The Photoassimilation of Succinate to Hexose by *Rhodospirillum rubrum*

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1. A pathway for the synthesis of hexose from succinate by *Rhodospirillum rubrum* is proposed. 2. With 2,3-¹⁴C₂-labelled succinate and fumarate as substrates in experiments with chromatophores and a soluble enzyme fraction of *R. rubrum* it was found that the products of succinate metabolism by the extracts were the same as in whole cells. It was also found that the light-dependent oxidation of succinate was catalysed by the chromatophores, but that all the other enzymes involved were in the soluble fraction. 3. By using specific assays the presence of all the enzymes required for the proposed pathway was demonstrated in the extracts and their specific activities were measured. 4. The overall rate of succinate assimilation was measured manometrically. The activities of the enzymes assayed were sufficient to account for the overall rate of assimilation. It is concluded that the proposed pathway represents the major mechanism for synthesis of hexose from succinate in *R. rubrum*. 5. The formation of alanine and aspartate was observed in experiments with isotopically labelled substrates, and possible synthetic pathways for these compounds are discussed.

Stanier, Doudoroff, Kunisawa & Contopoulou (1959) have shown that washed cells of *Rhodospirillum rubrum* assimilate succinate to a glycogen-like polysaccharide. By using ¹⁴C-labelled succinate as substrate they showed that the labelling pattern in the glucose units of the polysaccharide was consistent with the synthesis of glucose by the pathway shown in Scheme 1. Elsdon & Ormerod (1956), as a result of studies of the effect of inhibitors on the metabolism of organic acids by *R. rubrum*, had suggested that succinate was assimilated through phosphoenolpyruvate by the same reaction sequence, and that propionate was metabolized through succinate. Knight (1962) studied the metabolism of propionate by *R. rubrum* by using the short-exposure technique. He found that propionate was carboxylated to succinate and that this is oxidized to fumarate and then to malate. Glutamate, alanine and aspartate were also early products of succinate metabolism.

It has been shown (Evans, 1965) that the coupled photo-oxidation of succinate and photoreduction of NAD by chromatophores results in the formation of fumarate but no other intermediates of succinate assimilation. The steps involved in the further metabolism of succinate carbon and the synthesis of hexose from succinate by *R. rubrum* have been investigated with chromatophores and a soluble enzyme fraction.

MATERIALS AND METHODS

R. rubrum was grown and chromatophores were prepared as described by Evans (1965). A soluble enzyme fraction was prepared by ultracentrifugation. Cells washed once in 0.1M-tris-HCl buffer, pH 7.5, were resuspended at 300–400 mg. wet wt./ml. in 0.1M-tris-HCl buffer, pH 8.0. The cells and extract were kept at 0° throughout the preparation. The cells were disrupted by exposure to ultrasonic oscillation for 90 sec. by using a Mullard 500 w 25 kcy./sec. ultrasonic generator. The preparation was centrifuged for 10 min. at 10000g to remove cell debris and then for 2 hr. at 105000g to remove chromatophores. The resultant extract diluted to 10 mg. of protein/ml. contained not more than 12 µg. of bacteriochlorophyll/ml. For use in experiments with ¹⁴C-labelled substrates the buffer concentration was decreased to 0.01M and the pH of the extract corrected to 8.0 with NaOH before use. The extract was stored unfrozen on ice. Fresh extracts were used in all experiments and assays.

Manometric experiments on the photometabolism of succinate. Cells were harvested by centrifugation, washed once in cold 1% (w/v) KCl, resuspended in 1% KCl and diluted to 5 mg. dry wt./ml. in 0.025M-NaHCO₃. Dry weight was determined from the extinction at 680 mµ in a Unicam SP.600 spectrophotometer by using a calibration curve.

Warburg flasks contained 2 ml. of cell suspension and 0.8 ml. of water; side arms contained 0.2 ml. of 0.1M-sodium succinate or 0.2 ml. of water. Flasks were gassed with N₂ + CO₂ (95:5) and were incubated at 30° in an illuminated water bath. Gas uptake was followed for 60 min. after the

Scheme 1. Proposed pathway of hexose formation in *R. rubrum*, based on the work of Elsden & Ormerod (1956), Stanier *et al.* (1959) and Knight (1962).

contents of the side bulb were tipped. Activity was expressed as Q_{CO_2} , determined from the difference in rates between control and experimental flasks.

Experiments with ^{14}C -labelled substrates. Experiments with ^{14}C -labelled substrates were carried out under anaerobic conditions in Thunberg cuvettes designed to fit the cell compartment of the Unicam SP.500 spectrophotometer. They were made anaerobic by a series of four evacuations each followed by flushing with oxygen-free nitrogen (British Oxygen Co. Ltd.). After incubation for 30 min. at 30° in the light a fraction soluble in 80% (v/v) ethanol was prepared as described by Evans (1965).

