activities of glucose phosphate isomerase and aldolase in livers of guinea pigs exposed to bagasse dust, and this may indicate increased gluconeogenesis.

SUMMARY

1. Chemical analysis of the cellular constituents of guinea-pig liver revealed that, though there was a marked increase in the glycogen content and an appreciable decrease in the percentage of protein in dry tissue, no alteration in the total protein/100 g. of body weight and water content of the tissue as a result of exposure to bagasse dust could be noted.

2. Activities of α -glucan phosphorylase, glucose 6-phosphatase, glucose phosphate isomerase and aldolase in livers of normal guinea pigs and of guinea pigs exposed to bagasse dust were assayed. Though no significant change in the activities of the first two enzymes could be observed, a marked increase in that of the latter two enzymes as a result of exposure was apparent.

3. Excessive glycogen accumulation in the liver of guinea pigs exposed to bagasse dust is discussed in the light of the enzymic findings.

Thanks are due to the Indian Council of Medical Research, New Delhi, for financial assistance.

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The Photometabolism of Propionate by *Rhodospirillum rubrum*

By M. KNIGHT*†

A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield

(Received 10 November 1961)

The studies of Elsdon & Ormerod (1956) on the effect of monofluoroacetate on the photometabolism of propionate and succinate by *Rhodospirillum rubrum* suggested that these compounds were metabolized by a common pathway. Subsequently, Ormerod (1956) found that the incorporation of ^{14}C into *R. rubrum* during the photometabolism of propionate in the presence of $^{14}\text{CO}_2$ was three or

four times as great as the net fixation of carbon dioxide measured manometrically, whereas when butyrate was photometabolized in the presence of $^{14}\text{CO}_2$ the net fixation of carbon dioxide measured manometrically was equal to the amount of ^{14}C incorporated. These results suggested that carbon dioxide plays a special role in the photometabolism of propionate. The role of carbon dioxide was further emphasized by the work of Clayton and his colleagues (Clayton, Dettmer & Robertson, 1957; Clayton, 1957), who showed that the metabolic

* Batchelors' Research Fellow.

† Present address: Department of Bacteriology, University of California, Davis, Calif., U.S.A.

pathway of propionate in *R. rubrum* in both light and dark probably involved a preliminary carboxylation to succinate. Elsdén (1958) found that when $[1-^{14}\text{C}]$ propionate was photometabolized by *R. rubrum* some 60% of the ^{14}C originally present in the propionate was recovered as $^{14}\text{CO}_2$ despite the fact that there was a net uptake of carbon dioxide, whereas with $n[1-^{14}\text{C}]$ -butyrate only 5% of the radioactivity was recovered as $^{14}\text{CO}_2$. These results, although not conclusive, strongly suggest that propionate is metabolized via succinate.

The carboxylation pathway of propionate metabolism was first demonstrated by Larsen (1951) in *Chlorobium thiosulphatophilum*. Subsequently Ochoa and his co-workers (Flavin & Ochoa, 1957; Flavin, Castro-Mendoza & Ochoa, 1956, 1957), working with preparations from animal tissues, showed that the substrate of the carboxylation reaction is propionyl-coenzyme A and that the product of the carboxylation reaction is methylmalonyl-coenzyme A. This latter compound is then converted by the action of the separate enzyme methylmalonyl isomerase into succinyl-coenzyme A.

A more detailed study of the photometabolism of propionate by *R. rubrum* has now been made by the short-exposure techniques of Calvin (see Bassham & Calvin, 1957). The results of these experiments, which are described below, show that propionate is indeed assimilated via succinate. In addition it has been found that extracts of *R. rubrum* contain a propionyl-carboxylating system similar to that found in animal tissues and propionibacteria; a preliminary account of the enzymic studies has already been published (Gibson & Knight, 1961).

MATERIALS AND METHODS

Organisms and cultural procedure

Photosynthetic bacteria. Two species of photosynthetic bacteria were studied: *Rhodospirillum rubrum* S1, obtained from Dr C. B. Van Niel, Hopkins Marine Station, Pacific Grove, Calif., U.S.A., and *Rhodopseudomonas capsulata* N.C.I.B. no. 8254. Stock cultures of these organisms were maintained and tube cultures for inocula grown as described by Elsdén & Ormerod (1956), except that (a) Oxoid yeast extract or yeast autolysate was replaced by 0.5% of Difco Bacto yeast extract, and (b) citrate was omitted from the medium as it was found to inhibit the growth of *R. rubrum*. For metabolic experiments *R. rubrum* was grown either in 400 ml. lots in 16 oz. medical flat bottles or in 1 l. lots in Roux bottles on the following medium: 0.5% Difco yeast extract, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% CaCl_2 and 0.5% m-potassium phosphate buffer, pH 7.0. To prevent the formation of a precipitate the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and CaCl_2 were sterilized separately as 10% (w/v) solutions and added to a cold, sterile solution of the remaining constituents. The cultures were incubated in the light-cabinet for 36–44 hr. for the

medical flat bottles and 60 hr. for the Roux bottles; the temperature was approx. 30°. Cultures were harvested in an International refrigerated centrifuge at 2° (10 min. at 3000g), washed twice with 20 mM-potassium phosphate buffer and resuspended in the same buffer containing 18 mM- NaHCO_3 (for manometric experiments) or in glass-distilled water (for short-exposure experiments). Dry weights of suspensions were measured turbidimetrically as described by Elsdén & Ormerod (1956). For the preparation of cell-free extracts large volumes of culture were harvested at room temperature in a Servall SS-1 centrifuge with the two-place Szent-Gyorgyi & Blum continuous-flow attachment (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) (23 000g; flow rate 150–200 ml./min.). The cells were washed once with 2% of the original culture volume of 20 mM-potassium phosphate buffer, pH 7.0, and resuspended in an equal volume of 0.1 M-tris-HCl buffer, pH 7.6.

Rhodopseudomonas capsulata was grown in the same way except that, since the organism grew more rapidly, cultures in Roux bottles were harvested after incubation for 36–44 hr. Cells washed with 20 mM-phosphate buffer were inactive, and they were washed instead with a salts mixture consisting of the inorganic components of Lascelles (1956) mixture no. 1. This mixture had been shown to be essential for the preparation of active washed suspensions of *Rhodopseudomonas spheroides* (Lascelles, 1956). With this exception the methods of harvesting and resuspension were similar to those described above. Dry weight was determined turbidimetrically at 680 m μ in a Unicam SP. 600 spectrophotometer.

Other bacteria. Other organisms used were: a strain of *Veillonella gazogenes*, isolated in this Department by Dr J. N. Ladd; *Lactobacillus arabinosus* 17-5 (N.C.I.B. no. 6376); *Nocardia globetula* (N.C.I.B. no. 8852). *V. gazogenes* was maintained on the semi-solid lactate-yeast-extract medium described by Walker (1958) for the maintenance of the rumen organism LC. The medium used for large-scale culture (800 ml.) was that described by Johns (1951). Freshly autoclaved medium cooled rapidly to about 37° was inoculated with the whole of a 24 hr. tube culture and incubated under $\text{H}_2 + \text{CO}_2$ (95:5) for 15–18 hr. at 37°. The organisms were harvested (temperature 10°; 15 min. at 3000g), washed once, resuspended in 0.01% $\text{Na}_2\text{S}_2\text{O}_9$ in freshly boiled distilled water, pH 7.6, and stored under H_2 until used.

L. arabinosus was maintained on slopes containing (per 100 ml.): 10 ml. of yeast autolysate (Barker & Beck, 1942), 1 g. of glucose, 2 g. of CaCO_3 and 2 g. of agar. Inocula were grown in portions (15 ml.) of the same medium without agar or CaCO_3 . Large quantities of the organism were grown on a modification of the medium used by Nossal (1951) (personal communication from Dr D. E. Hughes). This medium was prepared as follows: 20 g. of Oxoid tryptone (Oxo Ltd., London, E.C. 4), 2 g. of Oxoid yeast extract, 60 g. of sodium acetate dihydrate, 1 g. of KH_2PO_4 and 1 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 700 ml. of water and autoclaved for 10 min. at 10 lb./in.². When cool, 100 ml. of sterile 10% (w/v) glucose and 100 ml. of sterile malate solution (containing 13.4 g. of DL-malic acid and 10.6 g. of Na_2CO_3) were added. The inoculum used was 1 tube/l. of medium. After incubation for 8–9 hr. at 30°, a further 100 ml. of sterile malate solution was added.

