by the formation of amino acyl-RNA. A method is described for the separation of amino acyl-RNA from enzyme reaction mixtures by chromatography on DEAE-cellulose.

3. The pyrophosphate-exchange reaction catalysed by the synthetases from the protein-body preparation showed an apparent inhibition with added ATP. The pyrophosphate exchange catalysed by the enzymes from the supernatant preparation was almost completely dependent on ATP and stimulated by the addition of ATP. The formation of amino acyl-RNA by enzyme preparations from both the protein-body and supernatant preparations was stimulated by the addition of ATP.

4. An enzyme fraction obtained from the protein-body preparation after treatment with ultrasonic vibration catalysed the incorporation of amino acids into amino acyl-RNA and soluble protein.

5. The localization of enzymes and RNA associated with the initial stages of protein synthesis in the two preparations from wheat endosperm, and the significance of these findings in relation to the independent synthesis of storage and cytoplasmic proteins of wheat endosperm, is discussed.

Grateful acknowledgement is made of the financial support from the Wheat Industry Research Fund. We thank Mr D. Morton and Miss M. Ryder for their able technical assistance.

REFERENCES

Beinert, H., Green, D. E., Hele, P., Hift, H., Korff, R. W. von & Ramakrishnan, C. V. (1953). *J. biol. Chem.* **203**, 35.
Clark, J. M. (1958). *J. biol. Chem.* **233**, 421.
Elliott, W. H. & Coleman, G. (1961). *Biochim. biophys. Acta*, **57**, 236.
Graham, J. S. D., Morton, R. K. & Raison, J. K. (1963). *Aust. J. biol. Sci.* **16**, 375.
Graham, J. S. D., Morton, R. K. & Raison, J. K. (1964). *Aust. J. biol. Sci.* **17**, 102.
Hallowes, K. H. & Winteringham, F. P. W. (1958). *Nature, Lond.*, **181**, 336.
Herberg, R. J. (1960). *Analyt. Chem.* **32**, 42.
Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.
Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A. & Merrill, S. H. (1961). *J. biol. Chem.* **236**, 200.
Holley, R. W. & Goldstein, J. (1959). *J. biol. Chem.* **234**, 1765.
Lee Peng, C. H. (1956). *Biochim. biophys. Acta*, **22**, 42.
Morton, R. K., Palk, B. A. & Raison, J. K. (1964). *Biochem. J.* **91**, 522.
Morton, R. K. & Raison, J. K. (1963). *Nature, Lond.*, **200**, 429.
Morton, R. K. & Raison, J. K. (1964). *Biochem. J.* **91**, 528.
Ofengand, E. J., Dieckmann, M. & Berg, P. (1961). *J. biol. Chem.* **236**, 1741.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.

Biochem. J. (1964), **91**, 546

Photoassimilation of Acetate and the Biosynthesis of Amino Acids by *Chlorobium thiosulphatophilum*

BY D. S. HOARE* AND JANE GIBSON†
Agricultural Research Council Unit for Microbiology, University of Sheffield

(*Received* 12 *September* 1963)

The green sulphur bacteria *Chlorobium limicola* and *Chlorobium thiosulphatophilum*, unlike other photosynthetic bacteria, are both strict anaerobes and strict autotrophs. With the exception of the green organism isolated from the symbiotic aggregate *Chlorochromatium* by Mechsner (1957) and *Chloropseudomonas ethylicum* isolated by Shaposhnikov, Kondratieva & Fedorov (1960), growth of the green sulphur bacteria has not been obtained in media containing organic compounds in place of reduced compounds of sulphur. However, Larsen (1953) found that washed suspensions of *C. thiosulphatophilum* carboxylated propionate to succinate, and Sadler & Stanier (1960) found that *Chlorobium limicola* grew in the presence of low concentrations of some simple organic compounds, added to the standard basal medium for autotrophic growth, and that this resulted in a greater final cell yield. Acetate was the most effective of the organic compounds tested in promoting increased cell yield and chlorophyll formation. It was shown that acetate was assimilated by growing cultures only if both bicarbonate and a source of reducing power such as sulphide were available.

* Present address: Department of Microbiology, University of Texas, Austin, Texas, U.S.A.

† Present address: The Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

Under these conditions the gross distribution of acetate carbon atoms in the cell fractions was virtually the same as that found with bicarbonate carbon atoms. The sulphide requirement for acetate assimilation suggested that *C. limicola* had a very limited ability to oxidize acetate.

The photoassimilation of acetate in resting cell suspensions of the photoheterotroph *Rhodospirillum rubrum* has shown that glutamate is an early product and that it cannot be formed by the conventional pathway involving citrate and other intermediates of the tricarboxylic acid cycle (Hoare, 1963). The investigations of Cutinelli, Ehrensvard, Reio, Saluste & Stjernholm (1951) also showed that acetate was assimilated into a number of amino acids, and the isotope distribution in alanine and aspartic acid was not in accord with that established in *Escherichia coli* and other aerobic micro-organisms.

Investigations were therefore carried out with another closely related species of green sulphur bacterium, *C. thiosulphatophilum* (Larsen, 1952), with the following main objectives: to see whether the observations of Sadler & Stanier (1960) with *C. limicola* also applied to *C. thiosulphatophilum*, to extend the observations to resting cell suspensions, since the bicarbonate requirement for the photoassimilation differed from *Rhodospirillum rubrum* in which acetate is photoassimilated in the absence of bicarbonate to form poly-β-hydroxybutyrate, to establish, in growing cultures, whether acetate was incorporated into any of the amino acids of the cell proteins of *C. thiosulphatophilum* and, if so, to determine whether the acetate was incorporated into the amino acids in a specific manner. The experiments of Cutinelli *et al.* (1951) with *Rhodospirillum rubrum*, those of Tomlinson (1954*a*, *b*) with *Clostridium kluyveri*, and the more recent studies with *Ruminococcus flavefaciens* (Allison, Bryant & Doetsch, 1962; Allison & Bryant, 1963) all indicate that the biosynthesis of some amino acids in anaerobes proceeds by mechanisms which differ from those so thoroughly established in *E. coli* and often assumed to occur in most, if not all, other micro-organisms. A preliminary account of some of the results has been reported (Gibson & Hoare, 1963).

MATERIALS AND METHODS

Growth and maintenance of micro-organisms. Two strains of *Chlorobium thiosulphatophilum* NCIB nos. 8346 and 8327 were obtained from Dr J. Lascelles. One of the strains 8327 (OC) had also been maintained continuously in Sheffield for 8 years but appeared to differ slightly in growth rate from the strain freshly supplied in 1961; this strain was used for most of the experiments. Organisms were maintained in stab cultures of Larsen's thiosulphate medium (Larsen, 1952) kept in the light at 26–29° and were transferred monthly. Liquid cultures were grown in glass-stoppered bottles completely filled with thiosulphate medium with the addition of sodium acetate where indicated. Some irregularities in growth were encountered occasionally when transferring from stab cultures to liquid cultures. Only cultures growing normally and of a bright deep-green colour were used as inocula for growth curves and for manometric experiments. In all cases the chemicals used for making media were A.R. grade and the sodium thiosulphate was recrystallized three times; both thiosulphate and sulphide solutions were made up fresh each time media were prepared. *Rhodospirillum rubrum* strain S1 was grown photosynthetically under semi-anaerobic conditions on 'malate medium' (Hoare, 1963). *Rhodomicrobium vannielii* was grown anaerobically on the medium of Duchow & Douglas (1949) with acetate in place of ethanol. *Nocardia globerula* NCIB no. 6376 was maintained and grown as described by Crawford (1958).