Chromatography and radioautography. Whatman no. 4 and 3MM papers were washed before use as described by Knight (1962). The fraction soluble in 80% ethanol was analysed by two-dimensional chromatography in the phenol-water and butan-1-ol-propionic acid-water system of Benson *et al.* (1950).

Dicarboxylic acids were identified by co-chromatography in the two-dimensional system, and in the AnalR pentan-1-ol-5M-formic acid solvent of Buch, Montgomery & Porter (1952). The latter was used one-dimensionally on unwashed Whatman no. 1 paper.

Amino acids were identified by co-chromatography in the two-dimensional system and by one-dimensional chromatography in the propanol-ammonia system of Hirsch & Schlegel (1963).

2,4-Dinitrophenylhydrazones of trioses were identified by

chromatography in the 2-methylbutan-2-ol-ethanol-water system of Altmann, Crook & Datta (1951).

Radioautographs were made by exposing the chromatograms to Ilford Industrial G X-ray films. The length of exposure was determined by the total radioactivity on the chromatogram. The film was developed with Ilford ID19 X-ray developer.

Enzyme assays. A temperature of 30° was used for all enzyme assays. Assays involving direct measurements of extinction changes were done in a temperature-controlled Unicam SP.500 spectrophotometer with cuvettes with a 1 cm. light-path. For comparative purposes all assays were done at pH 8.0, the pH optimum of the photoreduction reaction, which is the first step in the assimilation of succinate. Rates were measured on three extracts for each reaction. Rates were expressed as μ moles of substrates metabolized/mg. of protein/hr.

Succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1]. This was assayed in the photoreduction reaction with NAD as acceptor, as described by Evans (1965), by using Thunberg cuvettes for the Unicam SP. 500. The reaction mixture contained (in 3 ml.): sodium succinate, 20.0 μ moles; NAD, 2.0 μ moles; $MgCl_2$, 5.0 μ moles; tris-HCl buffer, pH 8.0, 100 μ moles; chromatophores (50 μ g. of bacteriochlorophyll) were added from the side arm of the cuvette to start the reaction. The mixture was incubated anaerobically under oxygen-free nitrogen for 30 min. in the light and the increase in extinction at 340 m μ read against a dark-control as a measure of the NAD reduced.

Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2). This was assayed by the method of Massey (1955). The reaction mixture contained (in 3 ml.): sodium fumarate, 50 μ moles; tris-HCl buffer, pH 8.0, 200 μ moles; soluble fraction (3.0 mg. of protein) was added to start the reaction. The decrease in extinction at 300 m μ was followed.

Malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37). This was assayed as described by Ochoa (1955a). The reaction mixture contained (in 3 ml.): sodium oxaloacetate, 4.0 μ moles; $NADH_2$, 0.4 μ mole; tris-HCl buffer, pH 8.0, 200 μ moles; soluble fraction (0.02 mg. of protein) was added to start the reaction. The oxidation of $NADH_2$ was followed by the decrease in extinction at 340 m μ .

Phosphoenolpyruvate carboxylase [GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32]. A qualitative demonstration of the presence of this enzyme was made by the CO_2 -exchange reaction described by Utter & Kurahashi (1955). A quantitative estimate of the activity was made by measuring the formation of phosphoenolpyruvate from oxaloacetate with ITP as energy source. This assay was based on that described by Siu, Wood & Stjernholm (1961) for 'phosphoenolpyruvic carboxytransphosphorylase'. The reaction mixture contained (in 2 ml.): sodium oxaloacetate, 10.0 μ moles; ITP, 5.0 μ moles; $MnSO_4$, 1.0 μ mole; tris-HCl buffer, pH 8.0, 100 μ moles; soluble fraction (1.0 mg. of protein) was added to start the reaction. After incubation for 5 min. the reaction was stopped by the addition of 1 ml. of 3N-perchloric acid. The precipitate was removed by centrifuging, the supernatant neutralized with K_2CO_3 , and a sample taken for phosphoenolpyruvate estimation. This assay gives a minimum rate for the reaction as some of the phosphoenolpyruvate may be further metabolized by phosphoenolpyruvate hydratase in the extract.

Phosphoenolpyruvate hydratase (D-2-phosphoglycerate hydro-lyase, EC 4.2.1.11) and phosphoglycerate phosphomutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1). Phosphoenolpyruvate hydratase was assayed by following the disappearance of phosphoenolpyruvate during incubation with the extract. The reaction mixture contained (in 2 ml.): phosphoenolpyruvate, 10.0 μ moles; $MgCl_2$, 10.0 μ moles; tris-HCl buffer, pH 8.0, 100 μ moles; soluble fraction (1.0 mg. of protein) was added to start the reaction. After incubation for 5 min. the reaction was stopped as described for the phosphoenolpyruvate-carboxylase assay, and the residual phosphoenolpyruvate estimated.