The cells were harvested after a total incubation of 36 hr. (Servall SS-1; 23 000g; flow rate 150–200 ml./min.),

washed once with water (15 min. at 3000g) and then freeze-dried. The dried cells were stored at -20° .

N. globerula was maintained and grown and an acetone-dried powder prepared as described by Crawford (1958).

Analytical methods

Propionate was estimated by steam-distillation in the Markham (1942) still and titration of the distillate with CO_2 -free alkali in a stream of CO_2 -free air.

Succinate was estimated manometrically either by the succinoxidase method of Krebs (1937), as described by Umbreit, Burris & Stauffer (1957), or by decarboxylation with a washed suspension of *V. gazogenes*.

L-Malate was estimated by decarboxylation with a suspension of dried *L. arabinosus* under conditions similar to those described by Ochoa (1955) for the assay of the malic enzyme.

L-Aspartic acid was estimated by decarboxylation with a suspension of an acetone-dried powder of *N. globerula* (Crawford, 1958).

Alanine was estimated by the chloramine-T method of Kemble & Macpherson (1954).

Lactate was estimated by the ceric sulphate method of Elsdon & Gibson (1954).

Protein was estimated by the turbidity method described by Sentheshanmuganathan (1960).

Paper chromatography

Before use, Whatman no. 4 and no. 3MM papers were soaked overnight in 0.2% EDTA, pH 8.5 (Eggleston & Hems, 1952), washed in six changes of water and dried in air. Whatman no. 1 paper was used without preliminary washing. Unless otherwise stated, chromatograms were developed in the descending direction at a temperature of $22 \pm 2^{\circ}$.

Ethanol extracts of cells prepared in the 'short-exposure' experiments described below were analysed by two-dimensional paper chromatography. Normally Whatman no. 4 paper was used but, for the isolation of intermediates in amounts sufficient for degradation, Whatman no. 3MM paper was used. The chromatograms were developed with the phenol-water and butanol-propionic acid-water mixtures described by Benson *et al.* (1950). The phenol solvent was prepared as described by Bassham & Calvin (1957). These solvents will be referred to as solvent system A. The phenol solvent is very sensitive to salts and the amount of extract applied to the paper was therefore severely limited. The product of up to 0.5 ml. of cell suspension was usually used, though up to six times this amount still gave satisfactory chromatograms. When ten times this amount was used, severe streaking occurred, but this quantity could be separated satisfactorily on Whatman no. 3MM paper.

Dicarboxylic acids were chromatographed in solvent system A or in two dimensions with Analara amyl alcohol-5M-formic acid (1:1, v/v), followed by butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1948). This solvent system will be referred to as solvent system B. (Analara amyl alcohol is a mixture consisting of 70–85% of 3-methylbutan-1-ol and 15–30% of 2-methylbutan-1-ol.) Succinic acid and methylmalonic acid were separated on Whatman no. 1 paper, with the ethanol-aq. NH_3 soln. solvent of Cheftel, Munier & Macheboeuf (1953) [ethanol-

aq. NH_3 soln. (sp.gr. 0.880)-water; 80:3:17, by vol.]. Acids were detected with the buffered methyl red spray of Kalbe (1954).

Amino acids were chromatographed on Whatman no. 4 paper in solvent system A. They were detected by spraying the paper with 0.1% (w/v) ninhydrin in butanol and heating in an oven at 110° for 10 min.

Radioactive areas on chromatograms were detected by radioautography. The dried chromatograms were clipped to Ilford Industrial G X-ray film and locating marks were made by punching holes in both film and chromatogram with an automatic centre punch. After exposure the film was developed in Ilford ID19 developer and fixed in IF2 fixer. The length of exposure was determined by use of the empirical formula: exposure (days) = $N/\text{activity (counts/min.)}$, where N is a constant varying with the counting equipment used. The values of N used were 40 000 (when many spots were expected) or 16 000 (when only one or two were expected). Total activity was estimated after development with the solvent (or with two-dimensional chromatograms, after the first development) rather than on the origin.

Compounds were eluted from chromatograms by methods similar to those of Dent (1947). The eluting solvent used was aq. 0.02% NH_3 soln., or, when the material eluted was required for immediate degradation, water.

Measurement of radioactivity

Counting equipment. Activity on paper chromatograms was measured by counting directly on the paper with a large-diameter (5 cm.) end-window Geiger-Müller tube (G.E.C. Type 2B2) in conjunction with a Panax D657 Scaler (Panax Equipment Ltd., Redhill, Surrey). The tube was supported 0.8 mm. above the surface of the paper by one of a series of cardboard masks, which also served to isolate the spot being counted from others close by. A Packard gas-flow counter (Packard Instruments, La Grange, Ill., U.S.A.) was used for the radioactive assay of all solid samples. The counting gas was propane supplied as '98% minimum stench propane' by Shell-Mex and B.P. Gases Ltd., London, W.C. 2. With propane the plateau in the counts/voltage characteristic of the counter was 4.5–5.0 kv, as compared with that for methane of 3.8–4.3 kv. Apart from this the counting efficiency and background (25 counts/min.) was the same as that obtained with methane. The advantages of propane were the ease and regularity with which it could be obtained and the constancy of its composition.

Preparation of samples for radioactive assay. The material to be counted was either plated at infinite thinness or converted either wholly or in part into CO_2 and counted as BaCO_3 and the observed activity corrected for self-absorption. In general, this latter method was used when it was necessary to compare the radioactivity of different compounds, or of different carbon atoms within the same compound. Otherwise, the first method was used.

The procedure used for plating at infinite thinness was as follows. The planchets used were of stainless steel, and had wells 19 mm. in diameter and 0.17 mm. deep cut in them. When not in use the planchets were stored under ethanol to keep them free of grease. If the material to be plated were a volatile acid or a carbonate solution, the disks were dipped into a solution containing 1% (v/v) of Lissapol NDB (a non-ionic detergent supplied by I.C.I. Dyestuffs

Division, Blackley, Manchester 9), in ethanolic 0.1% (w/v) NaOH, drained and allowed to dry. This treatment spread the material being plated, and neutralized any residual acid from the cleaning process. If the material were non-volatile, or if it were necessary to remove residual $^{14}\text{CO}_2$, this process was omitted. The volume of material plated was kept as low as possible and 30 μl . was the usual maximum. The sample was spread evenly over the planchet with a platinum wire and dried under an infrared lamp. Measurements of activity were referred to the volume applied to the plate.

For plating as BaCO_3 , compounds were first subjected to combustion quantitatively to CO_2 by a method based closely on that of Sakami (1955). The differences from Sakami's procedure are as follows: 10% (w/v) KI replaced 5% (w/v) quinol as iodine absorbent; the combustion reagent used was that of Van Slyke, Plazin & Weisiger (1951); the CO_2 produced was measured manometrically in the Warburg apparatus.

Barium carbonate plates were prepared as described by Sakami (1955). The final carbonate concentration used was in the range 0.05–0.06M, as opposed to the concentration (0.1M) used by Sakami. Plates made from 1 ml. samples of carbonate solution of this concentration were thinner and less fragile than those made from 0.1M solution.

Reagents

Solutions of sodium *n*-butyrate and sodium propionate were prepared by neutralizing the free acids, which had been purified by redistillation, with NaOH.

Fumaric acid, L-malic acid, L-aspartic acid, L-glutamic acid, L-alanine and EDTA were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Fumaric acid was recrystallized from water before use.

Succinic acid, methyl iodide and fuming H_2SO_4 (20% free SO_3) were obtained from Hopkin and Williams Ltd., London, E.C. 1. Amberlite CG-50 was obtained from British Drug Houses Ltd., Poole, Dorset. Celite 535 came from Johns Manville Ltd., London, E.C. 1; it was washed with ether before use.