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.

Procedure for growth experiments. Graded amounts of sterile sodium acetate solution were pipetted into duplicate sterile 25 ml. glass-stoppered bottles, which were then completely filled with inoculated thiosulphate medium. The bottles were incubated in a light-cabinet at 26–29° and at intervals one of each pair was opened and a sample removed for the estimation of extinction at 600 mμ with a Unicam SP. 600 spectrophotometer and a blue filter. The sample was then poured back into the bottle, which was topped up with a few drops of fresh medium, stoppered again and returned to the light-cabinet. The highly selective nature of the medium minimizes the risk of contamination. Furthermore, the growths in the duplicate bottles, the extinctions of which were measured at only two points during growth, were in good agreement with the bottles used for determinations of growth curves.

Manometric experiments. Since pronounced losses in photosynthetic activity were produced by washing the cells harvested at all stages of growth the cells were harvested by centrifuging for 5 min. at 15000*g* and were then suspended in a medium (medium A) containing: KH_2PO_4, 0·1%; $MgCl_2,6H_2O$, 0·05%; $CaCl_2$, 0·01%. The medium was gassed with $H_2 + CO_2$ (95:5) for 10 min. and was adjusted to pH 7·0 with N-NaOH. The rate of photosynthesis with thiosulphate as hydrogen donor varied greatly from suspension to suspension, but with H_2 consistently high rates were obtained. Warburg flasks contained 2–4 mg. dry wt. of cells (determined spectrophotometrically from the extinction at 600 mμ and calculated from a calibration curve prepared with a cell suspension of known dry weight) in 1·8 ml. of medium A with 0·2 ml. of 4 N-H_2SO_4 in the side bulb. The acid served both to give a measure of the bound bicarbonate and to inactivate the cells at the end of the experiment. The flasks were gassed with $H_2 + CO_2$ (95:5) and were incubated at 30°. The experiment was started by switching on the lights of the photosynthesis bath at 'zero time'.

Isolation of poly-β-hydroxybutyrate. The procedure of Stanier, Doudoroff, Kunisawa & Contopoulou (1959) was used. The ready isolation of white amorphous material soluble in hot chloroform was taken as presumptive evi-

dence for the presence of poly-β-hydroxybutyrate in *Rhodospirillum rubrum* and in *Rhodomicrobium vannielii*. The procedure of Law & Slepecky (1961) was used in an attempt to determine poly-β-hydroxybutyrate in the indigestible residues from *C. thiosulphatophilum*.

Experiments with isotopes

(1) *Isotope-incorporation experiments with resting cell suspensions.* Experiments were carried out in Warburg flasks as in the manometric experiments described above. The radioactive substrate was added to the cell suspension immediately before assembly of the manometers. At the end of some of the experiments in which radioactive acetate was used, the CO_2 in the reaction vessels was flushed out for 40 min. with CO_2-free air and was trapped in 3·5 ml. of CO_2-free 2 N-NaOH and plated as $BaCO_3$. The radioactivity incorporated into the cells was determined as follows. The cells were first washed twice by suspending in 1% acetic acid (5 ml.) and centrifuging, followed by resuspension in water and centrifuging. The cells (3 mg. dry wt.) were then suspended in water and filtered through Oxoid membrane filters and allowed to dry in air. Radioactivity was then estimated directly with a thin-end-window tube (General Electric Co. Ltd., type 2B2) connected to a Panax D657 Scaler; the membrane filters were held flat with a cardboard 'mask' with a circular hole in the centre and the end-window tube was placed centrally on top of the mask. In some of the earlier experiments the membrane filters were trimmed and stuck to filter-paper disks on metal blocks, in a similar way to that described by Sadler & Stanier (1960), and radioactivity was then measured with a Packard model 200A windowless gas-flow counter connected to a Baird–Atomic model 1035 scaler with 98% 'minimum stench' propane (Shellmex and B.P. Gases Ltd.) as the gas phase.

(2) *Isotope-incorporation experiments with growing cultures.* Larsen's basal thiosulphate medium with the addition of sodium acetate to a final concentration of 2·5 mM was used in completely filled glass-stoppered bottles of about 140 ml. capacity. The bottles were inoculated with a 'healthy' green culture of *C. thiosulphatophilum* grown in liquid medium. The bottles were incubated in a light-cabinet at 26–29° and when growth had started, as observed visually, a sample was removed for the determination of the extinction at 600 mμ and radioactive acetate (50 μc) was added. The cultures were then incubated for 18–24 hr. and the extinction of the cell suspension was measured at the end of the growth period. The cells were harvested by centrifuging for 30 min. at 20000*g* and were washed once by suspending in 25 ml. of water and centrifuging for 30 min. at 20000*g*. The washed cells from two of the experiments (series II and III with [2-^{14}C]acetate and [1-^{14}C]acetate respectively) were fractionated by the procedure of Roberts, Cowie, Abelson, Bolton & Britten (1955). In the first experiment (series I) a slightly modified procedure was used, to examine the radioactive products of the 'water-soluble' fraction by two-dimensional chromatography under conditions similar to those used with *Rhodospirillum rubrum* (Hoare, 1963). The cells were extracted twice with 25 ml. of ethanol–water–acetic acid (750:250:1, by vol.). The extract was evaporated to dryness by rotary evaporation at 30° to reduce the volume to 1–2 ml., followed by freeze-drying. The freeze-dried residue was then suspended in 2 ml. of water, left in a deep-freeze overnight, thawed out and centrifuged. The clear pigment-free supernatant comprised the 'water-soluble fraction', which is presumed to be comparable in composition with the cold 5% trichloroacetic acid fraction obtained by the procedure of Roberts *et al.* (1955). The pigmented residue from the freeze-dried material was dissolved in 10 ml. of ethanol, giving a dark-green solution comparable with the 'alcohol-soluble fraction' of Roberts *et al.* (1955). The residual material was then further fractionated by the procedure of Roberts *et al.* (1955) by successive extractions with ethanol–water–ether (3:1:4, by vol.) and hot 5% (w/v) trichloroacetic acid. The final cell-residue fraction was hydrolysed for 8 hr. in 6 N-HCl by boiling under a reflux condenser. The hydrolysate was evaporated to dryness on a boiling-water bath in a stream of air, the residue was dissolved in 10 ml. of water and samples were taken for total amino nitrogen estimation (Yemm & Cocking, 1954) and for radioactivity determinations. Radioactivity determinations on the various cell fractions were made by plating small samples at infinite thinness on stainless-steel planchets and counting with an end-window tube.

