Sedimentation and diffusion. Measurements of the sedimentation velocity of purified inhibitor AA, at five 8 min. intervals, showed a single symmetrical boundary. This single component in a solution containing 1 g./100 ml. had $S_{20,w}$ 2·3s and $D_{20,w}$ $9{\cdot}03 \times 10^{-7}$ cm.^{2}/sec. These sedimentation and diffusion coefficients, together with an assumed partial specific volume of 0·75 ml./g., indicate that purified inhibitor AA has mol.wt. 24 000.

Isoelectric point. Determinations of the solubility of the purified inhibitor in 0·1 M-sodium acetate buffers in the range pH 3·90–4·55 indicated an isoelectric point of 4·2.

Amino acid content. The tyrosine and methionine contents of an hydrolysate of purified inhibitor AA were 1·9 and 1·0 g. respectively/100 g. of inhibitor hydrolysed. The complete absence of tryptophan from purified inhibitor AA is of special interest as Kunitz's crystalline soya-bean trypsin inhibitor is apparently the only pure trypsin inhibitor that contains significant quantities of tryptophan (Laskowski & Laskowski, 1954). The non-identity of purified inhibitor A with the crystalline soya-bean trypsin inhibitor of Kunitz, which was established by the distinctly different behaviour on a DEAE-cellulose column (Birk, 1961*b*), is further substantiated by the present work.

SUMMARY

1. Further purification of an acetone-insoluble trypsin inhibitor on a carboxymethylcellulose column and its separation from a highly active amylase has been described.

2. The purity of the inhibitor has been established by rechromatography on carboxymethyl- and diethylaminoethyl-cellulose and by ultracentrifugal analysis.

3. The molecular weight of the pure inhibitor was determined as 24 000 and its isoelectric point was pH 4·2.

4. The non-identity of the pure inhibitor with the crystalline soya-bean trypsin inhibitor has been further substantiated by its higher specific activity (13-fold) against chymotrypsin and by the absence of tryptophan.

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The Photo-assimilation of Acetate by *Rhodospirillum rubrum*

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Rhodospirillum rubrum is a photosynthetic bacterium that grows anaerobically in the light in the presence of one of a number of simple organic compounds together with sodium hydrogen carbonate and growth factors. It was shown to be capable of growing on acetate as major carbon source with yeast autolysate as a source of growth factors. Under appropriate conditions growth was proportional to the concentration of acetate, and approximately 70% of the acetate carbon atoms were converted into cell carbon atoms (van Niel, 1944). Cutinelli, Ehrensvard, Reio, Saluste & Stjernholm (1951) examined the incorporation of isotopically labelled acetate and hydrogen carbonate into cell components of growing cultures of *R. rubrum*. The organism was grown anaerobically in the light on a medium containing yeast extract, acetate and inorganic salts (including hydrogen carbonate). Separate batches of cells were grown in media containing isotopically labelled acetate, as $^{13}CH_3 \cdot {}^{14}CO_2H$ in the presence of hydrogen carbonate, and in media containing $NaH^{14}CO_3$ in

the presence of acetate. Isotope incorporation into the amino acids appeared to proceed in a specific manner indicating that most of the acetate was assimilated directly, i.e. without oxidation of the acetate to carbon dioxide, followed by assimilation of the carbon dioxide as in other photosynthetic organisms. Similarly, hydrogen carbonate was incorporated, in the presence of acetate, in a specific manner and was not incorporated into every carbon atom of the amino acids as would be expected had the hydrogen carbonate been assimilated by a mechanism common to both autotrophic and heterotrophic photosynthetic organisms. This is implied in the general theory of photosynthesis proposed by van Niel (1941).

An early attempt to follow the course of the photo-assimilation of acetate by *R. rubrum* was undertaken by Glover, Kamen & van Genderen (1952). The organism was grown anaerobically in the light on a medium containing malate as major carbon source, and washed cell suspensions of *R. rubrum* were exposed, under photosynthetic conditions, to acetate or hydrogen carbonate containing ^{14}C for short periods (6–24 sec.) and the cells were fractionated into 'water-soluble', ether-soluble-'lipid' and 'insoluble-residue' fractions, which were assayed for radioactivity. The results showed that the bulk of the cellular carbon atoms were derived from acetate and that most of the acetate carbon atoms were incorporated into the 'lipid fraction'. Only after very short exposures (8 sec.) was a significant percentage of the [^{14}C]-acetate incorporated into the cells found in the 'water-soluble fraction'. Paper chromatography of this fraction showed that 50% of the ^{14}C incorporated was in an unknown compound (X) with R_F values close to those of α-oxoglutarate, 25% was present in a second unknown compound (Y) with R_F values close to those of phosphate esters and the rest was present in succinate, α-oxoglutarate and malate. The results suggest that acetate is not metabolized to any appreciable extent through the tricarboxylic acid cycle.

The pathway of the photo-assimilation of acetate by *R. rubrum* is thus far from clear. Moreover, Kornberg & Lascelles (1960) have failed to find isocitrate lyase (EC 4.1.3.1., formerly isocitratase) in cell-free extracts of cultures of *R. rubrum* grown photosynthetically or aerobically in the dark on media with acetate as major carbon source, although this enzyme was found in extracts of the related species *Rhodopseudomonas capsulata* and *Rhodopseudomonas palustris* grown under the same conditions. Since isocitrate lyase is an essential enzyme of the glyoxylate cycle, which has been shown to be concerned with the assimilation of compounds containing two carbon atoms in micro-organisms including *Escherichia coli* and *Pseudomonas ovalis* Chester (see review by Kornberg & Elsden, 1961), it would appear that *R. rubrum* has some other mechanism for assimilating acetate.

Experiments were therefore undertaken to investigate the course of photo-assimilation of acetate. Initial experiments were carried out with washed cell suspensions of *R. rubrum* that were exposed for short periods in the light to [^{14}C]acetate and hydrogen [^{14}C]carbonate under conditions similar to those used by Glover *et al.* (1952). A preliminary account of some of the results has been published (Hoare, 1962*a*, *b*; Elsden, 1962).

MATERIALS AND METHODS

Growth and maintenance of micro-organisms. *Rhodospirillum rubrum* strain S1, *Rhodopseudomonas capsulata* and *Rhodopseudomonas spheroides* were grown photosynthetically under semi-anaerobic conditions. One of the following two media was used for large-scale growth of these organisms. 'Acetate medium' contained, per l.: Difco yeast extract 5 g., $(NH_4)_2SO_4$ 1 g., M-phosphate buffer, pH 7·0 (KH_2PO_4–KOH mixture), 5 ml.; the following sterile solutions were then added: 10% (w/v) $MgSO_4,7H_2O$ 0·8 ml., 10% (w/v) $CaCl_2$ 0·4 ml., M-$NaHCO_3$ 10 ml. and M-sodium acetate 20 ml. 'Malate medium' contained, per l.: Difco yeast extract 2 g., DL-malic acid 2·7 g., $(NH_4)_2HPO_4$ 800 mg., K_2HPO_4 500 mg., KH_2PO_4 500 mg., $MgSO_4,7H_2O$ 200 mg., $CaCl_2$ 40 mg., adjusted to pH 6·8 with NaOH. The organisms were maintained in tubes containing 15 ml. of 0·5% Difco yeast extract for subsequent large-scale growth on 'acetate medium', or on 15 ml. of 'malate medium' for subsequent large-scale growth on 'malate medium'. Tubes were incubated in a light-cabinet at 25° and were transferred every 3 or 4 days. One tube was used to inoculate 1 l. of medium contained in a Roux bottle. The cells were harvested after 40 hr. in the light at 25°. Cell yields were in the range 500–700 mg. dry wt.

Veillonella gazogenes was grown under strictly anaerobic conditions on the following medium (Johns, 1951): Difco yeast extract 4 g., sodium lactate 10 g., water 1 l., adjusted to pH 6·8 with NaOH; 2 ml. of 10% $Na_2S,9H_2O$ was added after autoclaving. Tubes containing 15 ml. of medium were maintained at 37° in $H_2 + CO_2$ (95:5). One tube was used to inoculate 800 ml. of medium contained in a 1 l. Florence flask fitted with a delivery tube and Bunsen-valve; the flask was gassed for 3–5 min. with a gentle stream of $H_2 + CO_2$ (95:5) and was incubated for 16 hr. at 37°.

Clostridium tetanomorphum was maintained and grown under strictly anaerobic conditions, as for *V. gazogenes*, on the following medium, which is a modification of that used by Wachsman (1956): Difco yeast extract 10 g., sodium L-glutamate 5 g., sodium thioglycollate 0·5 g., phosphate buffer, pH 7·4 (KH_2PO_4–KOH mixture), to 0·05M final concentration, tap water to 1 l.; 0·2 g. of $MgCl_2$, was added after autoclaving.

Clostridium welchii SR12 was grown as described by Krebs (1950).

Manometric experiments on the photometabolism of acetate. Cells were harvested by centrifuging, washed twice with cold 0·02M-phosphate buffer, pH 7·0 (KH_2PO_4–KOH mixture), and suspended in 25 ml. of glass-distilled water. Dry weight was determined from the extinction at 680 mμ in a

Unicam SP. 600 spectrophotometer, a calibration curve being used.

Warburg flasks contained 2 ml. of cell suspension (5 mg. dry wt./ml.) in 25 mM-$NaHCO_3$ and 5 mM-phosphate buffer, pH 7·0 (KH_2PO_4–KOH mixture), and 0·3 ml. of water; side bulbs contained 0·2 ml. of 25 mM-$NaHCO_3$ (controls) or 0·2 ml. of 0·1 M-sodium acetate in 25 mM-$NaHCO_3$ (tests). Flasks were gassed with $H_2 + CO_2$ (95:5) and were incubated in an illuminated water bath at 30°. Gas uptake was followed for 40–60 min. after the contents of the side bulbs had been tipped. Activity was expressed as Q_{CO_2}, determined from the difference in rates between control and test flasks.

Radioactive compounds. Sodium [^{14}C]carbonate, sodium [1-^{14}C]acetate and sodium [2-^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [^{14}C]acetate preparations were purified by the procedure of Kornberg (1958) immediately before use in 'short-exposure experiments'.

Short-exposure experiments with radioactive compounds. Experiments were carried out in the apparatus described by Knight (1962). The reaction vessel contained 40 ml. of cell suspension (generally 5 mg. dry wt./ml.) in glass-distilled water with 400 μmoles of $NaHCO_3$. The suspension was illuminated with a 500 w Photoflood lamp at a distance of 9 in., was vigorously stirred with a magnetic stirrer and was aerated with a gas mixture (described with each experiment). The suspension was equilibrated for 3 min. before the addition of 100 μmoles of sodium acetate (if required) and equilibration was then continued for 10–15 min. The appropriate ^{14}C-labelled compound was then injected from a syringe and samples were withdrawn at intervals into weighed bottles containing 15 ml. of ethanol–water–acetic acid (950:50:1, by vol.). The bottles were reweighed to obtain the weight of sample.