Avidin (25 000 units/g.) was obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A., D-biotin from L. Light and Co. Ltd., coenzyme A from Sigma Chemical Co., St Louis 18, Mo., U.S.A. or C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, glutathione from The Distillers Co. (Biochemicals) Ltd., London, S.W. 19, and tetrasodium ATP from Zellstofffabrik Waldhof, Wiesbaden, Germany. Ribulose diphosphate (barium salt, about 70% pure) was a gift from Dr J. Hurwitz.

AnalaR KH_2PO_4 , from Hopkin and Williams Ltd., was recrystallized by precipitation from concentrated aqueous solution by the addition of ethanol.

Solvents. All solvents, with the exception of propionic acid, butan-2-one, acetone and ether, were AnalaR reagents. Propionic acid and butan-2-one were obtained from British Drug Houses Ltd.; acetone and ether came from May and Baker Ltd., Dagenham, Essex. Before use as chromatographic solvents or for extraction, these four solvents were distilled.

Radioactive compounds. Sodium [^{14}C]carbonate, sodium [^{14}C]butyrate, sodium [^{14}C]- and [^{14}C]-propionate and [^{14}C]- and [^{14}C]-succinic acid were obtained from The Radiochemical Centre. The organic materials were

dissolved in water to a final activity of 25 $\mu\text{Ci/ml}$. These stock solutions were diluted 100-fold by vol. with a solution of carrier of appropriate concentration for use in manometric experiments, but in the short-exposure experiments [^{14}C]-propionate was used undiluted. In preliminary experiments it was not purified and radiation-decomposition products were found on the chromatograms. In later experiments it was purified with the procedure described by Kornberg (1958) for the purification of radioactive acetate.

$\text{Na}_2^{14}\text{CO}_3$ was obtained as an aqueous solution at 1 mCi/ml. For short-exposure experiments it was used undiluted. For experiments with cell-free extracts it was diluted 100-fold by vol. with NaHCO_3 or KHCO_3 of the concentration required.

Chemical preparations. Methylmalonic acid was prepared by treating the sodium derivative of diethyl malonate with methyl iodide, saponifying the ester and extracting the acid with ether. The methods used were those described by Vogel (1948) for the preparation of *n*-propylmalonic acid. After saponification the free acid was removed by extraction with ether in a Kutscher-Steueld liquid-liquid extractor for 14 hr. It was recrystallized by precipitation from ethyl acetate by addition of benzene.

Propionyl-CoA was prepared by the method described by Stadtman (1957), freshly distilled propionic anhydride being used. The propionyl-CoA so obtained was purified by chromatography on washed Whatman no. 3MM paper, with the solvent and method of detection described by Stadtman. It was eluted at 0° with glass-distilled water and estimated in terms of its adenine content as described by Peel (1960).

Experimental procedures

Manometric experiments. The water bath used in manometric photometabolism experiments had a glass bottom and the vessels were illuminated from below by two banks of seven 100 w bulbs, one along each side of the bath. These bulbs were cooled with a current of air from a centrifugal blower, and the tank was insulated from direct heating by a second sheet of glass placed 2 cm. below the glass panel of the tank. These precautions ensured that the heat generated by the lamps did not affect the temperature of the bath.

The photometabolism of ^{14}C -labelled substrates was followed under $\text{N}_2 + \text{CO}_2$ (95:5) at 30°. The vessels used were modified double-side-bulb Warburg cups, in which the stopper of one side bulb was replaced by a capillary stopper to which was fixed a capillary tap. Each vessel contained 1 ml. of bacterial suspension in 18 mM- NaHCO_3 , 0.2 ml. of 0.05M substrate (sodium salt; [^{14}C]butyrate, [^{14}C]- or [^{14}C]-propionate, [^{14}C]- or [^{14}C]-succinate) in one side bulb and 0.2 ml. of 2N- H_2SO_4 in the other. Manometers were set up in duplicate, and one of each pair was used to estimate the initial NaHCO_3 content. When CO_2 uptake was complete in the second manometer the acid was tipped in and the final NaHCO_3 content was estimated. The CO_2 was collected from the manometer as described by Ormerod (1956).

Short-exposure experiments. Short-exposure experiments were carried out with both [^{14}C]propionate and $^{14}\text{CO}_2$ as substrates and the procedures used differed only in detail.

The apparatus used is illustrated in Fig. 1. It was made from a medical flat bottle (50 ml.), to the neck of which was cemented a B14 standard socket and a gas inlet and outlet.

The outflowing gas was led into a cylinder of water, about 20 cm. deep, to provide a slight positive pressure within the reaction bottle. A hole was cut into the bottom of the bottle to accommodate a tap (2 mm. bore) for sampling. A second hole cut in the side was closed with a Subaseal

rubber cap (turnover type, size 9, William Freeman and Co. Ltd., Subaseal Works, Barnsley, Yorks.) through which the substrate was injected with a hypodermic syringe. The flask was illuminated from the front with a 500 w Photo-flood bulb (Photolita no. 2, Phillips Electrical Ltd.,

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Reaction vessel for short-exposure experiments. A, Gas inlet; B, gas outlet; C, sample inlet; D, sample outlet.

Fig. 2. Apparatus for trapping of acetaldehyde (one-quarter actual size). A, Tube to pump; B, 3 ml. of 2% (w/v) NaHSO_3 .

Fig. 3. Arrangement of columns for the separation of organic acids by gradient elution with chloroform-butanol solvent system. A, Chloroform-butanol (1:1, v/v); B, chloroform (590 ml.); C and E, chloroform (10 ml.); D and G, Celite (10 g.) + 5 ml. of 0.18N- H_2SO_4 ; F, sample + Celite (1 g.) + 0.5 ml. of 0.18N- H_2SO_4 ; H, magnetic stirrer.

London, W.C. 2) placed 20 cm. from the vessel. The back of the vessel was covered with aluminium foil to reflect any light passing through the suspension. No provision was made for cooling the apparatus. The experiments were carried out at room temperature (about 20°) and it was found in a trial run that 4 min. after injection of the substrate the temperature had risen to 25° and after 8 min. to 27°. The contents of the vessel were stirred by a magnetic stirring bar rotating on the back wall of the vessel.

Suspensions to be used in short-exposure experiments were first tested manometrically for their ability to photo-metabolize propionate, and if active they were used without delay. The experiments with $[2-^{14}\text{C}]$ propionate (P series) were carried out as follows. The suspension (40 ml., containing either 5 or 10 mg. dry wt. of cells/ml.) was placed in the reaction vessel and stirred for 3 min. with a rapid flow of gas ($\text{N}_2 + \text{CO}_2$, 95:5) to displace the air. The gas flow was then reduced to a flow just sufficient to maintain a pressure of 20 cm. water within the apparatus, 60 μ moles of inactive propionate were injected and the light was turned on. After 10 min., 1 ml. of $[2-^{14}\text{C}]$ propionate, containing 25 μC of ^{14}C and 10–12 μ moles of propionate, was injected into the suspension. The first sample was taken as soon as possible after the addition of the substrate (at about 7 sec.) and others were taken at intervals up to 8 min. The samples were run from the reaction vessel directly into 1 oz. glass vials containing 15 ml. of ethanol with 0.02 ml. of propionic acid, cooled to -20° (Moses & Calvin, 1958). The propionic acid was added partly to dilute the labelled propionate remaining in the sample and partly because it was found that it helped to coagulate insoluble material. The samples were about 5 ml. in volume, but no attempt was made to standardize the volume taken. Instead, the vials were weighed before and after sampling and comparisons between samples were made on a basis of activity per unit weight of sample.

In experiments in which $^{14}\text{CO}_2$ was used as substrate (C series), conditions were similar except that N_2 was used as the gas phase and 50 μl . of $\text{Na}_2^{14}\text{CO}_3$ soln. (50 μC of ^{14}C ; 8.5 μ moles of Na_2CO_3) was added at the end of the 10 min. preincubation period.

The samples were left overnight at -20° . The insoluble material was then centrifuged and re-extracted with 1% (v/v) of propionic acid in 80% (v/v) ethanol for 4 hr. at room temperature. The supernatants from these two extractions were combined, concentrated in a rotary evaporator to a small volume under reduced pressure (temp. 35°) and finally freeze-dried. The residue was extracted with 1 ml. of water, the insoluble material packed down by centrifuging, and a sample analysed by two-dimensional paper chromatography as described above.