Separation and isolation of amino acids

Since the total amino acid nitrogen in the hydrolysates of the cell residues was comparatively low (160, 250 and 140 μg.atoms in series I, II and III respectively), amino acid separations were carried out on a microscale by paper-chromatographic and paper-electrophoretic techniques. Whatman 3MM papers were used throughout. A primary separation of amino acids was effected by applying samples as a line 20 cm. from the anode end of a 60 cm. strip of paper which was subjected to electrophoresis for 90 min. at 1800 v in a pyridine–acetic acid buffer, pH 3·6 (Dixon, Kauffman & Neurath, 1958). This resolved the mixture into aspartic acid and glutamic acid, which were well separated and moved towards the anode, the 'neutral amino acids', remaining on or close to the line of application, and the basic amino acids, which moved towards the cathode. The basic amino acids were partly resolved: arginine was separated from histidine and lysine. Radioactive bands revealed by radioautography were eluted from the paper strips with water. The basic amino acids were then resolved by descending paper chromatography in butan-2-ol–aq. 3% NH_3 soln. (5:2, v/v) (Hausman, 1952). In this way histidine was separated from lysine. The neutral amino acids were then partially separated by descending paper chromatography for 32–36 hr. in propan-1-ol–water (4:1, v/v) (Kemble & McPherson, 1954). Diaminopimelic acid and phenylalanine were completely separated from other amino acids. Glycine and serine overlapped and in some cases alanine was not completely separated from threonine. These pairs of amino acids were then separated by paper electrophoresis in pyridine–formic acid buffer, pH 2·2 (Richmond & Hartley, 1959). The amino acids were applied as a line 20 cm. from the anode and the paper was subjected to electrophoresis for 90–100 min. at 1800 v.

Other amino acids which overlapped (proline with tyrosine, valine with methionine and leucine with isoleucine) were resolved by descending chromatography for 36–40 hr. in 2-methylpropan-1-ol–ethyl methyl ketone–water (7:5:3, by vol.) (Kemble & McPherson, 1954). Each

isolated amino acid was finally purified by paper chromatography in the butan-1-ol–propionic acid solvent system of Benson *et al.* (1950).

Preparation of amino acid solutions for degradations

All eluates from paper chromatograms and paper-electrophoresis strips were concentrated to dryness *in vacuo* with P_2O_5 as desiccant. The final eluates containing resolved amino acids were made up to a standard volume (2 or 4 ml.) with water to form a 'master solution'. Samples were then taken for amino acid estimation by the procedure of Yemm & Cocking (1954), with corresponding standard amino acid solutions, and for the different degradation procedures.

Radioactive degradation procedures

Combustion of organic compounds was carried out by the procedure of Sakami (1955*a*) with the 'combustion reagents for general use' of Van Slyke, Plazin & Weissiger (1951). Decarboxylation of amino acids with chloramine-T was carried out essentially as described by Kemble & McPherson (1954). Warburg flasks contained the amino acid solution (generally 10 μmoles) in the side bulb, the main compartment contained 1·5 ml. of fresh 10% (w/v) chloramine-T, 0·1 ml. of 10% formaldehyde and 0·5 ml. of M-citrate buffer, pH 2·5. The reaction was carried out at 30°. The evolved CO_2 was passed into 3·5 ml. of 2N-NaOH in a gas bubbler (20 min.), the alkali was made up to 10 ml. with the addition of 1·2 ml. of 0·48M-Na_2CO_3 carrier, and 1 ml. samples were taken for plating as $BaCO_3$ and assay of radioactivity.

Degradation of alanine was carried out by a modification of the procedure of Knight (1962), suggested by H. J. Somerville (personal communication). The decarboxylation with ninhydrin was carried out in the apparatus described by Knight but the apparatus used for trapping acetaldehyde contained 8 ml. of 5M-CrO_3 in 25% (v/v) H_2SO_4 in place of the sodium bisulphite. In this way the acetaldehyde produced as a result of the reaction between alanine and ninhydrin was oxidized directly to acetic acid. When the reaction was complete and the evolved CO_2 had been trapped, the contents of the bead tower containing acetic acid and excess of chromic acid reagent were washed out into a round-bottomed flask and the acetic acid was recovered by steam-distillation in the apparatus of Elsden & Gibson (1954) and purified by ion-exchange chromatography on Amberlite CG 50 (Seki, 1958).

Aspartic acid was degraded by a modification of the procedure of Knight (1962), washed cell suspensions of *Nocardia globerula* being used for the decarboxylation of aspartic acid (Krebs & Bellamy, 1960). The alanine recovered from the reaction products as described by Knight (1962) was then further degraded as described above.

Several procedures were used for the partial and complete degradation of glutamic acid. For the complete and unequivocal degradation of glutamic acid the procedure of Mosbach, Phares & Carson (1951) was used with minor modifications to adapt it to a semi-microscale. The reaction was carried out in a pear-shaped vessel of 50 ml. capacity. The vessel contained 2–3 ml. of glutamate soln. (100 μmoles of glutamate) adjusted to pH 6·5–7·0 with N-NaOH (Koeppe & Hill, 1955). The solution was gassed for 10 min. with a gentle stream of O_2-free N_2. A solution (1–2 ml.) containing 100 μmoles of chloramine-T (freshly prepared) was then added drop by drop. Gassing with O_2-free N_2 was continued throughout the reaction. Five minutes after the addition of the chloramine-T, the temperature was raised to 50° for 40 min. Potassium hydroxide (10%, w/v), 1 ml., freshly redistilled diethylene glycol, 3 ml., and 90% (w/v) hydrazine hydrate, 0·5 ml., were then added, and the mixture was heated under a reflux water condenser for 75 min. The condenser was removed and the solution heated to remove water. An air condenser was then attached and the temperature of the reaction mixture was slowly raised to 160° and maintained between 160° and 170° for 75 min. The reaction mixture on cooling was transferred to a Kutscher–Steudel extractor and extracted for 2 hr. with peroxide-free ether (100 ml.). The ether phase was discarded and the aqueous phase was acidified and steam-distilled from the lactic acid apparatus of Elsden & Gibson (1954). The distillate (200 ml.) was titrated with standard alkali, concentrated to dryness and the butyric acid recovered by chromatography on Amberlite CG 50 (Seki, 1958). Total acid recovered in the steam-distillate was about 100 μmoles but consisted of a mixture of acetate and butyrate. The recovery of butyrate after ion-exchange chromatography was 30–50 μmoles. The butyrate was then degraded stepwise by standard procedures (Sakami, 1955*b*).

The terminal carboxyl group (C-5) was removed by a direct Schmidt reaction on glutamic acid. This procedure was used by Cutinelli *et al.* (1951). The degradation of glutamate to succinate, followed by the decarboxylation of succinate to propionate, was carried out by the microscale procedure of Hoare (1963).