Fractionation of cell suspensions. Cell samples from the 'short-exposure experiments' were left overnight at −15°. They were then centrifuged for 15 min at 1000 *g* at 2°, and the clear supernatants were decanted into 250 ml. round-bottomed flasks. The residues were re-extracted with 10 ml. of ethanol–water–acetic acid (80:20:1, by vol.) for 2 hr. at room temperature (18–25°) and were centrifuged again. The supernatants were combined, concentrated to 2–3 ml. on a rotary evaporator at 30° and then freeze-dried. The freeze-dried samples were suspended in 1 ml. of water, added in two 0·5 ml. amounts, and transferred to small centrifuge tubes. After being left overnight in the deep-freeze the samples were thawed and centrifuged. The clear pale-yellow supernatant ('soluble fraction') was analysed by chromatography and radioautography. The dark-green residue from the freeze-dried material, which contained lipid and chlorophyll, was not examined. The cell residues from the acid-ethanol extractions were washed twice with 5 ml. of water and stored at −15°: this comprised the 'cell-residue' fraction. Poly-(β-hydroxybutyric acid) (hereafter called 'polymer') was isolated from the 'cell-residue' fraction by hydrolysis in 2 N-H_2SO_4 for 1 hr. at 100° to remove polysaccharides, followed by digestion with hypochlorite as described by Stanier, Doudoroff, Kunisawa & Contopoulou (1959).

Chromatography and radioautography. The 'soluble fractions' (200–300 μl.) were chromatographed on Whatman no. 4 papers washed in EDTA solution as described by Knight (1962). Two-dimensional chromatograms were run in the phenol–water and butan-1-ol–propionic acid solvent systems of Benson *et al.* (1950). Radioautographs were made by exposing the chromatograms to Ilford Industrial G X-ray film, the period of exposure being determined from the approximate total radioactivity on the chromatogram after running in phenol. The films were developed in Ilford ID19 X-ray developer.

Estimation of radioactivity. Radioactive areas on the chromatograms, outlined by comparison with the corresponding radioautographs, were assayed for radioactivity with a thin end-window tube (General Electric Co. Ltd. type 2B2) connected to a Panax D657 scaler. Plated samples, as $BaCO_3$, were assayed with a Packard model 200A windowless gas-flow counter connected to a Baird-Atomic model 1035 scaler; 98% 'minimum stench' propane (Shellmex and B.P. Gases Ltd.) was the circulating gas. Barium carbonate plates were counted at finite thickness and were corrected for self-absorption. 'Cell-residue' and 'polymer' fractions were plated by filtration through Oxoid membrane filters and were washed with 0·1% acetic acid, followed by water, and dried at room temperature; they were then assayed with the end-window tube and the counts were not corrected for self-absorption; cell-residue samples were prepared of approximately the same weight within the range 8–10 mg. dry wt. In some cases the polymer was dissolved in hot $CHCl_3$ and suitable samples were assayed by plating on stainless-steel planchets and counting at infinite thinness.

Paper electrophoresis. Radioactive samples eluted from chromatograms were analysed by paper electrophoresis in one of the following systems. 33 mM-Ammonium acetate buffer, pH 6·0, was used with Whatman 3 MM papers, 60 cm. in length and an applied voltage of 1000 v. The apparatus used was that described by Markham & Smith (1952), as used by Peel (1958). In some cases the pyridine–acetic acid–water buffer, pH 6·5, of Ryle, Sanger, Smith & Kitai (1955) was used with an applied voltage of 1800 v. Radioactive regions were located by radioautography or by 'scanning' with an end-window tube.

Purification of radioactive glutamate. Radioactive spots, identified as glutamate, were eluted from chromatograms with water. Eluates from a number of chromatograms of samples exposed for short periods (not greater than 60 sec.) were combined and concentrated *in vacuo* over P_2O_5 at room temperature and were then subjected to paper electrophoresis, together with a glutamate 'marker' run in parallel on the same sheet of paper. The dried paper strip, after electrophoresis, was exposed to X-ray film, which was developed after a suitable period. The radioactive spot corresponding with the glutamate marker was eluted with water and a suitable amount of carrier glutamic acid was added. In this way a 'master solution' of [^{14}C]glutamic acid was produced, from which samples were taken for total combustion and for degradation as set out below.

Degradation of radioactive glutamate. At the time this work was undertaken the only published method for the unequivocal degradation of glutamate was that devised by Mosbach, Phares & Carson (1951). More recently an alternative method has been described by Pigretti & Stoppani (1961), based on an earlier procedure by Wang, Christiansen & Cheldelin (1953). In the method of Mosbach *et al.* (1951) the recoveries in the initial steps resulting in the formation of butyric acid are rather low. A method of degradation that would result in higher recoveries of the partial degrada-

tion products was therefore sought. The method devised is shown in Scheme 1, but it has the disadvantage that it may not give an unequivocal degradation, since succinic acid, which is a symmetrical molecule, is an intermediate.

The procedure adopted is set out in detail below. In general the [^{14}C]glutamate solution was made up to 3 ml. with the addition of carrier glutamate (60 μmoles). A sample was then taken for total combustion and the remainder, or a sample of the remainder, was taken for the first step of the degradation on a micro scale or a macro scale. Each product of the degradation, apart from CO_2 (i.e. the succinate, propionate and ethylamine), was isolated and made up to 3 ml.; a sample was taken for total combustion and the remainder taken through the next step of the degradation procedure.

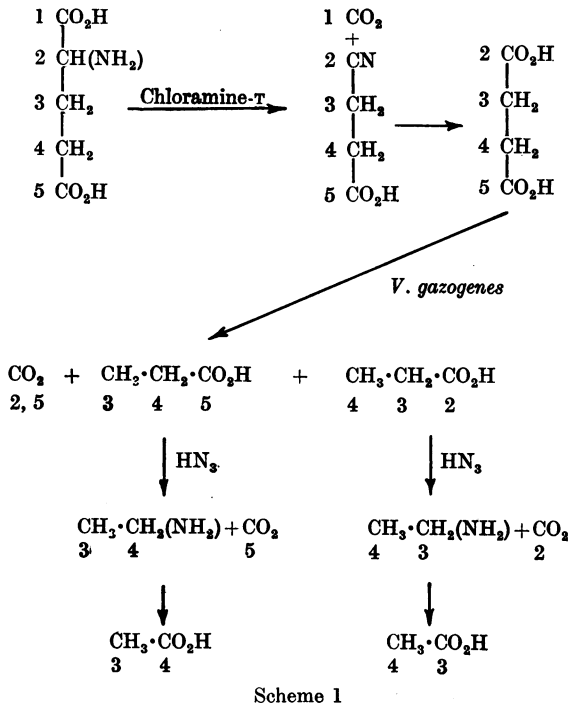
Degradation of glutamate by reaction with chloramine-T. This reaction was carried out under one of two sets of conditions. (*a*) The reaction was carried out on a micro scale, essentially according to the procedure of Kemble & McPherson (1954). Warburg vessels contained 1·5 ml. of 10% (w/v) chloramine-T, 0·8 ml. of 0·5M-acetate buffer, pH 4·0, and 0·1 ml. of 20% formaldehyde in the main compartment, with 0·3 ml. of glutamate (about 18 μmoles) in the side bulb; controls were run without glutamate. The reaction was carried out at 30° and was complete in 1–2 min. The evolved CO_2 was subsequently passed into CO_2-free N-NaOH (3·5 ml.) and was diluted with carrier Na_2CO_3 (to make a total of 600 μmoles of carbonate), made up to 10 ml., and 1 ml. samples were taken for plating as $BaCO_3$.

(*b*) The reaction was also carried out on a macro scale in a transfer unit similar to that described by Kornberg, Davies & Wood (1952). A round-bottomed flask of about 150 ml. capacity, fitted with a side bulb, contained 960 μmoles of glutamic acid in 2 ml. of water at pH 4·0 (in the side bulb) and 15 ml. of 10% (w/v) chloramine-T and 5 ml. of 0·5M-acetate buffer, pH 4·0, in the main flask. This was joined by a T-tube connexion fitted with a tap to a graduated tube containing 4 ml. of CO_2-free N-NaOH. The tap was closed and the contents of flask, side bulb and graduated tube were frozen. The T-tube was connected to a vacuum pump and the apparatus was evacuated. The tap was then closed and the system was thawed out. The graduated tube was kept ice-cold and the flask was immersed in a bath at 37°. The contents of the side bulb were added to the flask contents and the whole was shaken at 37° for 1 hr. A soda-lime tube was attached to the tap, which was then opened. The graduated tube was agitated, and then removed, and its contents diluted to 10 ml. with CO_2-free water. Formation of CO_2 was determined on 0·2 ml. samples in a Warburg flask by adding 1·0 ml. of 2N-H_2SO_4 in a total volume of 2·5 ml. and correcting for a 'blank' on a similar sample of the diluted CO_2-free N-NaOH. Barium carbonate plates were made from samples containing 60 μmoles of CO_2.

Isolation of succinate from the products of the chloramine-T reaction. This was carried out by the method of Cohen (1939). (*a*) Micro-scale procedure. Contents of Warburg flasks were transferred to test tubes with Pasteur pipettes together with washings of 1·5 ml. of hot water, followed by conc. HCl (1 ml.). The tubes were covered with glass marbles and heated for 20 min. on a boiling-water bath. The tubes were cooled, 1 ml. of 10N-NaOH was added and the tubes were left on ice for at least 1 hr. The material was centrifuged and the residue washed with 2 ml. of ice-cold water. The combined supernatants were neutralized, after addition of phenol red, brought to the boil and 0·5 ml. of 5% NH_4Cl was added. The solutions were cooled and made just alkaline to phenol red. The contents were transferred to a Kutcher–Steudel extractor and were extracted for 2–3 hr. with 100 ml. of peroxide-free ether (freshly prepared by shaking with 1% $FeSO_4,7H_2O$ in 0·1N-H_2SO_4, followed by washing with water). The ether extract was discarded; the aqueous phase was acidified with 8N-H_2SO_4 and extracted again with 100 ml. of fresh ether for 2½ hr. The ether extract was evaporated on a water bath with the addition of 1 ml. of water. The extract was evaporated to dryness twice after addition of 1 ml. of water to remove residual acetic acid. The final residue was made up to a standard volume in water.

(*b*) Essentially the same procedure was used on the larger scale with proportionally larger volumes of washings and acid for hydrolysis. The more bulky residue of toluene-*p*-sulphonamide was removed by filtration and the filtrates were concentrated to 10–15 ml. on a rotary evaporator at 30°. This resulted in the deposition of a further bulky precipitate, which was again filtered ice-cold and washed with ice-cold water. The filtrate was made just alkaline to phenol red and was divided into two parts for ether extractions.