After development of the radioautographs the radioactive areas were marked on the original chromatograms and counted as described above.

Degradation of labelled compounds

In general, the procedure followed was to estimate the specific activity of the compound after the addition of carrier, to remove successive C atoms as CO_2 , and to measure the specific activity of this CO_2 . Each intermediate in a degradation sequence was isolated and purified before the next step. Specific activities were estimated so that the losses inherent in an extended sequence of manipulations could be neglected. More than 90% of the total

activity could usually be accounted for. In some cases, it was convenient to use a biological decarboxylation as the first step in a degradation. When this was done, additional carrier was added before the chemical stage of the degradation, and the new specific activity measured. The activity of C atoms removed after this second addition of carrier was referred to this second specific activity.

Degradation of succinate. Succinate was degraded through the sequence of reactions (1)–(3).

The procedures used for both the decarboxylation of succinate and the collection of CO_2 were those described by Ormerod (1956). Succinate was eluted directly into a weighed manometer vessel, and the volume of eluate determined by re-weighing as suggested by Kemble & Macpherson (1954).

The propionate formed in the above reaction was recovered from the residue by distillation in the Markham (1942) still, and purified by column chromatography on Amberlite CG-50 as described by Seki (1958). After addition of about 400 μ moles of inactive propionate and estimation of the new specific activity, the propionate was decarboxylated (reaction 2), as described by Sakami (1955) with the modifications introduced by Ladd (1959).

The ethylamine thus produced was liberated by the addition of 3 ml. of saturated NaOH and transferred to 3–4 ml. of $\text{N-H}_2\text{SO}_4$ contained in the apparatus used for trapping acetaldehyde by aeration for 15 min. at 100° . The ethylamine solution was evaporated to dryness and subjected to combustion.

Degradation of fumarate. The first step in the degradation of fumarate was its reduction to succinate as described by Krebs, Smyth & Evans (1940). The succinate thus obtained was purified by column chromatography on Celite, gradient elution with a butanol- CHCl_3 mixture as described by Saz & Vidrine (1959) being used. The author is indebted to Dr H. J. Saz (Johns Hopkins University School of Hygiene and Public Health) for the details of the method used, and for permission to publish them. The apparatus used is shown in Fig. 3. The separation took place on the lower of the two columns; the upper column served to equilibrate the organic solvent with the aqueous phase (0.18N- H_2SO_4). Each column consisted of 10 g. of ether-washed Celite 535 and 5 ml. of 0.18N- H_2SO_4 , packed dry. The material to be separated, dissolved in about 0.5 ml. of water, was made acid to Congo red with 10N- H_2SO_4 , mixed with 1 g. of Celite plus 0.5 ml. of 0.18N- H_2SO_4 and packed on top of the lower column. Before the apparatus was assembled, CHCl_3 was run through the upper column, and then through the lower column until the first drop came out; the volume of CHCl_3 above each column was then adjusted to 10 ml. The double column was then attached to the mixing chamber, which contained 590 ml. of CHCl_3 , and to this was attached the reservoir, which contained 50% (v/v) butan-1-ol in CHCl_3 . Fractions (5 ml.) were collected and titrated with methanolic 5 mN-KOH in a stream of CO_2 -free air. Methanol (2 ml.) was added to each fraction before titration to ensure a clear end point (Moyle, Baldwin & Scarisbrick, 1948). The succinate was then degraded as described above.

Degradation of malate. Malate was degraded by the sequence of reactions (4)–(8).

Malate was eluted into a tared manometer vessel as described for the degradation of succinate, and decarboxylated as described by Ochoa (1955). The amount of CO_2 produced was measured and then transferred to an absorber in the usual way. The lactate was recovered from the reaction mixture by extraction with ether by the method of Strecker (1958). The volume of the solution was reduced, where necessary to less than 4 ml., the pH brought below 2 by the addition of 3N- H_2SO_4 , and the mixture added to 10 g. of ether-washed Celite 535 and 1 ml. of 3N- H_2SO_4 . The powder, after thorough mixing, was packed into a column (18–25 mm. int. diam.) and eluted with ether, 50 ml. of which eluted the lactate quantitatively. The lactate was then re-extracted into aqueous solution by shaking with 1 ml. of 0.1N-NaOH and purified by chromatography on a Celite column.

After addition of about 600 μ moles of inactive sodium DL-lactate and estimation of the specific activity, the lactate was oxidized to acetaldehyde and CO_2 as described by Sakami (1955) (reaction 5). Sakami's apparatus was modified by replacing his acetaldehyde trap by one based on the design of Long (1946) (Fig. 2), thereby reducing the volume of bisulphite from 15 to 4 ml. The CO_2 formed was collected in CO_2 -free NaOH and counted as BaCO_3 . The acetaldehyde-bisulphite compound was decomposed and the acetaldehyde treated with hypiodite and the formate produced oxidized by the procedures described by Sakami (1955) (reactions 6 and 7). The iodoform was recovered by filtration on to a double thickness of glass paper (Whatman GF/A; H. Reeve Angel Ltd., London, E.C. 4). The filter was transferred to a combustion tube, dried for 48 hr. over CaCl_2 and oxidized with the standard combustion reagent.

Degradation of aspartic acid. Reactions (9) and (10) were employed in the degradation of aspartic acid.

The acetaldehyde was then degraded by reactions (6)–(8). The decarboxylation of aspartate was carried out in a manometer as described by Crawford (1958), in 0.1M-ammonium acetate buffer, pH 5.5. At the end of the experiment 2N-HCl was added from the second side bulb to liberate the bound CO_2 . After the removal of the insoluble material on the centrifuge, the supernatant was repeatedly evaporated to dryness to remove the excess of HCl and the ammonium acetate buffer. The α -alanine formed was purified by electrophoresis on Whatman no. 3MM paper in the pyridine-acetic acid-water buffer, pH 6.5, of Ryle, Sanger, Smith & Kitai (1955) (1 hr.; 52v/in.). It was then eluted from the paper, about 600 μ moles of inactive DL- α -alanine were added and the specific activity was estimated. The alanine so obtained

was decarboxylated with ninhydrin in the apparatus described by Sakami (1955) for the oxidation of lactate, and by the procedure described by Sakami for the decarboxylation of glycine, except that it was found necessary to boil the reaction mixture for 30 min. rather than for 1 min. as suggested by Sakami. The acetaldehyde produced was degraded in the same way as that produced during the degradation of malate.

Degradation of alanine. Alanine recovered from short-exposure experiments was degraded in the same way as that produced by the decarboxylation of aspartic acid.

RESULTS

Manometric photometabolism experiments

The first experiments in the present study were designed to confirm and extend those of Elsdén (1958). In addition to [$1\text{-}^{14}\text{C}$]propionate and n [$1\text{-}^{14}\text{C}$]-butyrate, [$2\text{-}^{14}\text{C}$]propionate and both carboxyl- and methylene-labelled succinate were used as substrates (Table 1). These results obtained with n [$1\text{-}^{14}\text{C}$]-butyrate and [$1\text{-}^{14}\text{C}$]propionate confirm those of Elsdén. In addition, results obtained with [$2\text{-}^{14}\text{C}$]propionate show that only the carboxyl group of propionate exchanges during the photometabolism of propionate. The succinate results show a similar effect, though the recoveries of activity are rather high. This may be connected with the fact that the amounts of CO_2 produced were very high. Ormerod's (1956) figure for net production of CO_2 with succinate as substrate was 0.74–0.77 mole of CO_2 /mole of succinate. In these experiments the observed production of CO_2 was nearly twice this.

Certain of these experiments were repeated with *Rhodospseudomonas capsulata*, since Van Niel (1944) reported that this organism grew well with propionate as carbon source. Production of $^{14}\text{CO}_2$ from carboxyl-labelled propionate and succinate was similar to that found in *R. rubrum* (Table 2).