Phenylalanine was partially degraded to benzoic acid by the procedure of Gilvarg & Bloch (1952) with minor modifications. The reaction was carried out on a semi-microscale. A 150 ml. round-bottomed flask with two necks contained phenylalanine (1 m-mole) dissolved in 25% (w/v) H_2SO_4 (15 ml.). The vessel was immersed in an ice bath and finely pulverized $K_2Cr_2O_7$ (4 g.) was added. A reflux water condenser was attached and a gassing inlet tube was inserted in the other neck. A tube of CaO–NaOH was attached to the gassing tube and the top of the condenser was connected to a trap containing 1% (w/v) of 2,4-dinitrophenylhydrazine in 2N-HCl (5 ml.). The trap retained small amounts of benzaldehyde that were formed during the oxidation of the phenylalanine. The apparatus was then flushed with CO_2-free N_2 for 30 min. A bubbler containing CO_2-free 2N-NaOH (5 ml.) was then attached to the assembly and the reaction vessel was immersed in a water bath. Gas (CO_2-free N_2) was passed through the reaction vessel at a slow and steady rate (2–3 bubbles/sec.) throughout the reaction. The temperature of the water bath was raised slowly to boiling point and the vessel was shaken periodically. Oxidation generally started before the water in the bath reached boiling point. After 2 hr. at 100° the reaction vessel was removed from the bath and CO_2-free N_2 was bubbled through for a further 30 min. Yields of CO_2 were invariably greater than theoretical and although samples were plated and counted the results are considered to be only qualitatively valid in comparisons of incorporation patterns from [1-^{14}C]- and [2-^{14}C]-acetate.

Benzoic acid was recovered from the reaction mixture by three extractions with 50 ml. of light petroleum (b.p.

30–40°); the extracts were filtered and distilled to dryness from a water bath. The residue of benzoic acid was recrystallized from hot water. Samples of benzoic acid were taken for total combustion and for decarboxylation by the Schmidt reaction. The Schmidt reaction was carried out on 600 μmoles of benzoic acid under conditions identical with those used for the decarboxylation of fatty acids and yields of CO_2 were 95–100%. Aniline was recovered from the reaction products by ether extraction from alkaline solution. Ether was removed by evaporation in a stream of air and the oily drop of aniline was acetylated by the addition of acetic anhydride (0·2 ml.) and a drop of conc. H_2SO_4. The mixture was left overnight at 37°. About 3 ml. of water and a small amount of decolorizing charcoal were added and the mixture was boiled and filtered hot. Acetanilide crystallized from the filtrate and was dried and subjected to combustion.

RESULTS

Growth of Chlorobium thiosulphatophilum *in the presence of acetate.* Preliminary experiments showed that the addition of 5 mM-sodium acetate to the basal thiosulphate medium increased the cell yield of *C. thiosulphatophilum* in a similar way to that found with *C. limicola* by Sadler & Stanier (1960). The increase in cell yield with *C. thiosulphatophilum* strain 8346 was less than that with the two cultures of strain 8327 (Table 1). Growth curves were similar for all strains and showed that acetate (final concn. 1–5 mM) increased the rate of growth as well as the final cell yield (Fig. 1). Acetate concentrations greater than 10 mM reduced first the rate of growth and finally the cell yield (below yields of cultures without acetate).

Influence of acetate on photosynthesis by resting cell suspensions. In manometric experiments with hydrogen gas as a source of reducing power, the rate of gas uptake was not affected by the presence of acetate (5–10 mM) whether the cells were grown on thiosulphate or thiosulphate with acetate. The manometric technique may not be sufficiently sensitive to show any influence of acetate on carbon dioxide uptake. In experiments with $Na_2{}^{14}CO_3$ there was a progressive fixation of radioactivity by resting cell suspensions in the presence and absence of acetate (Table 2). Resting cell suspensions grown with or without acetate also assimilated sodium [1-^{14}C]acetate (Table 3). The presence of light, a source of reducing power and of bicarbonate were essential. Radioactivity was not

Table 1. *Effect of acetate on growth of* Chlorobium thiosulphatophilum

Sodium acetate was added to the basal thiosulphate medium to the final concentrations indicated. Media in 25 ml. glass-stoppered bottles were inoculated and growth was measured by determining the extinction at 600 mμ after all cultures had reached maximum growth. The final extinctions remained constant (±0·02) for at least 24 hr. after maximum growth had been reached.

Concn. of acetate (mM)	Final cell yield (E at 600 mμ)		
	Strain 8327 (OC)	Strain 8346	Strain 8327
0	0·638	0·680	—
1	0·780	—	—
2	0·960	0·880	—
5	1·270	0·895	—
10	1·130	0·905	—
15	0·980	—	—
0	0·750	0·660	0·760
2	1·020	0·825	0·920
5	1·190	0·925	1·280
10	1·240	0·880	1·230

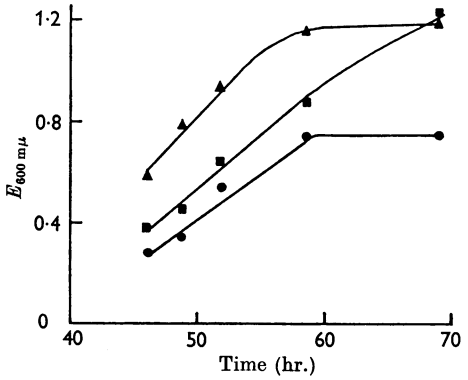


Fig. 1. Effect of acetate concentration on the growth of *C. thiosulphatophilum* 8327 (OC). Basal thiosulphate medium (25 ml.), with acetate added to the concentrations indicated, was inoculated and illuminated at 26–29°; growth was followed by measuring extinction at 600 mμ. ●, No acetate present; ▲, 5 mM-acetate; ■, 10 mM-acetate.

Table 2. *Effect of acetate on the assimilation of sodium* [^{14}C]*carbonate by resting cell suspensions of* Chlorobium thiosulphatophilum

Warburg flasks contained 1 ml. of cell suspension (3 mg. dry wt./ml.), 0·8 ml. of medium A and 10 μl. of $Na_2{}^{14}CO_3$ (10 μc, 10 μmoles). Gas phase, H_2. Temp., 30°. When acetate was present it was added in 10 μl. of 0·5 M-sodium acetate. Reaction was initiated by illuminating the system. Organisms were filtered, washed and counted on Oxoid membrane filters as described in the Materials and Methods section.

Incubation time (min.)	$10^{-3} \times Na_2{}^{14}CO_3$ incorporated (counts/min.)	
	Acetate absent	Acetate present
0	Not determined	0·05
5	1·34	0·37
10	5·20	0·91
20	7·25	3·1
40	24·1	17·5
60	31·2	28·1
60 (dark)	0·06	Not determined

detected in the carbon dioxide at the end of manometric experiments with either [1-^{14}C]- or [2-^{14}C]-acetate.

Products of the photoassimilation of acetate by resting cell suspensions. Stanier *et al.* (1959) found that washed suspensions of *Rhodospirillum rubrum* assimilated acetate in the absence of carbon dioxide to form poly-β-hydroxybutyrate. Since carbon dioxide was essential for the assimilation of acetate by resting cell suspensions of *C. thiosulphatophilum* it seemed possible that the latter could not form poly-β-hydroxybutyrate. Accordingly cells of *C. thiosulphatophilum* grown on thiosulphate medium and on acetate with thiosulphate were examined for poly-β-hydroxybutyrate by the procedure of Stanier *et al.* (1959). No polymer could be obtained, or detected by the method of Law & Slepecky (1961), in 250 mg. dry wt. of cells, although good yields were obtained under identical conditions with *Rhodomicrobium vannielii* grown on the medium of Duchow & Douglas (1949) with acetate in place of ethanol, and with *Rhodospirillum rubrum* grown on 'malate medium' (although polysaccharide is the major storage product in *Rhodospirillum rubrum* under these conditions; Stanier *et al.* 1959).