Degradation of succinate with Veillonella gazogenes. Cells harvested from 1 l. of culture were washed in 0·1% $Na_2S,9H_2O$, pH 7·4, and were suspended in 5 ml. of 0·1M-phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 5·8, containing 0·1% of $Na_2S,9H_2O$. The suspension was kept on ice in an atmosphere of H_2 unless used immediately. Such cell suspensions were used for the micro-estimation and degradation of succinate (Swim & Krampitz, 1954). Warburg flasks



Scheme 1

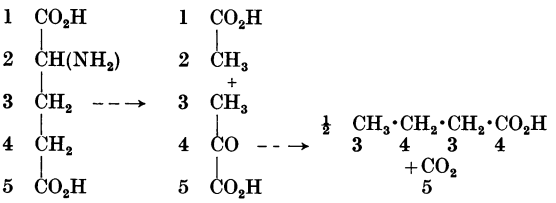
with two side bulbs contained: succinate solution and 0·1 M-phosphate buffer, pH 5·8, to 2·2 ml. in the main compartment, 0·3 ml. of cell suspension in one side bulb and 0·2 ml. of 2 N-H_2SO_4 in a second side bulb. Controls were run without succinate. The flasks were aerated with O_2-free N_2 and the reaction was carried out at 30°. After addition of acid, recoveries of CO_2 from standard succinate solutions were 98–99%. In micro-degradations of succinate, the CO_2 was passed into 3·5 ml. of N-NaOH and converted into $BaCO_3$ after the addition of carrier Na_2CO_3, as with CO_2 produced in the chloramine-T reaction. Decarboxylation of succinate on the macro scale was carried out in a similar apparatus to that used for the chloramine-T reaction. The reaction vessel was fitted with two side bulbs, one of which contained 1 ml. of 4 N-H_2SO_4 and the other the succinate solution; the main flask contained 10 ml. of 0·1 M-potassium phosphate buffer, pH 5·8, and 8 ml. of washed cell suspension of *V. gazogenes* (50 mg. dry wt./ml.). The other limb of the transfer unit again contained 4 ml. of CO_2-free N-NaOH. The system was evacuated and the reaction carried out at 30° with shaking for 60–80 min., followed by treatment with acid from the side bulb. A control was included that contained the reaction mixture without succinate. Carbon dioxide was estimated and plated from suitable samples of the alkali, as with the CO_2 from the chloramine-T reaction.

Micro-estimation of succinate with succinoxidase. In some cases succinate was estimated manometrically with a pigeon-breast-muscle succinoxidase preparation as described by Cohen (1939); see Umbreit, Burris & Stauffer (1951).

Isolation and degradation of propionate. On the macro scale the reaction products were transferred to a centrifuge tube containing 1 ml. of 8 N-H_2SO_4; the flask was washed with 5 ml. of water and the washings were added to the tube. The tube was centrifuged for 20 min. at 3000 *g* and the clear supernatant decanted into a clean tube. The residue was resuspended in 5 ml. of water and centrifuged again. The supernatants were combined, 2 drops of phenol red added and then 10 N-NaOH until the solution was just alkaline. The solution was then concentrated to 5–6 ml. by evaporation on a boiling-water bath in a stream of air. The sample was acidified and steam-distilled in the lactic acid apparatus of Elsden & Gibson (1954), with the addition of 1 ml. of $MgSO_4,7H_2O$ in N-H_2SO_4. Distillate (300 ml.) was collected and titrated with standard NaOH with a microburette. The contents of 'control' flasks were treated in the same way. The distillates were concentrated to dryness by evaporation on a boiling-water bath in a stream of air. Propionate was degraded by the Schmidt reaction, as described by Sakami (1955*a*), with the modifications used by Knight (1962). A similar procedure was used to isolate propionate on the micro scale. The reaction products were transferred from the Warburg flasks to centrifuge tubes together with washings of 2–3 ml. of water. The cells were centrifuged for 20 min. at 3000 *g*, the clear supernatant was decanted off and acidified and steam-distilled as before, except that two 100 ml. amounts of distillate were collected and titrated.

Total combustion of radioactive compounds. Organic compounds were subjected to combustion by the procedure of Sakami (1955*b*), with the 'combustion reagents for general use' of Van Slyke, Plazin & Weissiger (1951).

Glutamate degradation with Clostridium tetanomorphum. Washed cell suspensions of *Clostridium tetanomorphum* convert glutamic acid quantitatively into NH_3, CO_2, acetate and butyrate (Wachsman & Barker, 1955; Wachsman, 1956). The CO_2 is derived exclusively from C-5 of glutamate, the butyrate is derived from C-3 and C-4 of glutamate and the acetate is derived mainly from C-1 and C-2 of glutamate, although some is derived from C-3 and C-4 of glutamate. Under ideal conditions 1 mol.prop. of glutamate yields 1 mol.prop. of acetate, 0·5 mol.prop. of butyrate and 1 mol.prop. of CO_2, as illustrated in Scheme 2.



Scheme 2

In practice, however, it has been found that washed cell suspensions convert glutamate into a mixture of volatile fatty acids in which acetate is in far greater excess over butyrate than would be obtained under ideal conditions. However, under the conditions set out below, there is a quantitative yield of CO_2 from glutamate and so washed cell suspensions of *Clostridium tetanomorphum* have been used to obtain C-5 of glutamate unequivocally.

Cells harvested from two 1 l. cultures were washed twice in 1% (w/v) Na_2SO_4 containing 0·05% of $Na_2S,9H_2O$, and were suspended in 5 ml. of 0·02 M-phosphate (KH_2PO_4–KOH mixture) buffer, pH 7·0, containing 0·1% of $Na_2S,9H_2O$. Cell suspensions were kept on ice in an atmosphere of H_2 unless used immediately. Warburg vessels contained, in 1 ml. of total reaction mixture: glutamate solution (in the side bulb), 0·5 ml. of cell suspension and 0·02 M-phosphate buffer, pH 7·0, containing 0·1% of $Na_2S,9H_2O$. The reaction was carried out at 37° in an atmosphere of H_2 and 0·3 ml. of 4 N-H_2SO_4 was tipped in from a second side bulb to release bound CO_2. Controls were included without glutamate and also a further control with standard glutamate and with 0·2 ml. of 10% (w/v) KOH in the centre well to check for any evolution of H_2. Carbon dioxide was trapped in N-NaOH and plated as $BaCO_3$ as already described. Volatile fatty acids were recovered from the reaction products in the same way as propionate was recovered on the micro scale from the products of the action of washed cell suspensions of *V. gazogenes*.

RESULTS

Photo-assimilation of acetate by washed cell suspensions of Rhodospirillum rubrum *grown on 'malate medium'.* Experiments were designed to follow the time-course of the photo-assimilation of acetate over short periods. In the first experiment a single batch of malate-grown cells was used which, when tested manometrically for the photometabolism of acetate, had Q_{CO_2} 27. A washed cell suspension (40 ml.; 200 mg. dry wt. of cells) was equilibrated in the light in the presence of acetate and hydrogen carbonate in an atmosphere of nitrogen +

carbon dioxide (95:5) and sodium [2-^{14}C]acetate (approximately 50 μc) was injected. The first sample was withdrawn as soon as possible, and thereafter samples were withdrawn at intervals up to 120 sec. The cell samples were fractionated and the 'soluble fractions' were analysed by two-dimensional chromatography and radioautography. The developed radioautographs showed the formation of about 15 radioactive compounds, but one prominent radioactive compound was present in all samples. The major radioactive compound (Fig. 1, spot *A*) was identified as glutamic acid on the basis of the following criteria.

(i) When test chromatograms were dipped in ninhydrin solution (0·1 %, w/v, in acetone), after heating for 3–5 min. at 110° a purple spot developed that was coincident with the radioactive area as developed on the corresponding X-ray film; this established that the compound was an amino acid. Traces of other ninhydrin-positive areas were detectable on chromatograms but none corresponded to any of the other radioactive compounds.

(ii) The radioactive spot was eluted, carrier glutamic acid was added and the mixture was chromatographed again two-dimensionally in the solvent systems of Benson *et al.* (1950); the developed chromatogram was exposed to X-ray film and the radioactive area on the developed X-ray film was coincident with the ninhydrin-positive spot on the chromatogram.

(iii) Further eluates with carrier glutamic acid were examined by paper electrophoresis: (*a*) in pyridine–acetic acid buffer, pH 6·5 (Ryle *et al.* 1955), for 1 hr. at an initial potential of 20 v/cm.; (*b*) in 33 mM-ammonium acetate buffer, pH 6·0, for 1 hr. at 30 v/cm.; (*c*) in pyridine–acetic acid buffer, pH 3·6 (Dixon, Kauffman & Neurath, 1958), for 1 hr. at 30 v/cm. In all cases the ninhydrin-positive spot on the paper was coincident with the radioactive spot on the X-ray film.

The soluble fraction of the sample taken after the shortest exposure (3 sec.) contained three radioactive compounds, including glutamate, 'compound *B*' (Fig. 1) with high R_F values in both solvent systems and which is probably β-hydroxybutyrate, and a further unknown 'compound *C*'. It is particularly noteworthy that intermediates of the tricarboxylic acid cycle were not detected in the sample from the shortest exposure. Such intermediates, including malate, succinate, fumarate and citrate were, however, detected in samples from longer exposures, as shown in Fig. 1, but the amount of radioactivity incorporated into them was at all times very much less than that incorporated into glutamate (Table 1). The distribution of radioactivity in the soluble fraction after increasing exposures to sodium [2-^{14}C]acetate is illustrated in Fig. 2. 'Compound *C*' shows an initial negative slope, as also does 'compound *B*', which suggests that these compounds may be earlier products of the photo-assimilation of acetate and intermediates in the biosynthesis of glutamate from acetate.

A further feature of the radioautographs of this series was the low incorporation of acetate carbon atoms into phosphate esters. This was found also in studying the time-course of incorporation of sodium [1-^{14}C]acetate into the soluble fraction of *R. rubrum*.