Short-exposure experiments

Succinate, fumarate, malate, aspartate, glutamate and alanine were conspicuous products in experiments of both the P and C series. Many other compounds, which were not identified, were labelled very quickly. For example, in a P series experiment, 8 sec. after the addition of the labelled propionate, all the compounds listed above, except alanine, were present, as well as seven unidentified compounds. In a C series experiment, all the listed compounds, with four unidentified ones, could be detected 18 sec. after the addition of the $^{14}\text{CO}_2$. The similarity of the radioautographs from the two series of experiments was most striking; the only major difference was the more rapid and heavy incorporation of ^{14}C into the area near the origin (referred to as the 'phosphate ester area') in the experiments of the C series.

Table 1. *Formation of $^{14}\text{CO}_2$ during photometabolism of ^{14}C -labelled substrates by Rhodospirillum rubrum*

Warburg manometers and cups with two side bulbs were used; one side bulb was closed by a capillary stopper bearing a tap. Duplicate cups were made up as follows: main compartment, 1 ml. of cell suspension (10 mg. dry wt. of cells/ml.) in 18 mM- NaHCO_3 and 0.1 ml. of water; first side bulb, 0.2 ml. of 50 mM-substrate; second side bulb, 0.2 ml. of 2N- H_2SO_4 . Gas phase $\text{N}_2 + \text{CO}_2$ (95:5); incubation temp. 30° . For details of procedure see text.

Substrate	CO_2 fixed/ substrate metabolized (moles/mole)	Activity of substrate (counts/min.)	Activity recovered in CO_2 (counts/min.)	Recovery of activity (%)
[1- ^{14}C]Butyrate	0.456	137 000	4 860	3.5
	0.495	137 000	6 400	4.7
[1- ^{14}C]Propionate	0.223	28 700	18 750	65.3
	0.319	28 700	19 550	68.1
	0.232	28 700	15 350	53.5
[2- ^{14}C]Propionate	0.454	99 700	3 110	3.1
	0.362	99 700	2 220	2.2
	0.263	99 700	2 060	2.1
[carboxy- ^{14}C]Succinate	-1.14	48 400	35 850	74.2
	-1.21	48 400	37 900	78.3
[2- ^{14}C]Succinate	-1.32	44 600	6 890	15.4
	-1.22	44 600	6 110	13.7

Table 2. *Formation of $^{14}\text{CO}_2$ during photometabolism of labelled substrates by Rhodopseudomonas capsulata*

Experimental details as for Table 1.

Substrate	CO_2 fixed/ substrate metabolized (mole/mole)	Activity of substrate (counts/min.)	Activity recovered in CO_2 (counts/min.)	Recovery of activity (%)
[1- ^{14}C]Propionate	0.266	21 150	10 000	47.3
	0.161	21 150	9 410	44.5
[2- ^{14}C]Propionate	0.254	36 800	1 590	4.3
	0.264	36 800	1 540	4.2
[carboxy- ^{14}C]Succinate	-0.500	48 400	24 600	50.8
	-0.598	48 400	22 550	46.7
[2- ^{14}C]Succinate	-0.443	44 600	2 060	4.6
	-0.471	44 600	2 120	4.8

Identification of intermediates. Compounds were tentatively identified by their positions on the radioautographs by comparison with published maps (Bassham & Calvin, 1957). The identification of succinate and fumarate was confirmed by their R_F values in the amyl alcohol-formic acid solvent (R_F succinate marker, 0.68; R_F suspected succinate, 0.69; R_F fumarate marker, 0.82; R_F suspected fumarate, 0.82) and then by co-chromatography with inactive succinate or fumarate on Celite (Swim & Krampitz, 1954). The acid peak eluted from the column in both cases coincided exactly with the peak of radioactive material eluted. The elution diagram for succinate is shown in Fig. 4.

Malate was identified by co-chromatography with inactive malate on paper in solvent systems A and B. In both cases there was exact coincidence between the radioactive and the spray-sensitive areas.

Fig. 4. Elution diagram of carrier succinate plus active succinate from P series. ●—●, $\mu\text{equiv.}$ of acid; —, radioactivity of sample.

The identifications of aspartic acid, glutamic acid and alanine were also confirmed by co-chromatography in two dimensions. In this case, however, enough material was available for only one solvent system (A) to be used.

Kinetic analysis. The differences observed between chromatograms of the two series of experiments, and the changes occurring in time in the samples from the same experiment, were given a more objective basis by determination of the activities of individual spots on chromatograms. Total activity in the sample was obtained by summing the activities of all compounds in the sample. When corrected for sample size, these figures show that the rate of incorporation of [2^{14}C]propionate into the soluble fraction was linear for at least 2.5 min.

When the activity of each compound, expressed as a percentage of the total activity on the chromatogram (referred to as the relative activity), is plotted against time, the curve of relative activity for the compound formed first from the labelled substrate has an initial negative slope. A very short time after the introduction of the labelled substrate, only one compound will be labelled; the relative activity of this compound will decrease with time, whereas that of all others will increase. Plots relating relative activity to time are given in Figs. 5 and 6. Curves for 'phosphate esters' were obtained by summing the activities of all spots falling within an arbitrarily defined area near the origin of the chromatograms.

In both these kinetic analyses, the succinate curve has an initial negative slope, which suggests that it is the first compound formed from propionate or CO_2 in the presence of propionate. In Fig. 5, the malate curve rises to a peak within 20 sec., and then falls away, a slope characteristic of an early intermediate. A similar effect in the fumarate curve is obscured by the low relative activity. In Fig. 6 the malate and fumarate curves both have negative initial slopes. This may be due either to the fact that the first sample in this experiment was taken at 18 sec., by which time the malate curve could have reached a maximum, or it may be due to the incorporation of $^{14}\text{CO}_2$ into malate, perhaps through an exchange reaction catalysed by the malic enzyme.

The results obtained suggest that succinate is the first product of propionate photometabolism, and that malate and fumarate are intermediates in the further metabolism of succinate. Samples of these compounds isolated from chromatograms were degraded to determine whether the labelling pattern within the molecule was consistent with their formation in this way. It will also be seen from Figs. 5 and 6 that $^{14}\text{CO}_2$ is incorporated more rapidly into the phosphate area than is [2^{14}C]propionate.

Fig. 5. Time-course of incorporation of [2^{14}C]propionate into intermediate by illuminated suspensions of *R. rubrum*; the propionate ($25\text{ }\mu\text{C}$; $10.9\text{ }\mu\text{moles}$) was added at 0 sec. and samples were taken at suitable intervals. The activities of non-volatile radiation-decomposition products were ignored in calculation of relative activities of intermediates. $\circ$, Succinate; $\bullet$, phosphate area; $\blacktriangle$, malate; $\blacksquare$, fumarate.

Fig. 6. Time-course of incorporation of $^{14}\text{CO}_2$ by suspensions of *R. rubrum* assimilating sodium propionate in the light. $\text{Na}_2^{14}\text{CO}_3$ ($50\text{ }\mu\text{C}$; $8.5\text{ }\mu\text{moles}$) was added at 0 sec. and samples were taken at suitable time-intervals. $\circ$, Succinate; $\bullet$, phosphate area; $\blacktriangle$, malate; $\blacksquare$, fumarate.

Table 3. *Degradation of succinate*

	P series		C series	
	Specific activity (counts/min./ μ mole)	%	Specific activity (counts/min./ μ mole)	%
Succinate	49.8	(100)	1060	(100)
Succinate C-1	0.18	0	522	49.3
Propionate (recovered and diluted with carrier)	2.82	(100)	3.82	(50.7)
Propionate C-1	0.07	2.5	3.08	41.0
Ethylamine (propionate C-2 + C-3)	2.73	97.0	0.06	1
Total recovery	—	99	—	91

Labelling patterns of intermediates

The compounds chosen for degradation were succinate, fumarate, malate, aspartic acid and alanine. Succinate, fumarate and malate were selected because the kinetic analysis showed that they were formed early and that they were probably intermediates in the photometabolism of propionate. Aspartic acid was chosen because it is known, in some organisms, to be derived from oxaloacetate by transamination, and it was thought that the labelling pattern might give some information about the labelling pattern in oxaloacetate. Alanine was chosen because it is known to be formed from pyruvate in other organisms, and if pyruvate were formed from propionate by way of a symmetrical intermediate such as succinate, then alanine formed from [2-¹⁴C]propionate should be evenly labelled in C-2 and C-3.