The distribution of radioactivity incorporated into resting cell suspensions was examined in the following way. Cells grown on thiosulphate with 5 mM-acetate were harvested and suspended (1·8 mg. dry wt./ml.) in 25 ml. of medium A. The suspension was transferred to a 'reaction vessel for short-exposure experiments' (Knight, 1962), was stirred with a magnetic stirrer and gassed with hydrogen + carbon dioxide (95:5), and was illuminated with a 500 w photoflood lamp. Sodium [1-^{14}C]acetate (25 μc, 75 μmoles) was added and after 30 min. incubation the suspension was centrifuged, washed with 25 ml. of water, centrifuged again and fractionated by the procedure of Roberts *et al.* (1955). Radioactivity was found in all the cell fractions and the distribution was similar to that found subsequently with growing cultures (Table 4).

Acetate assimilation by growing cultures and its distribution in different cell fractions. Cultures were grown on the basal medium with acetate added to a final concentration of 2·5 mM. After several transfers on this medium, completely filled glass-stoppered bottles were inoculated and incubated in an illuminated cabinet at 25–30°. When growth had started, sodium [1-^{14}C]acetate or sodium [2-^{14}C]-acetate was added and, after a period of 18–24 hr. when the culture gave an extinction of approx. 1·0, the culture was removed from the light-cabinet. A sample of the culture was assayed to determine the total radioactivity in the system. The cells were then removed by centrifuging for 30 min. at 20000*g* and a sample of the cell-free supernatant was assayed for radioactivity. The total counts in the cells were then determined by difference. The data and results for three separate experiments are given in Table 4. The results show that 80–90 % of the radioactivity in the system was incorporated into the cells under the above growth conditions. An attempt was made in one of the experiments (series III, with sodium [1-^{14}C]acetate) to determine the total radioactivity in the bound bicarbonate (carbon dioxide) in the system as this would give an index of the extent of acetate oxidation by growing cultures. A portion (100 ml.) of the freshly harvested cell-free culture medium was acidified in a closed system and the gas passed into 3·5 ml. of 2N-sodium hydroxide; the alkali was diluted to 10 ml. and samples were taken for

Table 3. *Assimilation of sodium* [1-^{14}C]*acetate by resting cell suspensions from cultures grown in the presence and absence of acetate*

Warburg flasks containing cell suspension (3·5 mg. dry wt./ml.), 1 ml., medium A, 0·8 ml., 0·5 M-sodium acetate, 10 μl., and sodium [1-^{14}C]acetate (10 μmoles, 32300 counts/min.), 10 μl., with 10 % trichloroacetic acid, 0·2 ml., in the side bulb. Gas phase, $H_2 + CO_2$ (95:5) or H_2; in the latter case the centre wells of the Warburg flasks contained 10 % (w/v) NaOH, 0·2 ml. The flasks were equilibrated for 10 min. at 30° in the dark. The reaction was started by turning on the light and was stopped by tipping in the contents of the side bulbs. The cells were washed and assayed for radioactivity as described in the Materials and Methods section. Radioactivity measurements were with a gas-flow counter, and are expressed as counts/min.

Incubation time (min.)	Grown with acetate		Grown without acetate	
	Gas phase $H_2 + CO_2$	Gas phase H_2	Gas phase $H_2 + CO_2$	Gas phase H_2
0	45	Not determined	38	Not determined
5	413	8	400	35
10	1063	32	—	15
20	2850	43	2766	25
40	7468	17	7154	34
60	11527	25	8384	—
60 (dark control)	137	Not determined	—	Not determined

recovery of carbon dioxide (2100 μmoles) and for radioactivity assay by plating as barium carbonate (total radioactivity $1{\cdot}7 \times 10^4$ counts/min. corrected for self-absorption). This accounts for less than 0·1 % of the total radioactivity in the system and therefore confirms that *C. thiosulphatophilum* has a very limited capacity to oxidize acetate.

The distribution of radioactivity in different cell fractions was investigated on the freshly harvested cells. These were washed once by suspending in 25 ml. of water and centrifuging for 30 min. at 20000*g*; this produced a clear pale-yellow supernatant. The washed cells were then fractionated by the procedure of Roberts *et al.* (1955) and the different cell fractions were assayed for radioactivity (Table 4). The total radioactivity in the cells estimated from the sum of that found in the different cell fractions was in all cases less than that estimated by difference between the activity of the whole culture and the culture supernatant. The reason for this discrepancy is not clear but may be due in part to a loss of radioactivity in the cell washings or there may have been some loss from the 'cell-residue' fraction during acid hydrolysis, since radioactivity measurements were made on samples of the hydrolysed material: the cell-wall material might be expected to contain acetyl groups (see review by Perkins, 1963), which would be lost during the acid hydrolysis. The distribution of radioactivity in the cell fractions is similar to that found by Sadler & Stanier (1960) with *Chlorobium limicola*. The 'cell residue', containing mainly proteins and cell-wall material, contained the highest proportion of the activity from both [1-^{14}C]- and [2-^{14}C]-acetate.

Incorporation of acetate carbon atoms into the amino acids of the cell proteins. The 'cell-residue' fractions were hydrolysed as described in the Materials and Methods section. Suitable samples of the hydrolysed material were then taken for assay of radioactivity, total amino acid content and for two-dimensional chromatography followed by radioautography. In all three series of experiments it was clear that acetate was incorporated into most of the amino acids. Individual amino acids were isolated as described in the Materials and Methods section. Samples of each amino acid were then taken for amino acid estimation and for total combustion to determine the approximate specific activities of the isolated amino acids (Table 5).

*Decarboxylation of isolated amino acids by reaction with chloramine-*T. To determine whether acetate is incorporated into the amino acids in a specific manner it would be desirable to degrade each of the isolated radioactive amino acids completely and unequivocally. Since such degradations have not yet been devised for all the common amino acids, this approach was limited to a few amino acids for which unequivocal degradations had been established. A good indication of the incorporation of acetate carbon atoms into all the amino acids could be obtained very simply by decarboxylation with chloramine-T (Kemble & McPherson, 1954). Under these conditions it was found that, in addition to the neutral amino acids studied by Kemble & McPherson (1954), the basic amino acids arginine,

Table 4. *Acetate assimilation by growing cultures and its intracellular distribution*

Stoppered bottles containing Larsen's basal medium (about 130 ml.) with 2·5 M-sodium acetate were inoculated and incubated in the light at 25°. Radioactive acetate (50 μc) was added when growth was established. Cell density (from *E* at 600 mμ) was measured when radioactive acetate was added (initial cell density) and after 24 hr. (final cell density) when cells were harvested and fractionated. The 'total' figures represent the sum of counts in all cell fractions and do not correspond to the amount estimated by difference between the whole culture and the culture supernatant. For series I the extraction procedure was slightly different (see text). Radioactivity measurements are expressed as counts/min.

