymes were therefore examined in extracts of Athiorhodaceae.

β-Ketothiolase. This enzyme was highly active in extracts of all the organisms tested and no difference was observed in preparations from cultures grown in the light or dark (Table 4). Even with crude extracts omission of CoA reduced the activity to about 15% of that in the complete system, whereas treatment with Dowex 1 reduced the activity in the absence of added CoA to about 5%. The inability to observe complete dependence upon CoA could have been due to traces of this substance in the acetoacetyl-CoA used as substrate.

Coenzyme A transferase. This enzyme was present in extracts of all three organisms tested. Its activity in extracts from both *R. spheroides* and *R. capsulata* grown anaerobically in the light was about 40% less than that found in preparations from aerobic cultures (Table 4). This difference in activity, though slight, was observed consistently.

The enzyme was examined in more detail with extracts of aerobically grown *R. spheroides*. The optimum pH was from 8·0 to 8·5, and succinate was not replaced by malate or fumarate. High concentrations of succinate were needed for maximum activity; this is in accord with observations made with purified preparations from pig heart (Stern *et al.* 1956*b*). In the present experiments the succinate concentration was 5 μmoles/0·4 ml., which permitted the enzymic reaction to proceed at nearly optimum rate. Higher concentrations of succinate interfered with the assay by chelating with Mg^{2+} ions (Stern *et al.* 1956*b*), thereby reducing their availability for complex formation with acetoacetyl-CoA, on which the sensitivity of the assay depends.

Enzymes concerned in the metabolism of β-hydroxy-β-methylglutaryl-coenzyme A

Key enzymes in the cycle proposed by Lynen *et al.* (1958) to account for the formation of free acetoacetate in liver are the HMG-CoA condensing enzyme (reaction 2) and the HMG-CoA cleavage enzyme (reaction 6). Evidence for the presence of these was sought in extracts of *R. spheroides* and *R. capsulata*.

Table 4. *β-Ketothiolase and succinyl-coenzyme A transferase in extracts of Athiorhodaceae*

Extracts were assayed under the standard conditions described in the Experimental section. The activities are expressed as units of enzyme/10 μg. of protein. The results are the mean values of several estimations with different extracts; the figures in parentheses show the range and the number of determinations.

Organism	Growth conditions	Activity: β-Ketothiolase	Activity: CoA transferase
R. spheroides	Anaerobic, light	40 (34–50; 3)	3·3 (2·6–4·0; 4)
	Aerobic, dark	40 (30–50; 3)	5·3 (4·0–5·7; 4)
R. capsulata	Anaerobic, light	35 (30–40; 2)	2·7 (2·3–3·4; 3)
	Aerobic, dark	30 (25–35; 2)	5·0 (4·0–6·0; 5)
R. palustris	Anaerobic, light	—*	2·7 (1 determination only)
	Aerobic, dark	—	4·1 (1 determination only)

* Not tested.

β-Hydroxy-β-methylglutaryl-coenzyme A condensing enzyme. This enzyme has been assayed in purified preparations from yeast (Ferguson & Rudney, 1959) and animal tissues (Lynen *et al.* 1958) by observing the rate of decline in $E_{310\ m\mu}$ due to condensation of acetoacetyl-CoA with acetyl-CoA. This method was applied to crude extracts of *R. capsulata* grown aerobically in the dark. However, the crude extracts, being rich in β-ketothiolase, rapidly decomposed acetoacetyl-CoA and the rate of decline in $E_{310\ m\mu}$ was not increased by addition of acetyl-CoA.

Treatment of extracts with Dowex 1 reduced the β-ketothiolase activity to about 5% in the absence of added CoA, but such extracts also failed to give evidence of the presence of HMG-CoA condensing enzyme.

β-Ketothiolase is more sensitive to inhibition by iodoacetamide than the HMG-CoA condensing enzyme and this differential effect has been used to eliminate β-ketothiolase activity from partially purified preparations of the condensing enzyme (Lynen *et al.* 1958; Ferguson & Rudney, 1959). Treatment of the bacterial extracts at 0° with iodoacetamide as described by Ferguson & Rudney (1959), or addition of the inhibitor (2–10 mM) directly to the assay mixture, was unsuccessful. The preparations were either completely inactivated or else there was no stimulation of acetoacetyl-CoA disappearance by acetyl-CoA.

Attempts to destroy differentially the β-ketothiolase by heat treatment also failed.

The presence of HMG-CoA could not be detected chromatographically (as the hydroxamate) in the incubation mixtures used to demonstrate free acetoacetate formation. In these experiments hydroxylamine (0·5 M, final concentration) was added after incubation for 60 min., and the hydroxamates were isolated and chromatographed (Stadtman & Barker, 1950); only acetohydroxamate was detected.