Material for degradation was taken from samples collected as early as was consistent with obtaining sufficient material for the degradation. Before degradation compounds were purified by co-chromatography with carrier on paper in one- or two-dimensional systems.

The results of these degradations are given in Tables 3–7. Because of the losses inherent in an extended series of manipulations it was not possible to recover completely the activity of the original material. It was found therefore preferable to work in terms of specific activity. In cases where a biological decarboxylation was used as the first step in degradation, the recovered decarboxylation product was diluted with carrier before degrading it further; this entailed an estimate of the diluted intermediate. Knowing the proportion of the total activity of the molecule recovered as CO₂, the activity of the recovered and diluted decarboxylation product could be determined by difference, thus making it possible to determine the proportion of the activity of the original compound in each atom of this fragment.

In general, the labelling patterns found were in agreement with those predicted. That is [2-¹⁴C]-

Table 4. *Degradation of fumarate from C series of experiments*

	Specific activity (counts/min./ μ mole)	%
Succinate	47.7	100
Succinate C-1	20.5	43.0
Propionate (recovered and diluted with carrier)	0.770	(57.0)
Propionate C-1	0.661	49.0
Ethylamine (propionate C-2 + C-3)	0	0
Total recovery	—	92

propionate resulted in labelling in C-2 and C-3, whereas labelling derived from ¹⁴CO₂ was limited to the carboxyl groups.

The labelling pattern of succinate from both series of experiments was in complete agreement with prediction. Only a small amount of fumarate was recovered in the P series and a quantitative degradation was not possible. However, since the CO₂ recovered by decarboxylation was inactive, it may be assumed that the activity was confined to C-2 and C-3 of the fumarate. The fumarate from the C series was labelled as predicted by theory.

Malate derived from the P series of experiments was labelled in C-2 and C-3. That formed in the C series was labelled in the carboxyl groups with the specific activity of C-4 greater than that of C-1. This may have been due to an exchange reaction catalysed by the malic enzyme.

Aspartate isolated from the C series of experiments was labelled symmetrically in both carboxyl groups. This suggests that its formation by transamination of oxaloacetate is unlikely, since oxaloacetate would retain the asymmetrical labelling of the parent malate. The labelling pattern of P series aspartate was consistent with its formation from a dicarboxylic acid.

Alanine labelled in the C series of experiments was labelled only in the carboxyl groups. That formed in the P series was labelled both C-2 and C-3 with a preponderance in C-3.

Table 5. *Degradation of malate*

	P series		C series	
	Specific activity (counts/min./ μ mole)	%	Specific activity (counts/min./ μ mole)	%
Malate	103	(100)	490	(100)
Malate C-4	0	0	282	57.6
Lactate (recovered and diluted with carrier)	1.62	(100)	4.88	(42.4)
Lactate C-1	0.02	1	4.65	40.4
Lactate C-2	0.733	45.2	0	0
Lactate C-3	0.792	48.9	0	0
Total recovery	—	95	—	98

Table 6. *Degradation of aspartate*

	P series		C series	
	Specific activity (counts/min./ μ mole)	%	Specific activity (counts/min./ μ mole)	%
Aspartate	97.8	(100)	93.0	(100)
Aspartate C-4	0	0	43.3	46.5
Alanine (recovered and diluted with carrier)	1.42	(100)	0.490	(53.5)
Alanine C-1	0.02	1	0.463	50.6
Alanine C-2	0.620	43.6	0.02	2
Alanine C-3	0.669	47.2	0.02	2
Total recovery	—	92	—	102

Table 7. *Degradation of alanine*

	P series		C series	
	Specific activity (counts/min./ μ mole)	%	Specific activity (counts/min./ μ mole)	%
Alanine	3.03	(100)	5.66	(100)
Alanine C-1	0	0	4.72	83.4
Alanine C-2	1.11	36.6	0.05	1
Alanine C-3	1.84	60.7	0.17	3.0
Total recovery	—	98	—	87

Table 8. *Carboxylation of propionyl-coenzyme A by crude cell-free extracts of Rhodospirillum rubrum*

Basic system contained, in a total volume of 1.2 ml.: tris-HCl buffer, pH 7.0, 50 μ moles; $MgCl_2$, 4 μ moles; glutathione, 5 μ moles; $KH^{14}CO_3$, 10 μ moles (54 000 counts/min./ μ mole); ATP, 2 μ moles; 0.5 ml. of extract, containing 3.8 mg. of protein. Propionyl-CoA or propionate + CoA was added as stated. Incubation was for 1 hr. at 30° in open tubes. Reaction was stopped by addition of 0.3 ml. of 2N-HClO₄. Samples of 20 μ l. were plated and counted. Protein was estimated as described by Sentheshanmugathan (1960).

Additions to basic system	Total activity fixed (counts/min.)
None	2 920
Propionyl-CoA (125 μ m-moles)	10 400
Potassium propionate (10 μ moles) and CoA (1 μ mole)	6 640

Experiments with cell-free extracts

Suspensions (20–30 mg. dry wt./ml.) of either *R. rubrum* or *Rhodopseudomonas capsulata* in 0.1M-tris-HCl buffer, pH 7.6, were disintegrated by exposure for 90 sec. to ultrasonic vibration in a stainless-steel conical flask attached to the transducer of a Mullard 500w 25 kcyc. ultrasonic generator. The product was centrifuged (2°; 30 min. at 15 000g) to remove cell debris and unbroken cells. The supernatant was clear and dark red. The propionyl-CoA-carboxylating activity of the extract was then assayed, by an assay based on that of Flavin & Ochoa (1957). The amount of propionyl-CoA used was reduced from 1 μ mole to 250 or 125 μ m-moles and in some later experiments glutathione was omitted.

Table 9. *Effect of removing chromatophores on propionyl-coenzyme A carboxylation*

Basic system was as described in Table 8. Crude extract was prepared by ultrasonic treatment (90 sec.) of suspension of *R. rubrum*, followed by centrifuging at 15 000g for 30 min. High-speed supernatant was prepared by centrifuging the crude extract at 105 000g for 180 min.

Preparation	Additions to basic system	Total activity (counts/min.)	Protein (mg.)	Specific enzymic activity (counts/min./mg./hr.)
Crude extract	None	1 260	3.8	333
	Propionyl-CoA (125 μ m-moles)	6 920	—	1 820
High-speed supernatant	None	1 010	2.9	348
	Propionyl-CoA (125 μ m-moles)	13 200	—	4 550

The standard assay procedure adopted was to incubate the extract, with or without propionyl-CoA at 30° for 1 hr. in open tubes. The reaction was stopped by the addition of perchloric acid to a final concentration of 0.4M. The precipitated protein was centrifuged off and the $^{14}\text{CO}_2$ fixed estimated by plating a sample of the supernatant at infinite thinness.

The ability of crude extracts to fix CO_2 in the presence of propionyl-CoA, or of propionate and CoA, is demonstrated in Table 8.

Partial purification of crude extract. When the crude extract was centrifuged at 105 000g for 2 hr., the bulk of the chromatophores sedimented; the supernatant contained very little carotenoid, that which remained being probably released by breakage of chromatophores. This treatment enhanced the activity of the extract, and the specific enzymic activity was on some occasions more than doubled (Table 9).

The high-speed supernatant was treated with just sufficient $(\text{NH}_4)_2\text{SO}_4$ to cause a light precipitate to appear on standing for 5 min. after the last addition of $(\text{NH}_4)_2\text{SO}_4$ (16.8–17.5 g./100 ml. of supernatant: 25% saturation). After standing for a further 15 min., the precipitate was centrifuged off (2°; 30 min. at 15 000g) and redissolved in 1 ml. of 0.1M-tris buffer, pH 7.6. To the supernatant was added a further 25 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. (65% saturation). The precipitate formed was centrifuged off as before and redissolved in 5 ml. of tris buffer, pH 7.6. This material lost activity very rapidly, but this inactivation was reversed by dialysis against 10 mM-tris buffer, pH 7.6 (50 vol.; 2 hr. at 2°). The preparation could then be stored at –20° for 14 days with little loss of activity. Although a considerable amount of activity was precipitated with the first $(\text{NH}_4)_2\text{SO}_4$ fraction, this fraction was heavily contaminated with carotenoid, and was not used. The enzyme preparation used in the experiments described below was the dialysed second $(\text{NH}_4)_2\text{SO}_4$ fraction.