Phosphoenolpyruvate hydratase and phosphoglycerate phosphomutase were assayed together. Phosphoenolpyruvate formation from 3-phosphoglycerate was followed to obtain a measure of the overall rate of the two reactions. The reaction mixture contained (in 2 ml.): 3-phosphoglyceric acid, 20.0 μ moles; $MgCl_2$, 10.0 μ moles; tris-HCl buffer, pH 8.0, 100 μ moles; soluble fraction (1 mg. of protein) was added to start the reaction. After incubation for 5 min. the reaction was stopped as described for the phosphoenolpyruvate-carboxylase assay, and the phosphoenolpyruvate formed estimated.

Phosphoglycerate kinase (ATP-D-3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3) and triose phosphate dehydrogenase [D-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating), EC 1.2.1.12]. These two enzymes were assayed together by using an assay based on that of Lascelles (1960). The ATP-dependent oxidation of $NADH_2$ with 3-phosphoglycerate as substrate was followed. The reaction mixture contained (in 3 ml.): sodium 3-phosphoglycerate, 10.0 μ moles; ATP, 10.0 μ moles; $NADH_2$, 0.5 μ mole; GSH, 10.0 μ moles; soluble fraction (0.5 mg. of protein) was added to start the reaction.

Triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1). The presence of this enzyme was demonstrated qualitatively by trapping the product of the reaction described above for the assay of phosphoglycerate kinase and triose phosphate dehydrogenase with hydrazine. The product was then hydrolysed with *N*-NaOH at room temperature and the 2,4-dinitrophenylhydrazone prepared. After extraction into ethyl acetate this was identified by paper chromatography. Only one product was observed, the 2,4-dinitrophenylhydrazone of which had the same R_F as that of dihydroxyacetone. No spot was seen corresponding to that of the 2,4-dinitrophenylhydrazone of D-glyceraldehyde. This indicates that any D-glyceraldehyde formed was immediately converted into dihydroxyacetone phosphate by triose phosphate isomerase in the extract. A quantitative estimate of the activity of this enzyme was not made.

Aldolase (ketose 1-phosphate aldehyde-lyase, EC 4.1.2.7). This enzyme was assayed by the method of Taylor (1955), in which triose phosphate formed from fructose 1,6-diphosphate is trapped with cyanide. The reaction mixture contained (in 0.4 ml.): fructose 1,6-diphosphate, 10.0 μ moles; KCN, 20.0 μ moles; tris-HCl buffer, pH 8.0, 100 μ moles; soluble fraction (1.0 mg. of protein) was added to start the reaction. After incubation for 5 min. the reaction mixture was hydrolysed with *N*-NaOH for 15 min. at room temperature and the alkali-labile phosphate determined. A second assay, in which the arsenate-dependent reduction of NAD with fructose 1,6-diphosphate as substrate is followed (Warburg & Christian, 1943), was also used. This assay

depended on the triose phosphate dehydrogenase and phosphoglycerate kinase in the extract and gives a measure of the overall rate of reaction, not that of aldolase alone. The reaction mixture contained (in 3 ml.): fructose 1,6-diphosphate, 10.0 μ moles; NAD, 1.0 μ mole; Na_3AsO_4 , 50.0 μ moles; GSH, 10.0 μ moles; tris-HCl buffer, pH 8.0, 100 μ moles; soluble fraction (1.0 mg. of protein) was added to start the reaction.

The rates of reaction observed were similar for both assays.

Pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40). The assay used for pyruvate kinase was based on the method for the estimation of phosphoenolpyruvate described by Czok & Eckert (1963). Muscle lactate dehydrogenase was added as indicator enzyme and the oxidation of $NADH_2$ with phosphoenolpyruvate as substrate followed. The enzyme requires Mn^{2+} . The reaction mixture contained (in 3 ml.): phosphoenolpyruvate, 10.0 μ moles; ADP, 5.0 μ moles; $NADH_2$, 0.4 μ mole; $MnSO_4$, 1.0 μ mole; tris-HCl buffer, pH 8.0, 200 μ moles; muscle lactate dehydrogenase, 0.02 mg. of protein; soluble fraction, 0.5 mg. of protein. Lactate dehydrogenase was added, the extinction at 340 $m\mu$ read after 2 min. and then the soluble fraction added. If the soluble fraction was added without lactate dehydrogenase pyruvate accumulated.