β-Hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme. Extracts of *R. spheroides*, *R. capsulata* and *R. palustris* formed acetoacetate when incubated with HMG-CoA, suggesting the presence of the HMG-CoA cleavage enzyme (Bachhawat *et al.* 1955). Extracts from aerobically grown *R. spheroides* were about three times more active than those from photosynthetically grown bacteria (Table 5).

The enzyme system was examined in more detail in extracts of aerobically grown *R. capsulata*. Addition of Mg^{2+} ions or glutathione did not increase its activity though both are required by the purified preparations from pig heart (Bachhawat *et al.* 1955). The optimum pH was at least 9·1, the highest pH tested. The cleavage enzyme in pig heart is also favoured by alkaline conditions, maximum activity occurring at pH 10 (Bachhawat *et al.* 1955).

To confirm that the acetoacetate formed from HMG-CoA was due to the cleavage enzyme, evidence for the other product of the reaction, acetyl-CoA, was sought with extracts of aerobically grown *R. capsulata*. To demonstrate the presence of acetyl-CoA use was made of the malate synthetase which is present in *R. capsulata* (Kornberg & Lascelles, 1960). When assayed by the method of Dixon & Kornberg (1959) the extracts were shown to be capable of forming malate from acetyl-CoA and glyoxylate at the rate of 2·8 μmoles/hr./mg. of protein. Incubation of extracts with [1-^{14}C]glyoxylate and subsequent radioautography showed incorporation of isotope into two components and that this was dependent on the presence of HMG-CoA (Table 6). Elution of these areas from the chromatogram and subsequent co-chromatography established their identity with malate and citrate. The latter evidently arose from the further metabolism of malate.

Table 5. *Cleavage of β-hydroxy-β-methylglutaryl-coenzyme A by extracts of Athiorhodaceae*

Bacterial extracts were incubated with HMG-CoA under the conditions described in the Experimental section. Activity is expressed as μmole of acetoacetate formed/hr./mg. of protein, and each value is the mean of three estimations with each type of extract. The figures in parentheses show the range.

Organism	Growth conditions	Activity
R. spheroides	Aerobic, dark	0·15 (0·09–0·21)
	Anaerobic, light	0·04 (0·03–0·05)
R. capsulata	Aerobic, dark	0·10 (0·07–0·11)

Table 6. *Synthesis of* [^{14}C]*malate by extracts of* Rhodopseudomonas capsulata

The complete reaction mixture contained: extract of aerobically grown *R. capsulata*, 4·5 mg. of protein; tris buffer, pH 8·5, 400 μmoles; $MgCl_2$, 5 μmoles; sodium [1-^{14}C]glyoxylate, 0·2 μmole (0·5 μc, 50 000 counts/min.); HMG-CoA, 0·5 μmole; water to 1·0 ml. Incubation was for 1 hr. at 34° in 1 cm. tubes and the reaction terminated by addition of 2 ml. of ethanol. In the control, ethanol was added at zero time. The ethanol extracts were examined by radioautography as described by Kornberg (1958).

Experimental conditions	Counts incorporated into malate and citrate* (%)
Complete reaction mixture	16·4
Without HMG-CoA	0·80
Zero-time control	0·20

* Percentage of total counts applied to chromatogram appearing in the malate and citrate.

β-Hydroxybutyrate dehydrogenase

Extracts of *R. spheroides*, *R. capsulatus* and *R. palustris* reduced DPN in the presence of DL-β-hydroxybutyrate. Only the D-isomer was active in this respect (personal communication from Professor H. A. Krebs). The activity of all organisms was similar and there was no significant difference in extracts prepared from cultures grown under the different conditions (Table 7). This enzyme was freely reversible, oxidizing DPNH in the presence of free acetoacetate.

β-Hydroxybutyryl-coenzyme A dehydrogenase. The presence of this enzyme was demonstrated in extracts of *R. spheroides* by the decline in $E_{340\ m\mu}$, due to DPNH in the presence of acetoacetyl-CoA. The assay of the enzyme was complicated by DPNH oxidase and β-ketothiolase present in the bacterial extract and no accurate measure of activity could be obtained from crude preparations.