Requirements for fixation of carbon dioxide. The effect of omitting various components of the basic

Table 10. *Requirements for fixation of carbon dioxide*

Basic assay procedure as in Table 8, with components omitted as indicated. Reaction mixture contained 250 μ m-moles of propionyl-CoA and 0.1 ml. of extract containing 2.7 mg. of protein (second ammonium sulphate fraction).

Component omitted	Counts/min. fixed
None	11 350
MgCl_2	1 310
ATP	47
Propionyl-CoA	1 240
Glutathione	29 400
None (enzyme heated for 3 min. at 100°)	1 650

assay mixture is shown in Table 10. This shows that ATP, Mg^{2+} ions and propionyl-CoA are essential for activity. About 85% of the activity was destroyed by heating for 3 min. at 100°. Glutathione inhibited the reaction. Recently Wood & Stjernholm (1961) have found that aqueous solutions of glutathione become inhibitory to the oxaloacetate transcarboxylase. No other sample of glutathione was tested, but glutathione was omitted from the reaction mixture in all subsequent experiments.

Recovery of methylmalonate from reaction mixtures. An incubation mixture containing, in a final volume of 6 ml., tris-HCl buffer, pH 7.0 (500 μ moles); MgCl_2 (40 μ moles), ATP (20 μ moles), $\text{KH}^{14}\text{CO}_3$ (10 μ moles), propionyl-CoA (1 μ mole) and protein (13.4 mg.) was incubated for 40 min. at 30°. 2N-Perchloric acid (1.5 ml.) was added, the mixture made alkaline to phenol red with 5N-KOH and incubated for 90 min. at 37° to hydrolyse any thioesters of CoA. The precipitate of KClO_4 and protein was centrifuged off, and the supernatant evaporated to dryness at 45° in a current of air. The residue was then redissolved in 2 ml. of water, mixed with Celite and extracted from a column with 400 ml. of ether. The ether was evaporated off at room temperature and the residue dissolved in 0.5 ml. of ethyl acetate. It was chromatographed on paper for 100 hr. in the ethanol-aq. NH_3 soln.

Table 11. *Inhibition of propionyl-carboxylase activity by avidin*

Samples of a propionyl-carboxylase preparation (second ammonium sulphate fraction), each containing 2.7 mg. of protein in 0.1 M-tris buffer, pH 7.6, were treated as follows: (1) incubated for 15 min. at 30°; (2) incubated for 15 min. at 30° with 4 units of avidin; (3) incubated for 15 min. at 30° with a mixture, 4 units of avidin and 20 µg. of biotin, that had been incubated for 15 min. at 30° immediately before use; (4) incubated for 15 min. at 30° with 4 units of avidin, with 20 µg. of biotin then added and the mixture incubated for a further 15 min. at 30°. Final volume in all cases, 0.8 ml. After these various treatments samples were removed and assayed for propionyl-carboxylase activity as in Table 8 except that glutathione was omitted from assay mixture and 125 µm-moles of propionyl-CoA were added as required.

Additions to assay mixture	Total ¹⁴ CO ₂ fixed (counts/min.)			
	(1)	(2)	(3)	(4)
None	496	68	55	746
Propionyl-CoA (125 µm-moles)	10 250	79	11 900	5 100

Table 12. *Propionyl-coenzyme A carboxylation by crude cell-free extracts of malate-grown Rhodospirillum rubrum*

Standard assay procedure (Table 8), with glutathione omitted. Propionyl-CoA (187 µm-moles) was added as indicated.

Preparations	Additions to basic system	Total activity (counts/min.)	Protein (mg.)	Specific enzymic activity (counts/min./mg./hr.)
Crude extract	None	2980	10.2	292
	Propionyl-CoA (187 µm-moles)	1620	—	154
High-speed supernatant	None	980	4.5	218
	Propionyl-CoA (187 µm-moles)	1095	—	244

solvent of Cheftel *et al.* (1953). The radioautograph revealed three radioactive compounds formed in the experimental tube; none was produced in a similar incubation mixture lacking propionyl-CoA. The largest spot corresponded in position with succinate markers, and immediately behind this was a small spot running opposite methylmalonate markers. When this material was eluted, mixed with carrier methylmalonate and chromatographed in solvent system B, the radioactive and spray-sensitive areas coincided exactly, showing this material to be methylmalonate. A third spot appeared in the chromatogram, that ran in solvent system B in a position which suggested that it might be malate. Since these tubes were incubated aerobically, a little of the succinate produced could have been oxidized to malate.

Inhibition of propionyl carboxylase by avidin. Preincubation of the enzyme preparation for 15 min. with 2 units of avidin/1.34 mg. of protein completely inhibited the reaction (Table 11). This effect was prevented by pretreatment of the avidin with a fivefold excess of biotin. The inhibition could also be reversed to some extent by incubating the enzyme-avidin mixture with a similar excess of biotin. This experiment suggests that biotin is a cofactor for this enzyme, as it has been shown to be for other acyl thio ester carboxylases.

Carboxylation of propionyl-CoA by cell-free extracts of malate-grown Rhodospirillum rubrum. It was reported by Elsdon & Ormerod (1956) that suspensions of *R. rubrum* prepared from cultures grown on malate did not metabolize propionate in the light. It was of interest therefore to test the propionyl-carboxylase activity of crude cell-free extracts prepared from cultures grown on malate. The results shown in Table 12 indicate that extracts of malate-grown cells did not carboxylate propionyl-CoA. By contrast the specific activities of crude extracts of cells grown on yeast extract were in the range 1820–9030 counts/min. fixed/mg. of protein/hr.; the corresponding figures for high-speed supernatants were in the range 4590–13 300 counts/min. fixed/mg. of protein/hr.

Propionyl-CoA carboxylation by crude extracts of Rhodopseudomonas capsulata. A crude extract and a high-speed supernatant of *Rhodopseudomonas capsulata* prepared in the way described above catalysed the carboxylation of propionyl-CoA. The crude extract had a specific activity of 154 counts/min./mg. of protein/hr., which, in the presence of propionyl-CoA (187 µm-moles) was increased to 1240 counts/min./mg. of protein/hr. The corresponding values for the high-speed supernatant were 266 counts/min./mg. of protein/hr. and 745 counts/min./mg. of protein/hr. respectively.

DISCUSSION

Analysis of the short-exposure experiments with whole cells shows that propionate is converted into succinate and thence into fumarate and malate. The labelling patterns found in these compounds and also in aspartate and alanine are, in general, consistent with this scheme. Thus the succinate, malate and aspartate formed from [2-¹⁴C]propionate were approximately equally labelled on C-2 and C-3 (Tables 3, 5 and 6). The fumarate derived from [2-¹⁴C]propionate, although isolated in amounts insufficient for a complete degradation, contained no radioactivity in its carboxyl groups. On the other hand, the pattern of labelling found in the alanine produced in the P series of experiments (Table 7) is difficult to reconcile with any known mechanism of synthesis, for the C-3 of alanine contained almost twice as much isotope as C-2 (Table 7), which is in marked contrast with the C₄ dicarboxylic acids, which had an even distribution of the tracer between C-2 and C-3.

In the experiments with ¹⁴CO₂ only the carboxyl groups of the compounds studied were labelled. Since free succinate is a symmetrical molecule it is to be expected that succinate and the dicarboxylic acids derived from it, i.e. fumarate, malate and aspartate, would be equally labelled on both carboxyl groups. This was not the case. Whilst it is possible, because of the relatively small amounts available for degradation, that the discrepancies in the labelling of succinate, fumarate and aspartate are due to experimental error, the discrepancy with malate is considered to be too large to be accounted for in these terms. *R. rubrum* was shown by Vernon & Kamen (1953) to contain the malic enzyme and the effect of this enzyme, if it were active under the conditions of these experiments, would be to increase the degree of labelling on C-4 and hence to give the distribution of isotope observed.