Aminotransferases. Aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) and alanine aminotransferase (L-alanine-2-oxoglutarate aminotransferase, EC 2.6.1.2) were demonstrated qualitatively by incubating glutamate and either oxaloacetate or pyruvate with the extract. After incubation the amino acids formed were identified by chromatography. The enzymes were identified and assayed quantitatively by the method of Bergmeyer & Bernt (1963). In this assay the transamination of the amino acid with 2-oxoglutarate is coupled to the oxidation of $NADH_2$ by the keto acid formed with the relevant dehydrogenase. Aspartate aminotransferase was followed with the endogenous malate dehydrogenase, and alanine aminotransferase with added muscle lactate dehydrogenase. The reaction mixture contained (in 3 ml.): sodium 2-oxoglutarate, 20.0 μ moles; $NADH_2$, 0.5 μ mole; tris-HCl buffer, pH 8.0, 200 μ moles; soluble fraction (0.1 mg. of protein); either sodium aspartate, 100 μ moles, or sodium alanine, 100 μ moles, plus muscle lactate dehydrogenase (0.02 mg. of protein).

Malate dehydrogenase (decarboxylating) [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40]. This enzyme was assayed as described by Ochoa (1955b), the reduction of NADP with malate as substrate being followed. The reaction mixture contained (in 3 ml.): sodium malate, 20.0 μ moles; NADP, 0.5 μ mole; $MnSO_4$, 5.0 μ moles; tris-HCl buffer, pH 8.0, 200 μ moles; soluble fraction, 5.0 mg. of protein.

Oxaloacetate decarboxylase (oxaloacetate carboxy-lyase, EC 4.1.1.3). This enzyme was assayed manometrically as described by Herbert (1955). The reaction mixture contained (in 3 ml.): sodium acetate buffer, pH 5.4, 330 μ moles; $MnSO_4$, 3.0 μ moles; sodium oxaloacetate, 30.0 μ moles; soluble fraction, 10.0 mg. of protein. The gas phase was air. The CO_2 output was followed for 10 min. after tipping oxaloacetate from the side arm.

Aspartate ammonia-lyase (L-aspartate ammonia-lyase, EC 4.3.1.1). This enzyme was assayed as described by Virtanen & Ellfolk (1955). No activity was detected.

Photophosphorylation. The rate of photophosphorylation was measured by the method described by Geller & Lipmann (1960). The reaction mixture contained (in 1 ml.): ADP, 10.0 μ moles; $MgCl_2$, 5.0 μ moles; potassium phosphate buffer, pH 7.5, 10.0 μ moles; chromatophores (50 μ g. of bacteriochlorophyll) were tipped from the side arm to start the reaction. The gas phase was oxygen-free nitrogen. After incubation for 30 min. in the light the decrease in inorganic phosphate was measured as an estimate of photophosphorylation.

Analytical procedures. Inorganic phosphate was estimated by the method of Allen (1940). Bacteriochlorophyll was estimated by the method of van Niel & Arnold (1938) by using the extinction coefficient given by Lascelles (1956).

Protein was estimated by the method of Warburg & Christian (1942).

Phosphoenolpyruvate was assayed as described by Czok & Eckert (1963) with muscle lactate dehydrogenase and pyruvate kinase. Free amino groups were estimated by the method of Yemm & Cocking (1955), with glutamic acid as standard.

Fructose and fructose phosphates were estimated by the method of Roe, Epstein & Goldstein (1949).

Measurement of radioactivity. Radioactive spots on chromatograms were outlined by comparison with the radioautographs and counted directly on the paper with a large-diameter end-window Geiger-Müller tube (General Electric Co. Ltd., type 2B2) in conjunction with a Panax D657 scaler (Panax Equipment Ltd., Redhill, Surrey).

Reagents. ATP, ADP, NAD and NADP (all as sodium salts), 3-phosphoglyceric acid and fructose 1,6-diphosphate (both as barium salts), phosphoenolpyruvate (silver-barium salt), GSH, muscle lactate dehydrogenase and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. ITP and $NADH_2$ were from the Sigma Chemical Co., St Louis 18, Mo., U.S.A. Oxaloacetic acid was from the Sigma Chemical Co. or was prepared as described by Heidelberger (1953).

Radioactive succinic acid and fumaric acid were obtained from The Radiochemical Centre, Amersham, Bucks. They were neutralized with NaOH and dissolved in water before use. They were found to contain less than 3% of radioactive impurities in the chromatographic procedures used.