All chromatograms of samples exposed to acetate contained one or more unidentified radioactive compounds with high R_F values in both solvent systems, suggesting the formation of hydrophobic compounds, possibly non-volatile fatty acid derivatives. Experiments carried out by Stanier *et al.* (1959) with washed cell suspensions of 'depleted cells' of *R. rubrum* demonstrated that sodium hydrogen carbonate was essential for the formation of proteins and polysaccharides from added radioactive acetate under their experimental conditions; in the absence of hydrogen carbonate, acetate was photo-assimilated to poly-(β-hydroxybutyrate). In these experiments the cells were initially depleted of endogenous carbon reserves, and washed cell suspensions of the 'depleted cells' were then incubated in the light with radioactive acetate for fixed periods of time. It was therefore considered desir-

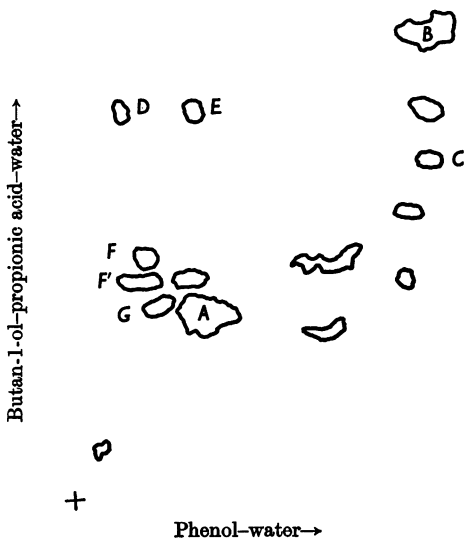


Fig. 1. Copy of radioautograph of 'soluble fraction' of washed cell suspension of *Rhodospirillum rubrum* exposed for 63 sec. in the light to sodium [2-^{14}C]acetate in the presence of $NaHCO_3$ in an atmosphere of $N_2 + CO_2$ (95:5). Major radioactive spots: *A*, glutamate; *B*, believed to be β-hydroxybutyrate; *C*, unknown; *D*, fumarate; *E*, succinate; *F* and *F'*, malate; *G*, citrate; other minor radioactive constituents have not been identified.

Table 1. *Incorporation of* ^{14}C *into glutamate and intermediates of the tricarboxylic acid cycle of washed cell suspensions of* Rhodospirillum rubrum *exposed to* [2-^{14}C]*acetate in the presence of hydrogen carbonate in the light*

Washed cell suspensions of malate-grown cells (40 ml.; 200 mg. dry wt. of cells) were exposed to sodium [2-^{14}C]-acetate (50 μc) in the presence of hydrogen carbonate in an atmosphere of N_2+CO_2 (95:5) under conditions described in the Materials and Methods section. Samples were taken after exposures for the times indicated. Radioactive compounds detected on chromatograms of the 'soluble fraction' of the cells were assayed for ^{14}C activity. Activity in each compound is expressed as a percentage of the total activity in the 'soluble fraction', and as counts/min. estimated directly on the chromatogram.

	Distribution of ^{14}C in 'soluble fraction'							
	Glutamate		Malate		Citrate		Succinate + fumarate	
Exposure (sec.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)
3	31·7	53	0	0	0	0	0	0
14	44·0	284	2·3	15	3·1	20	0	0
23	49·1	406	2·2	19	2·2	19	0	0
30	42·8	645	1·8	27	1·5	22	0	0
37	44·3	930	1·9	40	0·9	19	1·6	33
63	49·0	1822	1·1	39	0·6	21	1·8	70
120	38·1	3240	3·8	320	0·7	61	1·8	152

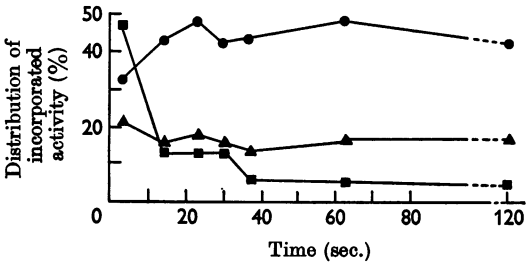


Fig. 2. Time-course of incorporation of sodium [2-^{14}C]-acetate into components of the 'soluble fraction' of washed cell suspensions of *Rhodospirillum rubrum* incubated in the light in the presence of $NaHCO_3$ in an atmosphere of N_2+CO_2 (95:5). ●, Glutamate; ■, unknown spot *C* on radioautograph illustrated in Fig. 1; ▲, spot *B*, believed to be β-hydroxybutyrate.

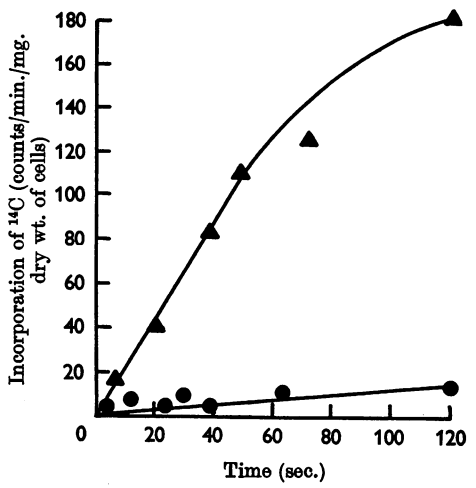


Fig. 3. Time-course of incorporation of sodium [1-^{14}C]-acetate into the 'soluble fraction' of *Rhodospirillum rubrum* in the light: ▲, in the presence, ●, in the absence, of $NaHCO_3$. Gas phase N_2+CO_2 (95:5) in the presence of $NaHCO_3$, or O_2-free N_2 in the absence of $NaHCO_3$.

able to investigate whether the flow of acetate carbon atoms into the soluble fraction of washed cell suspensions would be influenced by hydrogen carbonate. Accordingly a further series of experiments, with a single batch of malate-grown cells (with Q_{CO_2} 30 when tested manometrically for the photometabolism of acetate), followed the photoassimilation of sodium [1-^{14}C]acetate into the 'soluble fraction' in the presence and absence of hydrogen carbonate. Parallel systems were set up, each containing 40 ml. of washed cell suspension (200 mg. dry wt. of cells). In one case the cells were equilibrated for 10 min. in the light in the presence of acetate and hydrogen carbonate in an atmosphere of nitrogen + carbon dioxide (95:5); in the other case the cells were equilibrated in the presence of acetate alone in an atmosphere of oxygen-free nitrogen. Sodium [1-^{14}C]acetate (50 μc) was then injected and samples were taken for analysis after increasing exposures up to 120 sec. as before. The result is illustrated in Fig. 3. Quite clearly under these experimental conditions hydrogen carbonate is essential for the incorporation of acetate carbon atoms into the soluble fraction of the cells to any measurable amount. The most prominent component of the soluble fraction was glutamate, which became radioactive before intermediates of the tricarboxylic acid cycle. Irrespective of the period of exposure, the radioactivity incorporated into glutamate greatly exceeded that in any of the

intermediates of the tricarboxylic acid cycle. As with [2-^{14}C]acetate, compounds with high R_F values in both solvent systems were prominent and very little isotope was incorporated into phosphate esters such as 3-phosphoglyceric acid.

Photo-assimilation of [2-^{14}C]*acetate by washed cell suspensions of* Rhodospirillum rubrum *grown on 'acetate medium'*. Very similar results have been obtained as a result of following the time-course of photo-assimilation of sodium [2-^{14}C]acetate in the presence and absence of hydrogen carbonate, with washed cell suspensions of *R. rubrum* grown on 'acetate medium'. The major radioactive compound in the soluble fraction was identified as glutamate, and its formation preceded that of intermediates of the tricarboxylic acid cycle. The presence of hydrogen carbonate was essential for the incorporation of acetate carbon atoms into the soluble fraction. A preliminary qualitative examination of the 'cell residues' of the samples showed that an appreciable amount of acetate carbon atoms had been incorporated under both sets of conditions (i.e. in the presence and absence of hydrogen carbonate) and that this was mainly in material resistant to digestion by alkaline hypochlorite and soluble in hot chloroform. This suggests that an appreciable amount of acetate is photo-assimilated to poly-(β-hydroxybutyrate) under these conditions. Experiments with cells grown on 'acetate medium' were not pursued extensively since cells grown under these conditions were always found to be less active (Q_{CO_2} 10–12) when tested manometrically for the photometabolism of acetate. Attempts to grow the S 1 strain of *R. rubrum* on media with acetate as sole carbon source with the basal media described by Cohen-Bazire, Sistrom & Stanier (1957) and by Kornberg & Lascelles (1960) have not been entirely successful. Growth took place at a slower rate than on the standard 'malate medium' when fresh inocula of organisms grown on 'malate medium' were used. However, it was not found possible to maintain growth after repeated subcultures on media with acetate as sole carbon source with or without the addition of hydrogen carbonate. Cultures on 'acetate media' appeared 'milky' and were much paler in colour than cultures grown on the 'malate medium'. Microscopic examination showed that organisms growing on 'acetate media' were very distorted: few motile organisms were observed and most cells appeared as elongated bent rods containing refractile granules presumed to contain poly-(β-hydroxybutyric acid). Appreciable growth took place on 'acetate media' supplemented with fairly high concentrations of yeast extract.

Incorporation of sodium [^{14}C]*carbonate in the presence and absence of acetate*. Since hydrogen carbonate appeared to be essential for the incorporation of significant amounts of acetate carbon atoms into the 'soluble fraction' of washed cell suspensions of *R. rubrum* when grown on the 'acetate medium' or the 'malate medium', it was considered desirable to follow the incorporation of hydrogen carbonate into the 'soluble fraction' in the presence of acetate. Furthermore, a direct comparison was made of the incorporation of hydrogen carbonate under autotrophic conditions with hydrogen as reductant with that under heterotrophic conditions in the presence of acetate. *R. rubrum* grown on 'malate medium', having Q_{CO_2} 22 when tested manometrically for the photometabolism of acetate, was used under two sets of conditions. A washed cell suspension in water (7 mg. dry wt. of cells/ml.) was divided into two portions. One 40 ml. portion was equilibrated for 10 min. in the light in the presence of sodium acetate (200 μmoles) and sodium hydrogen carbonate (200 μmoles) in an atmosphere of oxygen-free nitrogen. Sodium [^{14}C]carbonate (250 μc), was then injected and samples were withdrawn after 5, 20, 40, 60, 90, 120, 240 and 480 sec. and analysed as before. The remaining portion of the cell suspension was gassed with hydrogen for 30 min. and 40 ml. was then transferred to the reaction vessel and equilibrated for 10 min. in the light in the presence of sodium hydrogen carbonate (200 μmoles) in an atmosphere of hydrogen. Sodium [^{14}C]carbonate (250 μc) was then injected and samples were withdrawn for analysis after the same periods of exposure as in the parallel experiment in the presence of acetate. It was thus possible to compare directly the patterns of incorporation of hydrogen carbonate into the soluble fraction of the cells in the presence and in the absence of acetate. The pathway of assimilation of hydrogen carbonate differed markedly under the above two conditions. Under 'autotrophic conditions', i.e. in the absence of added acetate, hydrogen carbonate was assimilated primarily into phosphate esters with somewhat lower incorporation into malate, glutamate and aspartate. However, in the presence of acetate there was a much greater incorporation of hydrogen carbonate into malate and glutamate and very much less incorporation into phosphate esters. Both in the presence and absence of acetate, aspartate, succinate and fumarate became radioactive when sodium [^{14}C]carbonate was assimilated. It should be recalled that these compounds and malate form a much smaller proportion of the total radioactive compounds when [1-^{14}C]- or [2-^{14}C]-acetate is assimilated in the presence of hydrogen carbonate. The only compound which became appreciably radioactive from [1-^{14}C]- or [2-^{14}C]-acetate in the presence of hydrogen carbonate and from hydrogen carbonate (injected as sodium [^{14}C]carbonate) in the presence of acetate was glutamate. The time-course of distribution of the major detectable compounds

formed from hydrogen carbonate in the presence and absence of acetate is illustrated in Figs. 4 and 5. The total incorporation of hydrogen carbonate into the soluble fractions under these two conditions is given in Table 2. Although the initial rates of incorporation of hydrogen carbonate are of the same order under the two sets of conditions it is clear that the presence of acetate very much reduces the net incorporation of hydrogen carbonate into the soluble fraction after longer exposures (120–240 sec.). Acetate was found to inhibit the net fixation of $^{14}CO_2$ in *R. rubrum*, under different experimental conditions, by Ormerod (1956).