	Series I Sodium [1-^{14}C]acetate	Series II Sodium [2-^{14}C]acetate	Series III Sodium [1-^{14}C]acetate
Radioactive compound added (μmoles)	4·14	5·0	4·14
Initial cell density	0·375	0·43	0·10
Final cell density	0·920	1·02	1·08
10^{-6} × Total radioactivity in culture	10·3	11·7	13·4
10^{-6} × Total radioactivity in supernatant	1·8	1·02	1·8
10^{-6} × Total radioactivity in cells (by difference)	8·5	10·68	11·6
% of ^{14}C incorporated into cells	83	91	87
Distribution of incorporated radioactivity (× 10^{-5}) in:			
(1) Cold 5% trichloroacetic acid-soluble fraction	4·2 (5·6 %)	2·7 (4·2 %)	3·8 (5·1 %)
(2) Ethanol-soluble fraction	15·0 (20·0 %)	11·7 (18·2 %)	25·7 (34·6 %)
(3) Ethanol–ether-soluble fraction	2·9 (3·9 %)	2·2 (3·4 %)	4·3 (5·8 %)
(4) Hot-5% trichloroacetic acid-soluble fraction	15·1 (20·0 %)	8·6 (13·4 %)	13·6 (18·3 %)
(5) Cell residue ('protein')	37·9 (50·6 %)	38·8 (61·0 %)	26·9 (36·2 %)
10^5 × Total	75·1 (100 %)	64·0 (100 %)	74·3 (100 %)

histidine and lysine were quantitatively decarboxylated, glutamic acid was decarboxylated at the α-carboxyl (C-1) position, and both aspartic acid and diaminopimelic acid were decarboxylated at both the α-carboxyl and the terminal carboxyl position. The results are given in Table 6.

Degradations of alanine, aspartic acid, glutamic acid and phenylalanine. Alanine, aspartic acid and glutamic acid formed from both [1-^{14}C]acetate and [2-^{14}C]acetate were completely and unequivocally degraded by standard procedures. The results are given in Tables 7–10.

Table 5. *Specific activities of isolated amino acids from 'cell-residue' hydrolysates of cells grown in the presence of sodium* [1-^{14}C]*acetate and sodium* [2-^{14}C]*acetate*

'Cell-residue' fractions from cultures treated as described in Table 4 were hydrolysed and the amino acids, after isolation (see the Materials and Methods section), were made up to a known volume and samples were taken for amino acid estimation (Yemm & Cocking, 1954) and for total combustion and radioactivity determinations. Values recorded are the totals recovered for each amino acid.

	With sodium [1-^{14}C]acetate			With sodium [2-^{14}C]acetate		
Amino acid	Amino N (μmoles)	10^{-3} × Total activity (counts/min.)	10^{-3} × Sp. activity (counts/min./μmole)	Amino N (μmoles)	10^{-3} × Total activity (counts/min.)	10^{-3} × Sp. activity (counts/min./μmole)
Aspartic acid	18·5	630	34·2	12·6	119·5	17·4
Glutamic acid	19·2	787	40·8	8·4	142·6	17·0
Diaminopimelic acid	1·2	64	53·3	0·9	36·5	40·0
Glycine	5·3	249	47·1	5·5	7·6	1·4
Serine	4·1	229	56·2	6·3	206	32·6
Alanine	11·3	447	39·6	21·6	285	13·2
Threonine	3·5	122	34·8	3·9	95	24·3
Proline	5·5	193	35·1	2·0	87	43·5
Valine	5·1	483	96·0	5·2	358	69·0
Tyrosine	1·3	248	185·0	1·9	200	104·0
Phenylalanine	2·6	247	93·7	2·3	346	150·0
Isoleucine	3·4	352	105·0	6·4	380	59·0
Leucine	4·9	447	91·5	4·3	497	115·0
Arginine	4·0	167	42·0	2·2	60·4	29·7
Histidine	0·8	130	174·0	1·6	150	110·0
Lysine	5·3	563	106·0	4·4	285	64·7

Table 6. *Decarboxylation of amino acids formed from sodium* [1-^{14}C]*acetate and sodium* [2-^{14}C]*acetate*

Samples of radioactive amino acids with the addition of carrier (10 μmoles) were decarboxylated in Warburg flasks according to the procedure of Kemble & McPherson (1954). All counts are corrected for self-absorption. Recoveries of CO_2 in the chloramine-T reactions were 98–103% of theoretical.

	Radioactivity (counts/min.)			
	With sodium [1-^{14}C]acetate		With sodium [2-^{14}C]acetate	
Amino acid	10^{-3} × Total activity in sample	10^{-3} × Activity in CO_2 recovered	10^{-3} × Total activity in sample	10^{-3} × Activity in CO_2 recovered
Aspartic acid	63·0	0·1	10·7	0·1
Glutamic acid	138·5	0·3	13·9	< 0·1
Diaminopimelic acid	8·0	0·2	9·1	< 0·1
Glycine	31·1	1·1	—	Not estimated
Serine	28·7	0·8	51·5	0·6
Alanine	55·8	0·7	35·6	0·1
Threonine	15·3	0·5	23·7	< 0·1
Proline	24·1	< 0·1	21·7	< 0·1
Valine	60·4	0·2	89·5	0·1
Tyrosine	31·1	0·5	50·1	0·8
Phenylalanine	30·9	0·7	86·5	0·2
Isoleucine	44·0	11·2	95·0	< 0·1
Leucine	55·8	15·3	124·2	< 0·1
Arginine	20·8	< 0·1	8·3	< 0·1
Histidine	17·0	0·5	21·6	3·4
Lysine	70·5	0·2	35·6	0·3

Table 7. *Degradation of alanine from cells grown in the presence of sodium* [1-^{14}C]*acetate and sodium* [2-^{14}C]*acetate*

Samples of a solution of alanine derived from [1-^{14}C]- and [2-C]^{14}-acetate diluted with added carrier L-alanine were degraded. Additional carrier acetate was added for the Schmidt-reaction step. All counts are as $BaCO_3$ and are corrected for self-absorption. Reaction steps: 1, ninhydrin reaction; 2, Schmidt reaction.

Series	Source of ^{14}C	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total counts	Sp. activity (counts/min./ μmole)	Product (μmoles)	Total counts	Corrected* sp. activity (counts/min./ μmole)	Origin of carbon atoms
I	[1-^{14}C]Acetate	1	Alanine (2·8)	100	111650	1084	CO_2 (100)	600	6·0	C-1
							Acetate (67)	71700	1070	
		2	Acetate (61)	700	64500	84·4	CO_2 (537)	38600	925	C-2
							$CH_3 \cdot NH_2$ (700)	5330	95	C-3
II	[2-^{14}C]Acetate	1	Alanine (4·0)	200	71250	349	CO_2 (177)	225	1·6	C-1
							Acetate (130)	42800	329	
		2	Acetate (117)	800	38520	42·0	CO_2 (703)	650	7	C-2
							$CH_3 \cdot NH_2$ (762)	34150	349	C-3

* Corrected for additions of carrier.

Table 8. *Degradation of aspartic acid from cells grown in the presence of sodium* [1-^{14}C]*acetate and sodium* [2-^{14}C]*acetate*

Samples of a solution of aspartic acid derived from [1-C^{14}]- and [2-^{14}C]-acetate diluted with added carrier L-aspartic acid were degraded. Additional carrier alanine was added for the ninhydrin reaction and further carrier acetate was added for the Schmidt reaction. Reaction steps: 1, decarboxylation with a washed-cell suspension of *Nocardia globerula*; 2, ninhydrin reaction; 3, Schmidt reaction.