RESULTS

The photo-oxidation of succinate by chromatophores results in the formation of fumarate (Evans, 1965). The further metabolism of succinate carbon has been investigated by using $[2,3-^{14}C_2]$ succinate in experiments with crude extracts or chromatophores plus a soluble fraction of the cell, or by using $[2,3-^{14}C_2]$ fumarate with the soluble fraction alone. In all these experiments the reaction products identified by two-dimensional paper chromatography were similar. The major products were malate and alanine with smaller amounts of aspartate and glutamate. Succinate was not metabolized by the soluble fraction alone under the conditions used, but it was formed as a product when $[2,3-^{14}C_2]$ fumarate was used as a substrate. In experiments with $[2,3-^{14}C_2]$ succinate (Table 1) it was found that

Table 1. *Metabolism of $[2,3-^{14}C_2]$ succinate by chromatophores and soluble fraction*

	Conditions ...			Light		Light		Light		Light		Light		Light		Dark		Dark	
	Additions ...			None		NAD		ADP		NAD + ADP		Soluble fraction + NAD		Soluble fraction + NAD + ADP		NAD + ADP		Soluble fraction + NAD + ADP	
	Radioactivity (counts/min.)	Distribution of radioactivity (%)		Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)
Succinate	11 113	96	4	6675	60	6675	60	12 500	96	8474	73	8950	88	8950	88	11 220	95.5	10 960	96.5
Fumarate	480			4456	40	4456	40	560	4	3052	27	560		560		535	4.5	535	4.5
Succinate	9800	91		8840	84	8840	84	10 705	93	10 705	93	10 705	93	10 705	93	10 960	96.5	10 960	96.5
Fumarate	158	1.5		208	2.0	208	2.0	152	1.3	152	1.3	152	1.3	152	1.3	77	0.7	77	0.7
Malate	548	5.1		840	8.0	840	8.0	540	4.6	540	4.6	540	4.6	540	4.6	73	0.6	73	0.6
Aspartate	114	1.0		122	1.5	122	1.5	86	0.7	134	1.3	134	1.3	134	1.3	27	0.2	27	0.2
Glutamate	60	0.5		192	1.8	192	1.8	100	0.8	155	1.5	155	1.5	155	1.5	160	1.4	160	1.4
Alanine	67	0.6		252	2.4	252	2.4	55	0.4	165	1.6	165	1.6	165	1.6	47	0.4	47	0.4

The experimental procedure was as described in the Materials and Methods section. The complete reaction mixture contained (in 3 ml.): $[2,3-^{14}C_2]$ succinate, 2.1 μ moles (5 μ Ci); $MgCl_2$, 5.0 μ moles; P, 5.0 μ moles; tris-HCl buffer, pH 8.0, 30 μ moles; chromatophores, 100 μ g. of bacteriochlorophyll. Additions, as indicated, were: NAD, 2.0 μ moles; ADP, 5.0 μ moles; soluble fraction, 13 mg. of protein. The gas phase was nitrogen and the temperature 30°. Incubation was for 30 min. After incubation an 80% ethanol-soluble fraction was prepared and the products were separated by two-dimensional chromatography.

the oxidation of succinate in the light was inhibited by the addition of either the soluble fraction or of a phosphorylating system. In the latter case inhibition was observed only in the presence of both ADP and inorganic phosphate, neither alone causing inhibition. As less than 10% of the succinate was metabolized in experiments with succinate as substrate in the presence of soluble fraction and the phosphorylating system, further experiments were done with [2,3-¹⁴C₂]fumarate. Table 2 shows the effect of various nucleotides on the metabolism of fumarate by the soluble fraction. The addition of NAD had little effect on the products formed, the addition of NADP resulted in an increase in the amount of alanine formed, and the addition of ATP resulted in the appearance of a number of slow-running spots on the chromatograms in the area where phosphorylated compounds might be expected to run. One of these compounds was identified as phosphoglycerate by chromatography in the two-dimensional system. The amount of phosphate esters formed was increased by the addition of GSH. These experiments suggested that the extracts catalysed the metabolism of succinate in a qualitatively similar way to that observed in whole cells

by Knight (1962). This has been confirmed by the use of specific assays to demonstrate the presence of the enzymes involved in the reaction sequence shown in Scheme 1 in the extract, and to measure the specific activities of these enzymes. The soluble fraction obtained by ultracentrifugation was used without further purification in these experiments. Table 3 shows the specific activities of the enzymes which, acting together, could convert succinate into fructose 1,6-diphosphate, and of those involved in the formation of the amino acids detected in the experiments with isotopically labelled substrate. All the assays were performed at pH 8.0 and it was found that all the enzymes except malate dehydrogenase showed optimum activity between pH 7.5 and 8.5 in the buffer used. Although the malate dehydrogenase was the most active of the enzymes assayed at pH 8.0 its optimum activity was at pH 9.0. At pH 8.0 the equilibrium position of the malate-dehydrogenase reaction favours oxaloacetate reduction, virtually no oxidation of malate occurring, although the oxidation of malate may be followed at pH 9.0 and above. It was found that the malate-dehydrogenase reaction could be coupled with enzymes catalysing the further metabolism of