DISCUSSION

The activities of the enzymes examined were similar in extracts of pigmented and non-pigmented organisms. There was no evidence that their levels contributed to the regulation of carotenoid synthesis by these bacteria. It has been suggested that formation of HMG-CoA is a key reaction in the synthesis of cholesterol and that at this point of formation of the basic branched carbon unit some control of cholesterol synthesis may be effected (Rudney & Ferguson, 1959; Lynen *et al.* 1958). This might also apply to carotenoid formation by the photosynthetic bacteria. The HMG-CoA condensing enzyme could not be demonstrated in crude extracts of these organisms, even after treatment with iodoacetamide, but its presence certainly cannot be excluded. This enzyme in liver (Lynen *et al.* 1958) can only be assayed spectrophotometrically in partially purified preparations. The presence in the bacterial extracts of a highly active β-ketothiolase, which would compete for the common substrate, acetoacetyl-CoA, accentuates the difficulty of demonstrating the HMG-CoA condensing enzyme. In addition, the HMG-CoA cleavage enzyme present in the extracts could also contribute to the inability to detect the condensing enzyme by the methods used. Recently evidence has been provided that the activity of the HMG-CoA reductase (reaction 3) is a major factor in the control of cholesterol formation by liver (Bucher, Overath & Lynen, 1960). Attempts to demonstrate this enzyme spectrophotometrically in extracts of *R. spheroides* by the decline in $E_{340\ m\mu}$ due to TPNH oxidation have been unsuccessful, but this negative result may not be significant since only crude extracts were used.

The formation of acetoacetate by extracts of Athiorhodaceae under the conditions used may be attributable to the action of β-ketothiolase (reaction 1) and CoA transferase (reaction 5). Stimulation of acetoacetate formation by succinate supports such a mechanism, which has been demonstrated in preparations from ox heart (Stern, Coon & del Campillo, 1956*a*). In liver there is evidence that acetoacetate also arises by cleavage of HMG-CoA (Lynen *et al.* 1958) and by deacylation of acetoacetyl-CoA (Drummond & Stern, 1960).

The level of β-ketothiolase in the photosynthetic bacteria is strikingly high, the activity (1·2 μmoles decomposed/mg./min.) being only slightly less than that in extracts of *Clostridium kluyveri* (2 μmoles/mg./min.), in which it is a key enzyme in the energy-yielding mechanism of the organism (Barker, 1956). The activity is three times as high as in *Mycobacterium tuberculosis* (0·4 μmole/mg./min.) (Goldman & Gelbard, 1959). The β-ketothiolase links the tricarboxylic acid cycle and fat metabolism. The high activity in the photosynthetic bacteria might be connected with the metabolism of the polymer of β-hydroxybutyric acid, an assimilation product which comprises up to 25 % of their dry weight under certain conditions (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959). It has been suggested that the β-hydroxybutyryl-CoA dehydrogenase participates in the formation of the polymer from acetoacetyl-CoA (Stanier *et al.* 1959) and this enzyme was found in the bacterial extracts. However, the D(−)-hydroxybutyrate dehydrogenase, examined in preparations from animal tissues (Wakeman & Dakin, 1909; Green, Dewan & Leloir, 1937; Lehninger, Sudduth & Wise, 1960) is highly active in extracts of Athiorhodaceae. It may possibly be connected with the metabolism of the polymer, thus affording an explanation of its relatively high activity.

β-Ketothiolase, L(+)-β-hydroxybutyryl-CoA dehydrogenase and a high level of crotonase have been shown in extracts of *Rhodospirillum rubrum*

Table 7. *β-Hydroxybutyrate dehydrogenase in extracts of Athiorhodaceae*

Extracts were assayed as described in the Experimental section. The results are expressed as units/μg. of protein. The activity shown is for one extract of each type.

Organism	Growth conditions	Activity
R. spheroides	Aerobic, dark	0·096
	Anaerobic, light	0·140
R. capsulata	Aerobic, dark	0·079
	Anaerobic, light	0·052
R. palustris	Aerobic, dark	0·090
	Anaerobic, light	0·140

(Stern, del Campillo & Raw, 1956); these enzymes might also reflect the ability of this organism to form polymer (Stanier *et al.* 1959).

SUMMARY

1. Cell-free extracts of photosynthetic bacteria of the Athiorhodaceae family form acetoacetate from acetyl phosphate in the presence of phosphotransacetylase and coenzyme A. Such extracts are rich in β-ketothiolase and coenzyme A transferase.

2. The extracts contained β-hydroxy-β-methylglutaryl-coenzyme A cleavage enzyme. Neither the formation of β-hydroxy-β-methylglutaryl-coenzyme A from acetoacetyl-coenzyme A and acetyl-coenzyme A, nor its reduction to mevalonic acid by reduced triphosphopyridine nucleotide, was detected.

3. The level of activity of these enzymes, and of β-hydroxybutyrate dehydrogenase and β-hydroxybutyryl-coenzyme A dehydrogenase, did not differ significantly in extracts prepared from pigmented and non-pigmented organisms.

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