Assimilation of sodium [^{14}C]*carbonate by washed cell suspensions of* Rhodopseudomonas capsulata *in the presence of acetate.* Since the preceding experiments with *R. rubrum* indicated that the assimilation of hydrogen carbonate differed markedly in the presence and absence of acetate it was decided to follow the course of assimilation of sodium [^{14}C]-carbonate in the presence of acetate in washed cell suspensions of another species of the Athiorhodaceae. *Rhodopseudomonas capsulata* was chosen since the course of assimilation of carbon dioxide under 'autotrophic conditions' in this organism has been investigated by Stoppani, Fuller & Calvin (1954). *Rps. capsulata* was grown on the malate medium and washed cell suspensions were prepared as with *R. rubrum*. Although *Rps. capsulata*, unlike *R. rubrum*, has been found to contain isocitrate lyase when grown on media containing acetate or butyrate (Kornberg & Lascelles, 1960), it would not be expected to contain isocitrate lyase when grown on media containing malate. Under these conditions therefore *Rps. capsulata* might be expected to show similar properties to *R. rubrum*. When tested manometrically for the photometabolism of acetate, the cells had Q_{CO_2} 11·0. Cell suspension (40 ml.; 10 mg. dry wt./ml.) was transferred to the reaction vessel and equilibrated for 15 min. in the light in the presence of sodium acetate (100 μmoles) in oxygen-free nitrogen. Sodium [^{14}C]carbonate (250 μc, 25 μmoles) was then injected and samples were withdrawn at intervals up to 480 sec. and were analysed as before. The distribution of radioactivity in the soluble fraction was very similar to that found in *R. rubrum* under the same conditions: the most prominent radioactive compounds were

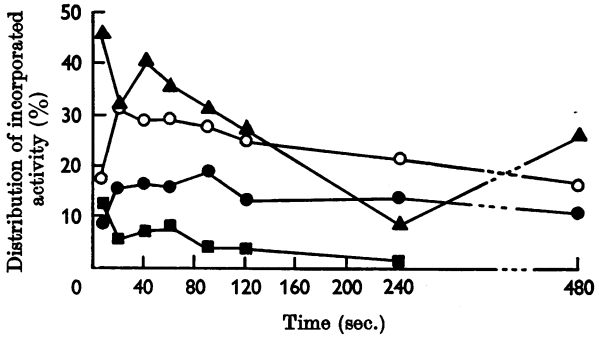


Fig. 4. Time-course of incorporation of $Na_2{}^{14}CO_3$ into components of the 'soluble fraction' of washed cell suspensions of *Rhodospirillum rubrum* incubated in the light in the presence of acetate in an atmosphere of O_2-free N_2. ○, Glutamate; ▲, malate; ●, phosphate esters; ■, aspartate.

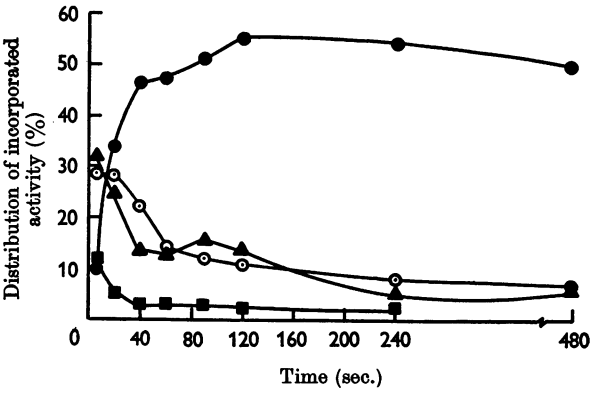


Fig. 5. Time-course of incorporation of $Na_2{}^{14}CO_3$ into components of the 'soluble fraction' of washed cell suspensions of *Rhodospirillum rubrum* incubated in the light in an atmosphere of H_2. ⊙, Glutamate; ▲, malate; ●, phosphate esters; ■, aspartate.

Table 2. *Total incorporation of sodium* [^{14}C]-*carbonate into the 'soluble fraction' of washed cell suspensions of* Rhodospirillum rubrum *in the presence and absence of acetate*

Washed cell suspensions of malate-grown *R. rubrum* (40 ml.; 280 mg. dry wt. of cells) were exposed, for the times indicated, to $Na_2{}^{14}CO_3$ (250 μc) in the light under two sets of conditions: (i) in the presence of sodium acetate (200 μmoles) in an atmosphere of O_2-free N_2; (ii) in the absence of acetate in an atmosphere of H_2. Further details of the experimental conditions are described in the text. ^{14}C activity was assayed in 'soluble fraction' after chromatography as described previously.

	Incorporation of ^{14}C (counts/min./mg. dry wt. of cells)	
Gas phase	Acetate absent H_2	Acetate present N_2
Exposure (sec.)		
5	25	42
20	28	45
40	53	68
60	94	87
90	120	85
120	140	99
240	209	114
480	263	167

malate, glutamate, aspartate and phosphate esters. Glutamate did not appear to become labelled quite as rapidly in *Rps. capsulata* as in *R. rubrum*, and the phosphate esters appeared to contain a higher percentage of the total radioactivity than was found with *R. rubrum*. The distribution of radioactivity in the major identified products of the soluble fraction is given in Table 3. A comparison with the results of Stoppani *et al.* (1954) indicates that, as with *R. rubrum*, the relative incorporation of hydrogen carbonate into the phosphate esters is much greater under autotrophic conditions than under heterotrophic conditions in the presence of acetate. The incorporation of acetate into washed cell suspensions of *Rps. capsulata* has not been studied in detail. Nevertheless it has been found that with both *Rps. capsulata* and *Rps. spheroides* grown on malate medium, washed suspensions incubated with sodium [2-^{14}C]acetate for 15 min. in the light incorporate acetate into the cell residue and soluble fractions. The major radioactive constituent of the soluble fraction was found to be glutamate and the isotope incorporation into glutamate was greater in the presence of hydrogen carbonate than in its absence. However, an appreciable incorporation of acetate carbon atoms into glutamate was obtained in the absence of hydrogen carbonate with both organisms.

Degradation of glutamate formed during the photometabolism of acetate in the presence of hydrogen carbonate by washed cell suspensions of Rhodospirillum rubrum. The radioactive glutamate formed in washed cell suspensions of *R. rubrum* was systematically degraded to locate the distribution of radioactive isotopic carbon atoms within the molecule. The radioactive glutamate obtained from the following experiments was degraded: (i) the photo-assimilation of sodium [^{14}C]carbonate in the presence of acetate; (ii) the photo-assimilation of [1-^{14}C]acetate in the presence of hydrogen carbonate; (iii) the photo-assimilation of [2-^{14}C]acetate in the presence of hydrogen carbonate. In (i) and (ii) pooled samples from chromatograms of soluble fractions of cells exposed for periods up to 1 min. were used. Insufficient radioactive material was available for duplicate degradations, and the results are summarized in Table 4. To obtain a larger amount of radioactive glutamate for degradation a washed cell suspension of *R. rubrum* (40 ml.; 10 mg. dry wt./ml.) was exposed to sodium [2-^{14}C]-acetate in the presence of hydrogen carbonate in the light for 1 min. and the whole suspension was fractionated as in previous experiments. The glutamate was eluted from the chromatograms and was purified by paper electrophoresis in the pyridine–acetic acid buffer, pH 6·5 (1 hr. at 30 v/cm.). A glutamate marker was run alongside. The dried paper strip was exposed to film. The developed film

Table 3. *Distribution of radioactive compounds formed from sodium* [^{14}C]*carbonate in the presence of acetate by washed cell suspensions of* Rhodopseudomonas capsulata

Washed cell suspensions of malate-grown *Rps. capsulata* (40 ml.; 400 mg. dry wt. of cells) were exposed to $Na_2{}^{14}CO_3$ (250 μc) in the light in the presence of sodium acetate (100 μmoles) in O_2-free N_2 for the times indicated. 'Soluble fraction' of cell samples was chromatographed and radioactive components were assayed for ^{14}C activity. Activity in each compound is expressed as a percentage of the total ^{14}C activity in the 'soluble fraction' and as counts/min. estimated directly on the chromatogram.

Exposure (sec.)	Distribution of ^{14}C activity in 'soluble fraction'											
	Glutamate		Malate		Fumarate		Succinate		Aspartate		'Phosphate esters'	
	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)
20	7·8	350	33·3	1490	7·7	345	12·1	540	7·7	345	16·0	730
40	12·0	545	32·2	1470	7·7	350	8·5	390	8·5	390	18·0	820
60	13·0	630	27·0	1305	6·3	305	5·7	275	5·1	250	23·5	1140
90	15·9	945	22·5	1335	5·9	350	6·6	390	3·6	215	23·0	1370
120	19·4	207	23·8	253	7·6	80	8·6	93	5·4	57	23·4	250
240	27·9	332	24·8	295	5·7	68	2·5	30	2·9	35	20·5	241
480	29·0	750	12·5	323	3·0	77	3·6	93	1·3	33	16·8	433

showed three radioactive spots. Each of these was assayed roughly with an end-window Geiger tube. The major spot that coincided with the glutamate marker moved 14 cm. anodically and assayed 10 500 counts/min. above background, a very weak spot (250 counts/min.) moved 22 cm. anodically and a third spot (1300 counts/min.) moved 28 cm. anodically. The spot with 1300 counts/min., which was thought to be pyrrolidone-α-carboxylic acid, was eluted and a portion was heated in a sealed tube in 3N-hydrochloric acid at 100° for 3 hr.; the tube was then opened, all traces of acid were removed and the sample was chromatographed on Whatman no. 4 paper in the butanol–propionic acid solvent system together with a glutamate marker. A single radioactive spot was found, coinciding with the ninhydrin-positive area of the glutamate marker with which it was mixed; no other radioactive spot was detected. This strongly suggested that the spot on the electrophoresis strip was due to pyrrolidone-carboxylic acid, which is readily formed from glutamate by mild chemical treatment at slightly acid pH (Wilson & Cannan, 1937; Foreman, 1914*a*, *b*). Hydrolysis with strong acid converts pyrrolidone-α-carboxylic acid quantitatively into glutamate (Meister & Bukenberger, 1962). The glutamate spot was eluted, mixed with carrier glutamate and systematically degraded. Sufficient material was available to carry out the degradation in duplicate. The results are given in Table 5. Although washed cell suspensions of *Clostridium tetanomorphum* were used to yield C-5 of glutamate as carbon dioxide, and in the cases where this method was used the gas was not radioactive, the volatile fatty acids were recovered and were radioactive. However, a quantitative determination of the radioactivity in the recovered volatile fatty acids was not carried out. In the experiment with [2-^{14}C]acetate the recovered volatile fatty acids were chromatographed on Amberlite CG-50 (Seki, 1958) and radioactive acetate and butyrate, in a ratio 5:1, were recovered. The disproportionately high ratio of acetate to butyrate indicates that considerable mixing of C-3 and C-4 and of C-1 and C-2 of glutamate had occurred. A rough estimation of the specific activity of the butyrate indicated that it was about double that of the acetate, and, since the butyrate is derived almost exclusively from C-3 and C-4 of glutamate, this would be consistent with the isotope distribution in glutamate in which C-3 and C-4 of glutamate are derived from [2-^{14}C]acetate.