Table 2. *Metabolism of [2,3-¹⁴C₂]fumarate by soluble fraction*

The experimental procedure was as described in the Materials and Methods section. The complete reaction mixture contained (in 3 ml.): [2,3-¹⁴C₂]fumarate, 2.1 μmoles (5.0 μc); MgCl₂, 5.0 μmoles; MnSO₄, 5.0 μmoles; tris-HCl buffer, pH 8.0, 30 μmoles; soluble fraction, 14 mg. of protein. Additions, as indicated, were: NAD, 2.0 μmoles; NADP, 2.0 μmoles; ATP, 10.0 μmoles; GSH, 10.0 μmoles. After incubation for 30 min. at 30° under nitrogen an 80%-ethanol-soluble fraction was prepared and the products were separated by two-dimensional chromatography.

	Additions ... None		NAD		ATP	
	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)
Succinate	1340	17.3	1215	16.5	1565	19.0
Fumarate	1170	15.1	978	13.2	820	10.0
Malate	3630	47.0	329	45.0	3630	43.0
Aspartate	443	5.7	322	4.4	454	5.5
Glutamate	160	2.1	430	5.8	160	1.9
Alanine	970	12.6	1105	15.0	1260	15.3
Phosphate esters	—	—	—	—	325	4.0

	Additions ... NAD+ATP		NADP+ATP		NAD+ATP+GSH	
	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)	Radioactivity (counts/min.)	Distribution of radioactivity (%)
Succinate	1365	20.2	1450	26.5	1425	26.5
Fumarate	525	7.8	335	6.2	145	2.7
Malate	2600	38.5	1615	29.6	1128	21.0
Aspartate	464	6.9	173	3.8	285	5.3
Glutamate	350	5.2	156	2.9	710	13.3
Alanine	1275	18.9	1547	28.4	1223	22.8
Phosphate esters	190	2.8	200	3.7	445	8.3

Table 3. *Specific activities of the enzymes involved in the metabolism of succinate*

Details of the assays used for each enzyme are given in the Materials and Methods section. The rates are the average values for three assays.

Enzyme	Rate of reaction	
	(μ moles/mg. of protein/hr.)	(μ moles/mg. of bacteriochlorophyll/hr.)
NAD-linked succinate dehydrogenase (photoreduction)		24.0
Photophosphorylation		280.0
Fumarate hydratase	99.0	
Malate dehydrogenase	590.0	
Phosphoenolpyruvate carboxylase	19.4	
Malate dehydrogenase + phosphoenolpyruvate carboxylase	7.8	
Phosphoenolpyruvate hydratase + phosphoglycerate phosphomutase	13.7	
Phosphoglycerate kinase + triose phosphate dehydrogenase	16.2	
Triose phosphate isomerase	Present	
Aldolase (chemical assay)	3.2	
Pyruvate kinase	9.5	
Aspartate aminotransferase	33.0	
Alanine aminotransferase	8.4	
Malate dehydrogenase (decarboxylating)	0.5

Fig. 1. Coupling of malate dehydrogenase and phosphoenolpyruvate carboxylase. The reaction mixture contained (in 3 ml.): sodium malate, 20 μ moles; NAD, 2.0 μ moles; $MnSO_4$, 1.0 μ mole; tris-HCl buffer, pH 8.0, 200 μ moles; soluble fraction, 1.0 mg. of protein. Additions, as indicated, were: ITP, 10.0 μ moles; ADP, 5.0 μ moles; muscle lactate dehydrogenase (LDH), 0.02 mg. of protein; pyruvate kinase (PK), 0.02 mg. of protein. The incubation was at 30°. The change in extinction at 340 $m\mu$ was followed.

oxaloacetate to result in a continuous oxidation of malate at pH 8.0. Two enzymes in the extract were found to couple in this way, phosphoenolpyruvate carboxylase and aspartate aminotransferase. In the presence of ITP and Mn^{2+} the extract catalysed the formation of phosphoenolpyruvate from malate by the combined action of malate dehydrogenase and phosphoenolpyruvate carboxylase (Fig. 1). In these experiments the reduction of NAD on the addition of ITP was followed to obtain a measure of the overall rates of reaction. After 10 min. incubation muscle lactate dehydrogenase, ADP and pyruvate kinase were added to confirm that phosphoenolpyruvate was being formed in the reaction. If glutamate was added instead of ITP a similar oxidation of malate was observed, presumably coupled to the formation of aspartate.