Series	Source of ^{14}C	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total counts	Sp. activity (counts/min./ μmole)	Product (μmoles)	Total counts	Corrected* sp. activity (counts/min./ μmole)	Origin of carbon atoms
I	[1-^{14}C]Acetate	1	Aspartic acid (40·7)	—	171200	4200	CO_2 (33·5)	150	4·5	C-4
							Alanine (40)	139400	3485	
		2	Alanine (35·6)	160	124066	634	CO_2 (160)	411	14·0	C-1
							Acetate (145)	90540	3434	
		3	Acetate (130·5)	800	74970	80·6	CO_2 (810)	61530	2960	C-2
							$CH_3 \cdot NH_2$ (523)	8840	654	C-3
II	[2-^{14}C]Acetate	1	Aspartic acid (22·4)	—	45500	2030	CO_2 (22)	120	5·5	
							Alanine (20)	34400	1720	C-4
		2	Alanine (17·9)	200	29788	138	CO_2 (200)	140	8·5	C-1
							Acetate (167)	20170	1452	
		3	Acetate (150)	800	18153	19·1	CO_2 (805)	4260	408	C-2
							$CH_3 \cdot NH_2$ (766)	10200	1024	C-3

* Corrected for additions of carrier.

Table 9. *Degradation of glutamic acid from cells grown in the presence of sodium* [2-^{14}C]*acetate*

Samples of a solution of glutamic acid derived from [2-^{14}C]acetate diluted with added carrier L-glutamic acid were degraded by a modification of the procedure of Mosbach *et al.* (1951). Additional carrier was added for the Schmidt reactions (steps 2–4).

Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total counts	Sp. activity (counts/min./μmole)	Product (μmoles)	Total counts	Corrected sp. activity (counts/min./μmole)	Origin of carbon atoms
1	Glutamate (21·7)	94	13900	120	Butyrate (42)	4800	562	
2	Butyrate (40)	950	4560	4·6	CO_2 (560)	Nil	Nil	C-5
					Propionate (481)	2370	590	
3	Propionate (433)	500	2133	2·28	CO_2 (600)	600	260	C-4
					Acetate (609)	615	263	
4	Acetate (540)	500	553	0·53	CO_2 (735)	321	218	C-3
					$CH_3 \cdot NH_2$ (960)	Nil	Nil	C-2
5	Glutamate (21·7)	—	13900	642	CO_2 (23·8)	Nil	Nil	C-1

Table 10. *Degradation of glutamic acid derived from sodium* [1-^{14}C]*acetate*

Glutamic acid was degraded by the procedure of Mosbach *et al.* (1951): (A) series I sample; (B) series III sample; (C) series I sample degraded via succinate (Hoare, 1963); (D) direct Schmidt reaction on series I sample. Specific activities of glutamate samples before addition of carrier were 5700 counts/min./μmole for series I and 4425 counts/min./μmole for series III.

	Reactant (μmoles)	Carrier added (μmoles)	Total counts	Sp. activity (counts/min./μmole)	Product (μmoles)	Total counts	Corrected* sp. activity (counts/min./μmole)	Origin of carbon atoms
(A)	Glutamate (24)	80	138500	1330	Butyrate (42)	52400	5420	
	Butyrate (40)	900	49780	50·6	CO_2 (500)	2220	450	C-5
					Propionate (270)	9220	3480	
	Propionate (253)	750	8280	8·28	CO_2 (472)	1620	1374	C-4
					Acetate (489)	2200	1830	
	Acetate (440)	400	1980	2·36	CO_2 (780)	1320	1310	C-3
					$CH_3 \cdot NH_2$ (810)	205	194	C-2
(B)	Glutamate (34)	165	150110	755	Butyrate (74)	64200	4340	
	Butyrate (70)	800	60990	70·1	CO_2 (900)	3014	243	C-5
					Propionate (547)	27300	3090	
	Propionate (492)	300	24570	31·2	CO_2 (800)	12963	1901	C-4
					Acetate (100)	Not determined		
	Acetate (100)	300	Not determined		CO_2 (400)	1568	1834	C-3
					$CH_3 \cdot NH_2$ (378)	67	83 (Nil)	C-2
(C)	Glutamate (24)	—	138500	5700	CO_2 (24)	300	12	C-1
					Succinate (19)	107778	5670	
	Succinate (16)	—	91000	5670	CO_2 (16)	8880	554	½(C-2 + C-5)
					Propionate (11·7)	22800	1949	
	Propionate (10·5)	900	20520	22·6	CO_2 (900)	2950	295	½(C-2 + C-5)
					Ethylamine (750)	10280	1200	
(D)	Glutamate (12	900	69250	76	CO_2 (857)	15730	1320	C-5

* Corrected for all additions of carrier.

Table 11. *Degradation of phenylalanine from cells grown in the presence of sodium* [1-^{14}C]*acetate and sodium* [2-^{14}C]*acetate*

Samples of a solution of phenylalanine derived from [1-^{14}C]- or [2-^{14}C]-acetate diluted with added carrier L-phenylalanine were degraded as described in the Materials and Methods section. Specific activities of CO_2 produced by the oxidation of phenylalanine were not calculated, since this includes an indeterminate amount derived from ring fission.

Source of ^{14}C	Reaction step	Reactant (μmoles)	Carrier added (μmoles)	Total counts	Sp. activity (counts/min./μmole)	Product (μmoles)	Total counts	Corrected* sp. activity (counts/min./μmole)
[1-^{14}C]Acetate	1	Phenylalanine (1·88)	2000	176000	88	CO_2 (8940)	83000	—
						Benzoic acid (474)	22600	47·6
	2	Benzoic acid (426)	180	20340	33·5	CO_2 (570)	640	1·6
						Acetanilide (126)	4270	47·0
[2-^{14}C]Acetate	1	Phenylalanine (1·15)	1000	154000	154	CO_2 (4690)	66500	—
						Benzoic acid (201)	29600	148
	2	Benzoic acid (180)	420	26640	44·4	CO_2 (596)	7270	40·4
						Acetanilide (72·5)	1920	88·2

* Corrected for additions of carrier.

Phenylalanine was partially degraded to determine whether the carbon atoms of acetate were incorporated into the aromatic ring system; the results are given in Table 11.

DISCUSSION

Although *C. thiosulphatophilum* is a strict autotroph, the cell yield can be increased by the addition of low concentrations of acetate to the culture medium and the growth response is similar to that found with the closely related *C. limicola* by Sadler & Stanier (1960). Acetate is assimilated by resting cell suspensions of *C. thiosulphatophilum* provided that bicarbonate and a source of reducing power (hydrogen gas) are present; this striking difference from *Rhodospirillum rubrum*, which assimilates acetate in the absence of bicarbonate to form poly-β-hydroxybutyrate, appears to be due to the inability of *C. thiosulphatophilum* to form this polymer. This may be a unique feature of the autotrophic green sulphur bacteria, since poly-β-hydroxybutyrate has been found in representative species of the other major groups of photosynthetic bacteria: namely *Chromatium okenii* (Schlegel, 1962; Schlegel & Gottschalk, 1962) and in *Rhodomicrobium vannielii*.

As with *C. limicola* it has been found that acetate is incorporated into all the major cell fractions of *C. thiosulphatophilum* (Table 4).