Effect of preincubation conditions on incorporation of [^{14}C]*acetate by washed cell suspensions of* Rhodospirillum rubrum. Benedict (1962) claimed that acetate was incorporated in the light into the soluble fraction of washed cell suspensions of *R. rubrum* in the absence of hydrogen carbonate. Further, the major products of the photo-assimilation of acetate after exposure for 5 sec. were found to be succinate and citramalate, and very little radioactivity was found in glutamate. However, the experimental conditions were slightly different from those employed in the work reported here. The main differences were (i) that the organism was

Table 4. *Degradation of* [^{14}C]*glutamate derived from* [1-^{14}C]*acetate in the presence of sodium hydrogen carbonate and from sodium* [^{14}C]*carbonate in the presence of acetate*

Degradation of [^{14}C]glutamate derived from [1-^{14}C]acetate was carried out on the macro scale (see Materials and Methods section). After recovery of propionate from the second stage of the degradation, additional carrier propionate was added (288 μmoles: equivalent to the propionate recovered) and a portion taken for total combustion and then specific-activity determination; a further amount of carrier propionate (460 μmoles: equivalent to the remaining propionate) was added for the Schmidt reaction step. Degradation of [^{14}C]glutamate derived from $Na_2{}^{14}CO_3$ in the presence of acetate was carried out on the micro scale: one portion was degraded by the chloramine-T reaction, and a second portion by decarboxylation with a washed cell suspension of *Clostridium tetanomorphum* (see Materials and Methods section).

Source of ^{14}C	Reactant	Amount (μmoles)	Total counts	Sp. activity (counts/min./μmole)	Product	Amount (μmoles)	Total counts	Sp. activity (counts/min./μmole)
Sodium [1-^{14}C]acetate	Glutamate	825	23 816	30·08	CO_2	806	2 377	2·95
					Succinate	689	18 465	26·80
	Succinate	408	10 934	26·80	CO_2	257	3 194	12·43
					Propionate	288	3 842	13·34
	Propionate	920	3 063	3·33	CO_2	747	1 942	(2·6)* 10·4†
					Ethylamine	652	Nil	Nil
$Na_2{}^{14}CO_3$	Glutamate	18·4	5 483	298	CO_2	18·4	4 195	228
					Succinate	16·4	Nil	Nil
	Glutamate	18·4	5 483	298	CO_2	13·5	Nil	Nil
					Total volatile fatty acids	26·3	Not determined	

* Specific activity determined.
† Specific activity corrected for additions of carrier.

Table 5. *Degradation of* [^{14}C]*glutamate derived from* [2-^{14}C]*acetate in the presence of sodium hydrogen carbonate*

The [^{14}C]glutamate sample was made up to 3 ml. with addition of carrier L-glutamic acid (60 μmoles); total counts in sample: 255 500. A portion (0·3 ml.) was taken for total combustion, 0·7 ml. for decarboxylation with washed cell suspension of *Clostridium tetanomorphum* and 1 ml. portions in duplicate (A and B) for degradation by the micro procedure. Carrier propionate (900 μmoles); equivalent to a dilution of 1 in 70 of recovered [^{14}C]-propionate was added for the Schmidt reaction step. There is no duplicate result for the products of the Schmidt reaction since sample B exploded.

Reactant	Amount (μmoles)	Total counts	Sp. activity (counts/min./ μmole)	Product	Amount (μmoles)	Total counts	Sp. activity (counts/min./ μmole)
Glutamate	20·7	85 167	4 114	(A) CO_2	20·7	534	25
				(B) CO_2	20·8	547	26
				(A) Succinate	18·4	75 100	3 890
				(B) Succinate	17·9	71 230	3 960
Succinate	16·5	64 185	3 890	(A) CO_2	16·5	2 270	123
				(B) CO_2	16·1	2 110	118
				(A) Propionate	14·4	56 300	3 910
				(B) Propionate	13·5	50 400	3 730
Propionate	913	50 670	55·5	(A) CO_2	720	1 180	(1·6)* 115†
				(A) Ethylamine	805	35 680	(44·3)* 3 100†
Ethylamine	268	11 890	44·3	(A) Acetate	190	7 730	(40·8)* 2 856†
Glutamate	14·0	57 596	4 114	CO_2	14·1	Nil	Nil
				Total volatile fatty acids	17·0	Not determined	

* Specific activity determined.
† Specific activity corrected for addition of carrier.

grown on a medium that contained malate and glutamate as major carbon sources (Kohlmiller & Gest, 1951) and (ii) that the cells were 'equilibrated' for 30 min. under nitrogen in the absence of acetate before addition of radioactive acetate. Accordingly an experiment was designed to compare, with malate-grown cells, the effects of the different 'equilibration conditions' on the products of the photo-assimilation of [^{14}C]acetate by the analytical procedures already described in this paper. *R. rubrum* grown on malate medium was harvested, washed and suspended in glass-distilled water to a cell concentration of 10 mg. dry wt./ml. When tested manometrically for the photometabolism of acetate the cell suspension had Q_{CO_2} 26. Two parallel cell suspensions were set up, each containing 20 ml. of cell suspension. Cell suspension (A) was 'equilibrated' in the light in the presence of acetate (200 μmoles); cell suspension (B) was 'equilibrated' in the light in the absence of acetate. The equilibration period was 10 min. and the suspensions were gassed with oxygen-free nitrogen throughout. Sodium [1-^{14}C]acetate (8 μmoles; $1{\cdot}4 \times 10^7$ total counts) was then added to each suspension and after exposure for 1 min. the cell suspension was run into 60 ml. of ethanol–water–acetic acid (950:50:1, by vol.) and the products were fractionated as before. Activity (^{14}C) in the soluble fraction was assayed by plating on stainless-steel planchets with the addition of 0·1% acetic acid to remove excess of [^{14}C]acetate; the addition of acetic acid was repeated until the count was constant. The whole cell residue was assayed as described in the Materials and Methods section, and the polymer fraction was assayed as for the 'soluble fraction', a suitable sample of the hypochlorite-indigestible, chloroform-soluble fraction of the cell residue being taken. The results are shown in Table 6. A part of the 'soluble fraction' of the cells equilibrated in the absence of acetate (i.e. under the conditions used by Benedict, 1962) was analysed by two-dimensional chromatography and radioautography. Under these conditions no citramalate was detected and only small amounts of succinate and other intermediates of the tricarboxylic acid cycle were present. One major radioactive spot was found to contain 55% of the total radioactivity in the 'soluble fraction'. This spot was identified as glutamic acid by the tests previously used. In addition the major radioactive spot was eluted from a chromatogram, and, without addition of carrier, was treated with chloramine-T reagent in excess, as used in the degradation of glutamate; the products were hydrolysed and extracted into ether, and the fraction extracted into ether from acid solution was radioactive. Samples of this fraction were chromatographed, together

with a [^{14}C]succinate marker, one-dimensionally in the phenol–water and butanol–propionic acid solvent systems of Benson *et al.* (1950). In both systems the radioactive spot derived from the products of the chloramine-T reaction had the same R_F as the succinate marker. A similar correspondence between succinate marker and radioactive reaction product was found after paper electrophoresis in 33 mM-ammonium acetate, pH 6·0, for 90 min. at 20 v/cm. These results show that succinate was the final product of the chloramine-T reaction and thus provide confirmation that the major radioactive spot was glutamate.

A further experiment was carried out with *R. rubrum* grown on the malate + glutamate medium of Kohlmiller & Gest (1951), which was used in the experiments of Benedict (1962). The washed cell suspension was somewhat less active when tested manometrically for the photometabolism of acetate (Q_{CO_2} 18 was found). Cell suspension (20 ml.; 10 mg. dry wt. of cells/ml. of water) was 'equilibrated' for 10 min. in the light in the absence of acetate in oxygen-free nitrogen. [1-^{14}C]Acetate was added as before and after exposure for 1 min. the reaction mixture was run into ethanol–water–acetic acid and the 'soluble fraction' was analysed by chromatography and radioautography. Glutamate was again the major radioactive compound and contained 35 % of the total radioactivity of the soluble fraction. Intermediates of the tricarboxylic acid cycle again formed only a small fraction of the radioactive constituents and no conclusive evidence was obtained for the formation of citramalate.

Attempts to demonstrate a net synthesis of glutamate. The rapid appearance of radioactivity from acetate into glutamate of the 'soluble fraction' without the appearance of precursors could be due to the very large pool size or 'steady-state concentration' of glutamate compared with a small pool size of its precursors. Indications that *R. rubrum* may contain a fairly high intracellular concentration of glutamate were obtained by chromatography of the 'soluble fraction' of a washed cell suspension incubated in the light with acetate and hydrogen carbonate (but without addition of radioactive material). Two-dimensional chromatograms were run as before and were developed by dipping in ninhydrin solution (0·1 %, w/v, in acetone) and heating for 2–3 min. at 110°. Several ninhydrin-positive spots were present but by far the most intense was in the glutamate position. Attempts were then made to estimate the concentration of glutamate in the 'soluble fraction' of washed cell suspensions incubated in the light under different conditions. Experiments were planned with two batches of cells to see if glutamate formation from acetate was stimulated by hydrogen carbonate under the conditions used in the short-term-exposure experiments with radioactive acetate. Glutamate concentration in the 'soluble fraction' was estimated with a washed cell suspension of *Clostridium welchii*

Table 6. *Effect of 'equilibration conditions' on the incorporation of* [1-^{14}C]*acetate by washed cell suspensions of* Rhodospirillum rubrum *in the absence of sodium hydrogen carbonate*

Washed cell suspensions of malate-grown *R. rubrum* (20 ml.; 200 mg. dry wt. of cells) were equilibrated for 10 min. in the light in an atmosphere of O_2-free N_2 under two sets of conditions: (*a*) in the presence of sodium acetate (200 μmoles); (*b*) in the absence of acetate. Sodium [1-^{14}C]-acetate ($1·4 \times 10^7$ total counts; 8 μmoles) was added and suspensions were incubated for 1 min. Cell suspensions were fractionated and ^{14}C activity was assayed in crude cell fractions as described in the Materials and Methods section. The values are not corrected for self-absorption.