A measure of the overall rate of succinate assimilation by *R. rubrum* may be obtained manometrically. The Q_{CO_2} observed in manometric experiments is not a direct measure of the rate of succinate assimilation as photosynthetic carbon dioxide fixation occurs during the photometabolism of succinate by this organism, as was shown by Ormerod (1956). The observed Q_{CO_2} can be used to calculate the rate of succinate assimilation if the following assumptions are made. First, that succinate is assimilated to a non-polar compound, as indicated by the experiments of Stanier *et al.* (1959). In this case for each molecule of succinate metabolized two carboxyl groups are neutralized, resulting in an uptake of two molecules of carbon dioxide by the system. Secondly, that one molecule of carbon dioxide is

Table 4. Rates of succinate assimilation by washed cells of *R. rubrum* based on manometric data, and minimum rates calculated to be required for the individual enzyme reactions

	Observed Q_{CO_2}		$Q_{\text{succinate}}$ ($\mu\text{moles/mg.}$ dry wt./hr.)	Required rates for enzyme reactions	
	($\mu\text{l./mg. dry}$ wt./hr.)	($\mu\text{moles/mg.}$ dry wt./hr.)		($\mu\text{moles/mg.}$ dry wt./hr.)	($\mu\text{moles/mg. of}$ bacteriochloro- phyll/hr.)
<i>R. rubrum</i> (Stanier)	12.0	0.54	0.36	2.7	22.5
<i>R. rubrum</i> (S1) (Elsden, 1962)	19.5	0.89	0.59	4.9	37.0

liberated on the conversion of one molecule of oxaloacetate into phosphoenolpyruvate. Thirdly, that one molecule of NADH_2 is available for carbon dioxide fixation/molecule of succinate assimilated. If this fixation occurred through the Calvin cycle, 0.5 mole of carbon dioxide would be fixed/mole of succinate assimilated. The experiments of Ormerod (1956) with $^{14}\text{CO}_2$ confirmed that 0.5 mole of carbon dioxide is fixed/mole of succinate. A carbon dioxide uptake of 1.5 moles/mole of succinate is therefore observed manometrically.

Two experiments gave Q_{CO_2} values of 11 and 13.5 $\mu\text{l./mg. dry wt./hr.}$ These values fall at the bottom of the range given by Elsden & Ormerod (1956) and Elsden (1962). The relationship between dry wt. of cells, chlorophyll and soluble protein in the bacteria was measured. The bacteria were found to contain (average of three determinations) 1.6 mg. of chlorophyll and 12.9 mg. of soluble protein/100 mg. dry wt. From these values and the rate of succinate metabolism observed manometrically, minimum rates were calculated for the enzyme reactions involved in succinate assimilation. Table 4 shows these rates calculated from the above Q_{CO_2} and also for the maximum Q_{CO_2} reported by Elsden (1962).

Qualitative attempts to demonstrate the synthesis of fructose from intermediates of the proposed pathway were successful only with 3-phosphoglycerate. When 3-phosphoglycerate, ATP and NADH were incubated with the soluble fraction, a material giving a positive reaction with the orcinol reagent was formed. It was not further identified. With phosphoenolpyruvate as substrate, a compound giving a yellow colour with the orcinol reagent was formed, preventing the detection of any fructose that may have been formed. No reaction with the orcinol reagent was observed when malate was the substrate.

DISCUSSION

The experiments with ^{14}C -labelled substrates confirm that the light-dependent NAD-linked succinate-dehydrogenase activity of the cell is confined to the chromatophores, and that the enzymes involved in the further metabolism of fumarate to malate, alanine, aspartate, glutamate and phos-

phate esters occur only in the soluble fraction of the cell. They also suggest that the soluble fraction contains fumarate-reductase activity, succinate being formed from fumarate. The formation of pyruvate and oxaloacetate, the precursors of alanine and aspartate, entails the oxidation of malate. The absence of a nucleotide requirement for these reactions suggests that in the supernatant the oxidation may be coupled to the reduction of fumarate. This suggestion is supported by the fact that the amount of succinate formed was approximately equal to the sum of the amounts of alanine and aspartate formed with fumarate as substrate.

All the enzymes required for the conversion of succinate into fructose 1,6-diphosphate were detected in the extracts of *R. rubrum*. It was found that all the enzymes, except triose phosphate isomerase, for which no rate measurements were made, had specific activities higher than the 2.7 $\mu\text{moles/mg. of protein/hr.}$ required for the overall rate of assimilation of succinate measured manometrically. The rate of the photophosphorylation reaction is also more than adequate to supply the 3.5 moles of ATP required/mole of succinate assimilated. Only one reaction in the sequence, the photo-oxidation of succinate, is directly dependent on light, all the others proceeding in the dark. Two steps are indirectly dependent on the light reaction, phosphopyruvate carboxylase, for the supply of ITP, assuming that this is formed by photophosphorylation directly or by the interaction of IDP and ATP, and 3-phosphoglycerate kinase for ATP.