The mechanism by which aspartate becomes labelled is not known. If it were synthesized from oxaloacetate derived from malate then one would expect the labelling pattern of aspartate to be similar to that of malate. This was not the case. It is not known whether *R. rubrum* contains aspartase, but if this enzyme is present then both the rapid appearance of the isotope in aspartate and the distribution of the tracer could be accounted for by the exchange reaction (11).

Glutamate was always formed in the early stages of both series of experiments but it was not obtained in amounts sufficient to permit a complete

degradation. On the other hand, citrate was labelled much more slowly than glutamate, which suggests that citrate is not a precursor of glutamate. This is, at first sight, incompatible with the work of Cutinelli, Ehrensward, Reio, Saluste & Stjernholm (1951) and Cutinelli *et al.* (1951), which suggested that glutamate is formed via citrate and 2-oxoglutarate. However, until the glutamate formed from specifically labelled propionate is degraded it is not possible to comment further.

The experiments with cell-free extracts show that both *R. rubrum* and *Rhodospseudomonas capsulata* contain a propionyl carboxylase similar to that found in animal tissues and *Propionibacterium shermanii* (Ochoa, 1959; Stadtman, Overath, Eggerer & Lynen, 1960; Kaziro, Leone & Ochoa, 1960). Thus propionyl-CoA and ATP were essential components of the system and methylmalonate and succinate were identified as products of the reaction catalysed by the crude preparations used. Further, the reaction was inhibited by avidin and the action of avidin was prevented by a preliminary incubation of the protein with excess of biotin.

Methylmalonate was not detected in whole cells, although the enzymic experiments strongly suggest that it is an intermediate in the photometabolism of propionate. The most probable explanation of this discrepancy is that, in contrast with succinate, fumarate and malate, methylmalonate occurs only as the CoA derivative and as such was present in amounts too small to be detected under the conditions in which these experiments were performed. It is, however, possible that methylmalonate might have been detected had propionate of very high specific activity been used as a substrate. Evidence from short-exposure experiments alone cannot be used to exclude a postulated intermediate from a pathway, since the amount of such an intermediate might be limited by the amount of enzyme or coenzyme available and so might be below the limit of detection.

Elsden & Ormerod (1956) found that suspensions of *R. rubrum* that had been grown on the malate medium do not metabolize propionate. The demonstration that cells grown on this medium do not contain the propionyl-CoA-carboxylase system provides an explanation for this observation; the reason why growth on this medium suppresses the formation of this system is unknown.

The initial steps in the photometabolism of propionate are thus clear, and presumably the rapid spread of propionate carbon atoms throughout the soluble compounds of the organism implies that these are derived, in part at least, from the succinate series of compounds. Stanier, Doudoroff, Kunisawa & Contopoulou (1959) showed that when starved suspensions of *R. rubrum* were incubated with either propionate or succinate substantial amounts

of a polyglucose were formed. Further, with labelled substrates they showed that the labelling pattern of the glucose derived from polysaccharide was consistent with its formation from a C_3 unit derived from succinate or a derivative thereof by decarboxylation. The fact that, despite a net uptake of carbon dioxide during the photometabolism of $[1-^{14}C]$ propionate, approximately 50 % of the carboxyl carbon atoms appears as carbon dioxide (Tables 1 and 2) is consistent with the observations of Stanier *et al.* (1959).

The experiments with $^{14}CO_2$ and unlabelled propionate showed that both the succinate group of compounds and the 'phosphate area' of the chromatogram become rapidly labelled, so much so that after only 18 sec. exposure to $^{14}CO_2$ some 25 % of the isotope fixed was found in the 'phosphate area'. However, little of the propionate carbon atoms was found in these compounds after a comparable time (Figs. 6 and 7). The chromatographic procedures were not used in a manner which resolved the individual phosphate esters so it is not possible to work out the kinetics of the formation of these compounds. Nevertheless it seems reasonable to assume that the ribulose diphosphate pathway of fixation of carbon dioxide is an important part in the photometabolism of propionate and that 3-phosphoglycerol phosphate is the hydrogen acceptor for the oxidation reactions that are associated with this process.

SUMMARY

1. When *Rhodospirillum rubrum* metabolizes $[1-^{14}C]$ propionate in the light, some 60 % of the ^{14}C can be recovered as $^{14}CO_2$ at the end of the experiment. Only 3 % of the activity of $[2-^{14}C]$ propionate can be similarly recovered. A similar effect is observed with $[1,4-^{14}C_2]$ - and $[2,3-^{14}C_2]$ -succinate.

2. Kinetic analyses of short-exposure experiments in which $[2-^{14}C]$ propionate or $^{14}CO_2$ and unlabelled propionate were used as substrates indicated that the first detectable product of propionate photometabolism is succinate, and that fumarate and malate are intermediates in the assimilation of succinate. These observations are confirmed by degradative studies.

3. Experiments with cell-free extracts show that the reactions involved in propionate carboxylation resemble those in animal tissues and in *Propionibacterium shermanii*. Propionyl-coenzyme A is the substrate of the carboxylation, adenosine triphosphate is required for its carboxylation, methylmalonyl-coenzyme A is the product of the reaction, and the reaction is inhibited by avidin.

4. Propionate photometabolism in *Rhodospseudomonas capsulata* probably follows the same pathway.

The author wishes to thank Professor S. R. Elsdén for his advice, encouragement and criticism during the whole course of this work and Dr J. Gibson for her help in the experiments with cell-free extracts. The work was supported in part by the Rockefeller Foundation and the Kellogg Foundation.

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The Regulation of Amino Sugar Metabolism in *Bacillus subtilis*

BY JULIET S. CLARKE AND C. A. PASTERNAK

Department of Biochemistry, University of Oxford

(Received 15 January 1962)

A primary stage in the biosynthesis of amino sugars is the amination of hexose 6-phosphate by L-glutamine to yield glucosamine 6-phosphate, a reaction demonstrated in extracts of *Neurospora crassa* (Leloir & Cardini, 1953), *Streptococcus haemolyticus* (Lowther & Rogers, 1956) and mammalian tissues (Castellani & Zambotti, 1956; Pogell & Gryder, 1957; Kizer & McCoy, 1959):

This investigation is concerned with the control of reaction (1) in bacteria. The organism chosen for study was *Bacillus subtilis*, since it is known to synthesize a polysaccharide rich in glucosamine and galactosamine (Sharon, 1957) and contains the enzyme uridine diphosphate N-acetylglucosamine epimerase (Glaser, 1959). Reaction (1) was demonstrated in extracts of *B. subtilis*, glucose 6-phosphate or fructose 6-phosphate serving as substrate. While the work was in progress, Ghosh, Blumenthal, Davidson & Roseman (1960) showed fructose 6-phosphate to be the substrate for purified enzymes from *Escherichia coli*, *N. crassa* and rat liver. The properties of the *E. coli* enzyme were so close to those of the crude *B. subtilis* enzyme that further purification seemed superfluous. Gram-positive organisms have been shown to contain higher amounts of aminotransferase (reaction 1) than Gram-negative ones (Strange & Dark, 1960).

When *B. subtilis* was grown in the presence of exogenous amino sugar the capacity to synthesize glucosamine 6-phosphate was decreased (Pasternak & Clarke, 1961; Clarke & Pasternak, 1961) owing to a reduced synthesis of aminotransferase. There was a concomitant induced synthesis of glucosamine 6-phosphate deaminase [2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating), EC 5.3.1.10] which destroys the product of the aminotransferase reaction (Roseman, 1959; Strange & Dark, 1960):

These two effects have now been separated and their significance clarified.

MATERIALS AND METHODS

Growth of organisms. A strain of *B. subtilis* derived from NCTC 1379 was maintained on slopes of Oxoid nutrient agar CM4 at 4° and used for all experiments. The growth medium was 1.3% (w/v) Oxoid nutrient broth CM1 sterilized by autoclaving at 15 lb./in.² for 20 min. Glucose and sodium L-glutamate were sterilized in the same way and added afterwards to give final concentrations of 0.1 and 0.5% respectively. Amino sugars were sterilized by filtration through Seitz filter pads or Oxoid membrane filters. The medium (500 ml. in 1 l. flasks) was inoculated with 5 ml. of a starter culture in logarithmic phase and shaken for 10–14 hr. on a reciprocal shaker at 37°. Growth