	10^{-4} × Incorporation of ^{14}C (counts/min.)	
Equilibration conditions	(*a*) Acetate present	(*b*) Acetate absent
Cell fraction		
Soluble fraction	2·4	46
Cell residue (including polymer)	5·0	41
Polymer	6·0	30

Table 7. *Glutamate estimations of the 'soluble fractions' of washed cell suspensions of* Rhodospirillum rubrum *exposed in the light in the presence of acetate under different conditions*

Washed cell suspensions (40 ml.) of *R. rubrum* grown on malate medium were incubated for 15 min. in the light with the additions and gas phase indicated below. Glutamate concentration of the 'soluble fraction' was determined manometrically with glutamate decarboxylase from *Clostridium welchii* SR 12.

Gas phase	Additions to system	Dry wt. of cells (mg.)	Glutamate (μmoles)	Glutamate (μmoles/100 mg. of cells)
N_2	Sodium acetate (200 μmoles)	436	5·13	1·18
$N_2 + CO_2$ (95:5)	Sodium acetate (200 μmoles) $NaHCO_3$ (400 μmoles)	436	6·46	1·48
N_2	Ammonium acetate (200 μmoles)	388	3·2	0·83
$N_2 + CO_2$ (95:5)	Ammonium acetate (200 μmoles) $NaHCO_3$ (400 μmoles)	388	4·2	1·08

as a source of glutamate decarboxylase, according to the procedure of Krebs (1950). The results are shown in Table 7.

DISCUSSION

The studies on the time-course of the photo-assimilation of acetate by washed cell suspensions of *Rhodospirillum rubrum* have shown that glutamate is a major early product and that it is derived from both [1-^{14}C]- and [2-^{14}C]-acetate. Furthermore, the formation of glutamate from acetate is greatly increased by the presence of hydrogen carbonate, which is itself incorporated into glutamate. The very rapid appearance of glutamate in the soluble fraction before the appearance of intermediates of the tricarboxylic acid cycle, in particular of citrate, suggests that glutamate is not formed via citrate. The failure to detect intermediates of the tricarboxylic acid cycle could be attributed to a small pool size for these intermediates, although the ready detection of malate, fumarate and succinate formed from [^{14}C]carbonate makes this unlikely. The degradation of the glutamate samples derived from [1-^{14}C]- and [2-^{14}C]-acetate and from sodium [^{14}C]carbonate has given an unequivocal isotope distribution and reveals a pattern of labelling that has not been found in any other micro-organism and which excludes the formation of glutamate by the conventional tricarboxylic acid cycle. The degradation data thus confirm the conclusions drawn from the earlier experiments on the time-course of acetate assimilation into soluble components of *R. rubrum*.

Studies on the photo-assimilation of propionate by washed cell suspensions of *R. rubrum* have been reported by Knight (1962). In these experiments glutamate was found in the soluble fraction of cells exposed for short periods to labelled propionate, and again citrate appeared to become labelled much more slowly than glutamate.

A comparison of the degradation data given in Tables 4 and 5 shows that the procedure employed for the degradation of glutamate has given an unequivocal isotope distribution, which can be illustrated thus:

$$\underset{\text{b}}{HO_2C}\cdot\underset{\text{c}}{CH(NH_2)}\cdot\underset{\text{m}}{CH_2}\cdot\underset{\text{m}}{CH_2}\cdot\underset{\text{c}}{CO_2H}$$

Where b is derived from hydrogen carbonate (or sodium [^{14}C]carbonate as used), c is derived from [1-^{14}C]acetate, and m is derived from [2-^{14}C]acetate.

This isotope distribution differs from that found in *Clostridium kluyveri* (Tomlinson, 1954), where glutamate is synthesized from acetate by an unknown mechanism. A reversal of the pathway for the degradation of glutamate in *Clostridium tetanomorphum* is also inconsistent with the observed isotope distribution.

It is fortunate that the degradation procedure, which involves the symmetrical molecule of succinic acid as an intermediate, has in this case given an unequivocal answer. With other isotope distributions it might not be possible to deduce an unequivocal answer by this method, since C-3 and C-4 of glutamate are indistinguishable from one another; C-2 and C-5 are also equivalent in succinate but C-5 can be obtained unequivocally by decarboxylation with washed cell suspensions of *Clostridium tetanomorphum* or by reaction with hydrazoic acid.

The isotope distribution in glutamic acid, apart from excluding its formation via the tricarboxylic acid cycle, also excludes its formation by a reversal of the reactions involved in the fermentation of glutamate by *Clostridium tetanomorphum*, in which citramalate is an intermediate. The isotope distribution is in agreement with the results of the partial degradation of the glutamate of the cell proteins of *R. rubrum* grown on media containing $^{13}CH_3\cdot{}^{14}CO_2H$ and sodium [^{14}C]carbonate (Cutinelli *et al.* 1951). These authors showed that C-1 of glutamate was derived from carbon dioxide and that C-5 of glutamate was derived from C-1 of acetate. Their degradation procedures did not give an unequivocal isotope distribution in C-2, C-3 and C-4 of glutamate, although it was possible to deduce that two of the three carbon atoms were derived from C-2 of acetate and one from C-1 of acetate. Since these authors had found alanine to be labelled specifically in the following manner:

$$\underset{\text{m}}{CH_3}\cdot\underset{\text{c}}{CH(NH_2)}\cdot\underset{\text{b}}{CO_2H},$$

and assuming the alanine to be derived directly from pyruvate, then a synthesis of glutamate from acetate via the tricarboxylic acid cycle would result in the following isotope distribution in glutamate:

$$\underset{\text{b}}{HO_2C}\cdot\underset{\text{m}}{CH(NH_2)}\cdot\underset{\text{c}}{CH_2}\cdot\underset{\text{m}}{CH_2}\cdot\underset{\text{c}}{CO_2H}$$

The results of the partial degradation of glutamate (Cutinelli *et al.* 1951) were consistent with this pattern of labelling and it was concluded that the tricarboxylic acid cycle was involved. Hug & Werkman (1957) subsequently showed that whole cells and cell-free extracts could transaminate α-oxoglutarate with a number of L-amino acids; this is consistent with the view that α-oxoglutarate, an intermediate of the tricarboxylic acid cycle, is a possible precursor of glutamate. Glutamate might also be formed from α-oxoglutarate by the action of a glutamate dehydrogenase (NAD- or NADP-linked). This seems unlikely in *R. rubrum*, since all attempts to demonstrate activity failed. This has included assays on acetone-dried cells, and fresh cell-free extracts with NAD or NADP as hydrogen

acceptors under anaerobic conditions, by following the reaction by measuring increases in extinction at 340 mμ or by formation of keto acids by the procedure of Friedeman & Haugen (1943).

A further examination of the degradation data, in particular of the results with the incorporation of [2-^{14}C]acetate (Table 5) since more radioactive material was available with this experiment, shows that very little ^{14}C (about 0·6 %) is incorporated into C-1 of glutamate. This indicates that very little of the acetate which is oxidized to carbon dioxide is re-assimilated. Although nearly all of the 2-^{14}C of acetate is incorporated into C-3 and C-4 of glutamate, there is nevertheless a small and significant incorporation into C-2 or C-5 or both (amounting to about 3 %). Since the incorporation into C-5 was negligible, it seems that the small incorporation is largely in C-2 of glutamate. This may be due to a small synthesis of glutamate from acetate via the tricarboxylic acid cycle, whereby the C-2 of acetate is incorporated into C-2 of glutamate (as discussed above). Nevertheless the major pathway of the biosynthesis of glutamate from acetate in *R. rubrum* is clearly one that does not involve the tricarboxylic acid cycle directly. The results of studies on the effects of fluoroacetate on the photometabolism of acetate by washed cell suspensions indicate that the tricarboxylic acid cycle is involved in the photometabolism of acetate (Elsden & Ormerod, 1956). More recently it has been claimed that *R. rubrum* has an 'anaerobic citric acid cycle' (Gest, Ormerod & Ormerod, 1962). These results are not necessarily in conflict with the results reported here. As pointed out by Stanier *et al.* (1959), the photoassimilation of acetate to cell material [in particular poly-(β-hydroxybutyrate)] is a reductive process and consequently some acetate must be oxidized to provide the necessary reducing power. It seems probable that the oxidation of acetate proceeds via the tricarboxylic acid cycle. The formation of glutamate from acetate depends on the presence of hydrogen carbonate, which is specifically incorporated. The degradation of the glutamate shows that this is derived (most probably indirectly) from two molecules of acetate and one of hydrogen carbonate. The overall conversion:

$$2CH_3\cdot CO_2H + CO_2(+NH_3) \rightarrow HO_2C\cdot CH(NH_2)\cdot CH_2\cdot CH_2\cdot CO_2H$$

involves a net reduction, and so the oxidation of some acetate via the tricarboxylic acid cycle may provide the necessary reducing power. The small amounts of ^{14}C-labelled malate, citrate and other intermediates of the tricarboxylic acid cycle found in these experiments may arise from the oxidation of acetate and have nothing directly to do with its assimilation to glutamate. Indeed, since the cells were malate-grown and were not deliberately 'depleted' (cf. Stanier *et al.* 1959), reserve polysaccharide might be oxidized as a source of reducing power, thus sparing the oxidation of [^{14}C]-acetate. In 'depleted cells', which should be entirely dependent on acetate oxidation as a source of reducing power, one might find intermediates of the tricarboxylic acid cycle more prominent.

Under the conditions of the experiments hydrogen carbonate is essential for the incorporation of acetate carbon atoms into the 'soluble fraction' in appreciable amounts. This agrees with the findings of Stanier *et al.*, with washed suspensions of 'depleted cells', that carbon dioxide is essential for the formation of cell materials other than poly-(β-hydroxybutyric acid). The experiments of Benedict (1962), showing acetate incorporation into the 'soluble fraction' in the absence of hydrogen carbonate, appear to be due to the different 'equilibration conditions' used. When cells are equilibrated in the presence of acetate, then acetate-acceptor molecules such as pyruvate and oxaloacetate may be used up, and when [^{14}C]acetate is added it cannot be incorporated into 'soluble components' unless hydrogen carbonate is supplied (Table 6). The failure of Benedict (1962) to find glutamate as a major component of the 'soluble fraction' conflicts with the results reported here. However, Benedict's analytical technique was different in detail and it is probable that the use of ion-exchange resins, e.g. Dowex 50 (H^+ form), would effectively remove amino acids (see, for example, Hulme & Wooltorton, 1958; Hulme, 1961). Citramalate and glycollate have never been observed as major constituents of the 'soluble fraction' of washed cell suspensions exposed to [1-^{14}C]- or [2-^{14}C]-acetate.