None of the enzymes assayed had sufficiently low activities to be obvious rate-limiting steps. The initial oxidation of succinate was one of the two slowest reactions measured. This reaction may control the overall rate, particularly as it is inhibited by the photophosphorylation system. It is possible that maximum rates of succinate oxidation occur only when the ATP/ADP ratio is high. However, comparison of the Q_{CO_2} for succinate and malate assimilation reported by Elsden (1962) suggests that these two compounds are assimilated at similar rates. If the rate of malate assimilation is calculated for the Q_{CO_2} , making the same assumptions as those described above for succinate, except that no

NADH₂ is available for carbon dioxide fixation, one mole of carbon dioxide will be taken up by the system/mole of malate metabolized. The Q_{CO_2} for succinate reported by Elsden of 19.5 μ l./mg. dry wt./hr. is equivalent to a rate of succinate assimilation of 0.59 μ mole/mg. dry wt./hr., and the Q_{CO_2} for malate of 12.0 μ l./mg. dry wt./hr. to a rate of assimilation of 0.54 μ mole/mg. dry wt./hr. It therefore seems that in washed cells the succinate dehydrogenase is not rate-limiting. With the chromatophore preparations used the photoreduction activity was quite labile, up to 50% of the activity being lost on standing overnight at 0°. It is possible that the rates measured are considerably below those occurring in the cell owing to the loss of components of the system during preparation of the chromatophores.

The other enzyme with relatively low activity was aldolase, but even with this enzyme the rate was slightly higher than the minimum required. *R. rubrum* thus differs from *Rhodospirillum spheroides*, in which Syzmona & Doudoroff (1960) found only very low aldolase activity. One other enzyme that might present a rate-limiting step is the malate dehydrogenase; Eisenberg (1953) found that extracts of *R. rubrum* would only oxidize malate at pH 9.0 or above, and this was confirmed in the assay used in these experiments. However, the fact that at pH 8.0 the malate dehydrogenase can couple with the phosphoenolpyruvate carboxylase, which overcomes the unfavourable equilibrium of the malate dehydrogenase by removing oxaloacetate, to synthesize phosphoenolpyruvate at a rate in excess of that required for the overall assimilation, eliminates this possibility. It is not therefore possible to select any of the steps investigated as the one controlling the overall rate of reaction.

The synthesis of fructose from malate by the reaction sequence proposed here has been demonstrated in an animal system by Mendicino & Utter (1962) by using purified glycolytic enzymes with mitochondria as an ATP-generating system. The failure to observe the formation of fructose (or fructose phosphates), except from 3-phosphoglycerate, in the present experiments was probably due to the interference of side reactions in the crude extracts used. Two reactions that would particularly interfere are the pyruvate-kinase reaction, which might convert any phosphoenolpyruvate formed into pyruvate, the pyruvate being transaminated to alanine, and the coupling of malate oxidation to fumarate reduction, which would decrease the amount of NADH₂ available for the triose phosphate-dehydrogenase reaction. In whole cells these reactions might be prevented by the maintenance of a high ATP/ADP ratio by photophosphorylation, which would prevent the pyruvate-kinase reaction, and of a high NADH₂ concentration by photoreduction.

The formation of three amino acids was observed in the experiments with ¹⁴C-labelled substrates. The mechanism of glutamate formation was not investigated.

It was found that the formation of aspartate could be accounted for by the transamination of oxaloacetic acid with glutamate. This reaction was possible in the experiments with ¹⁴C-labelled substrate as the extracts contained 0.40–0.46 μ mole of free amino groups/mg. of protein. Knight (1962) suggested on the basis of the different labelling patterns in malate and aspartate in his experiments that aspartate was formed from fumarate by aspartate ammonia-lyase, rather than through malate. No evidence for the presence of aspartate ammonia-lyase was found in the present experiments. However, Knight (1962) did not have glutamate in the medium in which he grew his cells, ammonia being the main nitrogen source, under which conditions aspartate ammonia-lyase might be induced.

It was also found that alanine formation could occur by transamination of pyruvate. Pyruvate could be formed from malate by three different pathways in the extract, oxidation of malate by malate dehydrogenase (decarboxylating), by decarboxylation of oxaloacetate, or from phosphoenolpyruvate by the action of pyruvate kinase.

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