In the presence of hydrogen, sodium [^{14}C]-carbonate is incorporated mainly into phosphate esters, as is characteristic of all autotrophic systems that have been examined. If acetate is present then there is a greatly reduced flow of sodium [^{14}C]carbonate into phosphate esters and an increased incorporation into malate and glutamate, and to a somewhat less extent into fumarate and asparate. A similar situation is found with *Rps. capsulata* (Table 3). Glutamate is the only common compound formed in appreciable quantity from both [^{14}C]acetate and sodium [^{14}C]carbonate. The different pattern of incorporation of sodium [^{14}C]-carbonate under these two sets of conditions suggests that acetate may inhibit carboxydismutase or other enzymes that are essential for the autotrophic assimilation of carbon dioxide. Cell suspensions of *R. rubrum* that have been 'equilibrated' in the light in the presence of acetate contain carboxydismutase (J. Lascelles, personal communication). However, despite the presence of carboxydismutase in the cells the enzyme may still not function. The

situation may be analogous to that in *Pseudomonas oxalaticus* when it is adapting itself to oxalate after growth on formate as sole carbon source (Quayle, 1962). This adaptation involves a long lag period with no growth; cells harvested during this lag period fix only small amounts of $^{14}CO_2$, by comparison with formate-grown cells, although carboxydismutase and other enzymes necessary for incorporation of carbon dioxide are present in the cells.

The presence of acetate does appear to decrease the net incorporation of carbon dioxide into the soluble fraction over longer periods of exposure (Table 2), even though, as has been demonstrated, carbon dioxide is necessary for the assimilation of acetate to form glutamate. Ormerod (1956) observed a suppression of fixation of carbon dioxide in the photo-assimilation of acetate by washed cell suspensions of *R. rubrum*. As Stanier *et al.* (1959) suggested, this may be due to a competition between acetate and carbonate for a limited supply of reducing power for their assimilation.

The results of the short-exposure experiments with [^{14}C]acetate show features in common with the earlier observations of Glover *et al.* (1952). Thus, of the ^{14}C incorporated into the 'soluble fraction', very little is present in the intermediates of the tricarboxylic acid cycle. This is true when either [1-^{14}C]- or [2-^{14}C]-acetate is photometabolized by washed cell suspensions, and holds for all periods of exposure in the experiments reported here (i.e. from 3 to 240 sec.). The compounds on chromatograms of the soluble fraction with high R_F values in both solvent systems, which are characteristically formed from acetate carbon atoms (and not from sodium [^{14}C]carbonate in the presence of acetate), may be precursors of lipid that Glover *et al.* (1952) showed to be the major product of the photo-assimilation of acetate. The experiments reported here also confirm the earlier observation that little or no acetate carbon atoms flow through phosphate esters. The unknown 'compound X', which formed such a high percentage of the ^{14}C-labelled compounds of the 'soluble fraction' in the experiments of Glover *et al.* (1952), must still be considered as unidentified. Under the conditions of the experiments reported here, the major component of the 'soluble fraction' is glutamic acid. Certainly 'compound X' cannot be glutamic acid, since 'compound X' was very close to α-oxoglutarate in the chromatography solvent system used by Glover *et al.* (1952). α-Oxoglutarate and glutamate are easily resolved from each other in these solvent systems [the organic phase from 2-methyl-butan-1-ol–90% formic acid–water (9:3:9, by vol.) and propan-1-ol–ammonia (sp.gr. 0·880)–water (6:3:2, by vol.), the solvent systems of Hanes & Isherwood (1949), were used]. It is similarly not possible to identify 'compound Y', which had nearly 25% of the ^{14}C activity of the 'soluble fraction' in the experiments of Glover *et al.* (1952).

Glutamate formation from acetate has been demonstrated in other photosynthetic organisms. Preliminary experiments mentioned above have shown that glutamate is the major radioactive component of the soluble fraction of washed cell suspensions of *Rps. spheroides* and *Rps. capsulata* exposed in the light to sodium [2-^{14}C]acetate. Losada, Trebst, Ogata & Arnon (1960) showed that glutamate was the major product of the photo-assimilation of acetate by *Chromatium*. This was established with cultures of *Chromatium* grown on acetate as sole carbon source. Under these conditions the cells contain isocitrate lyase (Fuller, Smillie, Sisler & Kornberg, 1961) and, unlike *R. rubrum*, it is conceivable that carbon dioxide may not be necessary for glutamate formation from acetate. Losada *et al.* (1960) showed that acetate assimilation, measured directly by uptake of [^{14}C]acetate, was insensitive to cyanide inhibition, which suggested that carboxydismutase was not involved.

Glutamate formation from acetate has also been demonstrated in *Chlorella pyrenoidosa*. Moses, Holm-Hansen, Bassham & Calvin (1959) supplied [2-^{14}C]acetate together with carbon dioxide to starved cells of *Chlorella pyrenoidosa* in the light and in the dark. After exposure for 3 min. the major constituent was glutamic acid, which accounted for 42·1% of the soluble components of cells exposed in the light, and 50·4% of that of cells exposed in the dark. Small amounts of acetate carbon atoms were incorporated into phosphate esters, but appreciable incorporation into intermediates of the tricarboxylic acid cycle was found. The soluble fraction from cells exposed in the light to [2-^{14}C]acetate in the presence of carbon dioxide had the following distribution in intermediates of the tricarboxylic acid cycle: malate 3·5%, citrate 22·5%, succinate 11·7%, fumarate nil. The values for citrate and succinate are considerably higher than that found in *R. rubrum* at any period of exposure to [^{14}C]acetate up to 4 min. The effect of acetate on $^{14}CO_2$ incorporation in the light was also investigated. After exposure for 3 min. the soluble fractions of *Chlorella pyrenoidosa* were examined. The most striking differences observed were the distribution of radioactivity in citrate and glutamate. Incorporation of $^{14}CO_2$ into citrate was increased 40-fold in the presence of acetate (from 0·1 to 3·9%), and its incorporation into glutamate was increased tenfold (from 0·3 to 3·0%). These findings would be consistent with the formation of glutamate from acetate through intermediates of the tricarboxylic acid cycle in *Chlorella pyrenoidosa*. However, more recent studies suggest that this may not be so. Bassham & Kirk (1960) and Smith,

Bassham & Kirk (1961) have made kinetic studies of the rates of appearance of ^{14}C in individual compounds formed by *Chlorella pyrenoidosa* during steady-state photosynthesis with $^{14}CO_2$. It was found that the rate of labelling of citric acid was far too slow for it to be a precursor of labelled glutamic acid. The rate of labelling of glutamate was 0·7 μ-mole/min., equivalent to 4·5 % of all ^{14}C assimilated from $^{14}CO_2$, whereas the rate of labelling of citrate was only 0·05 μmole/min. When unlabelled acetate was added to a suspension of *Chlorella pyrenoidosa* fixing $^{14}CO_2$ under steady-state conditions there was a large and immediate rise in total amount of glutamate without any significant change in the concentration of [^{14}C]glutamate being caused. The latter effect was interpreted as being due to the existence in *Chlorella pyrenoidosa* of separate metabolic pools of glutamic acid, one derived directly from acetate and one derived from photosynthetic intermediates. The addition of acetate did not cause significant increases in the concentrations of other amino acids. Whether or not *Chlorella pyrenoidosa* contains two pools of glutamic acid, one within and one without the chloroplast, it seems unlikely that the glutamate is derived via intermediates of the tricarboxylic acid cycle.

SUMMARY

1. The time-course of the photo-assimilation of [1-^{14}C]- and [2-^{14}C]-acetate by washed cell suspensions of *Rhodospirillum rubrum* has been studied. Glutamate is one of the most prominent early products in the soluble fraction and its formation from acetate depends on the presence of sodium hydrogen carbonate. Glutamate formation precedes the formation of intermediates of the tricarboxylic acid cycle.

2. The time-course of photo-assimilation of sodium [^{14}C]carbonate has been studied in the presence of hydrogen, and in the presence of acetate in an atmosphere of nitrogen. In the presence of hydrogen, sodium [^{14}C]carbonate is incorporated mainly into phosphate esters, whereas in the presence of acetate radioactivity is found mainly in malate and glutamate.

3. The glutamate obtained after short exposures of washed cell suspensions of *Rhodospirillum rubrum* to [1-^{14}C]acetate, [2-^{14}C]acetate and sodium [^{14}C]carbonate (in the presence of acetate) has been degraded and the isotope distribution indicates a novel mechanism for the biosynthesis of glutamate which cannot involve the tricarboxylic acid cycle directly.

4. Glutamate formation from acetate has also been demonstrated in washed cell suspensions of *Rhodopseudomonas capsulata* and *Rhodopseudomonas spheroides*.

5. The time-course of photo-assimilation of sodium [^{14}C]carbonate in the presence of acetate has been studied in malate-grown *Rhodopseudomonas capsulata* with similar results to those found with *Rhodospirillum rubrum* under comparable conditions.

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A Comparative Study of the Synthesis of Nicotinamide Nucleotides by Erythrocytes of some Vertebrates

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Kornberg (1950) and Rowen & Kornberg (1951) were the first to demonstrate that a partially purified enzyme preparation of rat liver catalysed the synthesis of NAD from nicotinamide, and they postulated ribosylnicotinamide and NMN as intermediates in the scheme of NAD synthesis. An enzyme capable of catalysing the synthesis of NMN from nicotinamide was also demonstrated in human erythrocytes by Leder & Handler (1951). However, the equilibria of the reactions brought about by nicotinamide–adenine dinucleotide pyrophosphorylase and nicotinamide mononucleotide pyrophosphorylase *in vitro* were not favourable for NAD synthesis under physiological conditions (Kornberg 1950; Rowen & Kornberg, 1951). Also, NMN synthesis from nicotinamide and glucose by human erythrocytes *in vitro* proceeded only at an extremely high and unphysiological concentration of nicotinamide (Leder & Handler, 1951).

The observation that human erythrocytes could synthesize NAD at low concentrations of nicotinic acid and that glutamine enhanced this synthesis led Preiss & Handler (1957) to postulate an alternative scheme for NAD synthesis from nicotinic acid which did not involve the formation of free nicotinamide. The investigators also studied, with partially purified enzymes from acetone-dried and powdered human erythrocytes, rat liver and autolysed yeast, the intermediate steps in NAD synthesis, wherein the synthesis started from nicotinic acid and proceeded through the formation of nicotinic acid mononucleotide and nicotinic acid–adenine dinucleotide, followed by amidation at the last stage (Preiss & Handler, 1958*a*, *b*).

In studies on the metabolism of nicotinamide nucleotides, Tulpule (1958) observed that washed rat erythrocytes were unable to synthesize NAD from nicotinamide under similar conditions to those employed by Leder & Handler (1951) in studies on human erythrocytes. An attempt was therefore made to study *in vitro* the synthesis of nicotinamide nucleotides by different mammalian species. The erythrocytes of most other vertebrates differ morphologically from the mammalian erythrocytes in that they are elliptical, nucleated and biconvex. They are also large in size but fewer in number. In the present investigation one avian species and one amphibian species were included to observe the