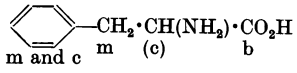
When *C. thiosulphatophilum* is grown in the presence of radioactive acetate, the acetate is incorporated into most of the amino acids of the cell proteins (Table 5). No clear indication of metabolic interrelationships between the different amino acids can be deduced from the data in Table 6. This is probably attributable to the fact that the carbon-atom skeletons of the amino acids are built up mainly from bicarbonate carbon atoms by autotrophic assimilation and acetate carbon atoms simply supplement this. Thus radioactivity incorporated into the isolated amino acids may represent the sum of two independent processes: autotrophic assimilation, presumably by the route established in green algae since all the enzymes of this mechanism have been shown to be present in *C. thiosulphatophilum* (Smillie, Rigopoulos & Kelly, 1962), and the assimilation of acetate by mechanisms not yet elucidated. The exceedingly low specific activity of glycine from cells grown in the presence of sodium [2-^{14}C]acetate suggests that there is very little incorporation of the methyl group (C-2) of acetate into glycine. The comparatively high specific activities of the aromatic amino acids suggests that acetate carbon atoms are incorporated into the aromatic ring and this is confirmed by the results of the partial degradation of phenylalanine (Table 11). The results of the

decarboxylation of the amino acids (Table 6) show that, with the exception of leucine and isoleucine derived from [1-^{14}C]acetate, the carboxyl groups of all the amino acids were virtually devoid of radioactivity. The small amounts of radioactivity found might be attributed to the very limited oxidation of acetate to carbon dioxide, which could then be assimilated into the carboxyl groups of the amino acids. The results indicate that acetate is assimilated into the amino acids in a direct and possibly specific manner which excludes any indirect mechanism involving the preliminary oxidation of acetate to carbon dioxide. The incorporation of [1-^{14}C]acetate into the carboxyl group of leucine would be consistent with the pathway for leucine biosynthesis established in *Salmonella typhimurium* (Jungwirth & Umbarger, 1962) and in the yeast *Torulopsis utilis* (Strassman, Locke, Thomas & Weinhouse, 1955; Strassman & Ceci, 1962). The incorporation of [1-^{14}C]acetate into the carboxyl group of isoleucine is not in accord with the well-established pathway for the biosynthesis of this amino acid in *E. coli* (for review see Umbarger & Davis, 1962), and suggests that a novel mechanism for isoleucine biosynthesis may occur in *C. thiosulphatophilum*. The complete degradations of alanine and aspartic acid (Tables 7 and 8) further establish that acetate is incorporated into these amino acids in a specific manner (summarized in Table 12). The isotope distribution is different from that established in a number of aerobic micro-organisms (see review by Ehrensvard, 1955) but is the same as that found in *Rhodospirillum rubrum* (Cutinelli *et al.* 1951) and in *Clostridium kluyveri* (Tomlinson, 1954*a*). The isotope distribution suggests, at least superficially, that condensation between a C_1 unit and a C_2 unit may occur to give pyruvate, from which alanine may be derived; a carboxylation of pyruvate or of a derivative such as phosphoenolpyruvate to yield oxaloacetate could then account for the isotope distribution in aspartic acid if this were derived from oxaloacetate. The occurrence of these isotope distributions in three anaerobic bacteria suggests that a common mechanism may be involved in the biosynthesis of alanine and aspartic acid in a number of anaerobes. It is clear that in aerobes the tricarboxylic acid cycle, which is of importance as a source of hydrogen donors which provide ATP through an oxidative electron-transport system, is also an important source of intermediates for the synthesis of amino acids and other cell materials. Thus α-oxoglutarate (at least in *E. coli*) derived from acetate via citrate gives rise to glutamate, which is a precursor of proline and arginine; oxaloacetate, which can be derived by the carboxylation of phosphoenolpyruvate, may also be considered to be derived via the tricarboxylic acid cycle or the glyoxylate cycle or both in situations where acetate is being assimilated; as in the experiments by Roberts *et al.* (1955), in which the incorporation of radioactive acetate into amino acids was studied in *E. coli* grown on glucose as major carbon source. Oxaloacetate then gives rise to aspartate, which is a precursor of threonine, isoleucine, lysine and methionine. It is, however, not well established that the tricarboxylic acid cycle functions in strict anaerobes. It has been found, for example, with *Pasteurella pestis* that the activities of many of the enzymes of the tricarboxylic acid cycle are very much less in anaerobic cultures than in aerobic cultures (Englesberg & Levy, 1955).

The degradation data for glutamic acid are not so clear, since the carboxyl group of acetate appears to be distributed between the C-3, C-4 and C-5 positions, although the methyl group of acetate is incorporated fairly specifically into the C-3 and C-4 positions. The latter is in accord with the isotope distribution in glutamate formed after short exposure of washed-cell suspensions of *Rhodospirillum rubrum* to sodium [2-^{14}C]acetate (Hoare, 1963). The glutamate degradations (Tables 9 and 10), especially of the two samples (series I and series III) derived from [1-^{14}C]acetate, cannot be considered entirely satisfactory since the recoveries were poor. Nevertheless it is clear that the α-carboxyl group (C-1) of glutamate is derived from bicarbonate and that the major part of the radioactivity from sodium [1-^{14}C]acetate is incorporated into C-3 and C-4 together with a significant amount in the terminal carboxyl group (C-5). This distribution differs from that found in other micro-organisms, including the comparatively closely related photosynthetic bacterium *R. rubrum*. The results with glutamate should be considered with reservation until the experiments have been

Table 12. *Summary of isotope distributions in amino acids*

m, Carbon atoms derived from [2-^{14}C]acetate; c, carbon atoms derived from [1-^{14}C]acetate; b, carbon atoms presumed to be derived from bicarbonate since insignificant amounts of [1-^{14}C]- or [2-^{14}C]-acetate were incorporated.

Alanine	$CH_3\cdot CH(NH_2)\cdot CO_2H$ m c b
Aspartic acid	$HO_2C\cdot CH(NH_2)\cdot CH_2\cdot CO_2H$ b c m b
Glutamic acid	$HO_2C\cdot CH(NH_2)\cdot CH_2\cdot CH_2\cdot CO_2H$ b (c) m(c) m(c) (c)
Glycine	$HO_2C\cdot CH_2(NH_2)$ b c
Phenylalanine	 m and c m (c) b

repeated on a larger scale. Since only [1-^{14}C]acetate is incorporated into glycine and the carboxyl group contained no radioactivity it can be inferred that the carboxyl group is derived from bicarbonate and the α-(methylene) carbon atom from the carboxyl group of acetate.

The partial degradation of phenylalanine (Table 11) was done to determine whether acetate was incorporated into the aromatic ring system and the isolation of acetanilide with a comparatively high specific activity from both samples of phenylalanine clearly demonstrates that both carbon atoms of acetate are incorporated. Unfortunately no conclusions can be drawn from the radioactivities found in carbon dioxide produced during the chromic acid oxidation of phenylalanine since the high recoveries suggest that some ring fission occurred. It is, however, clear from the decarboxylation of the benzoic acid samples that [1-^{14}C]-acetate is not incorporated into the carboxyl group (which is the β-carbon atom of the side chain of phenylalanine), whereas [2-^{14}C]acetate is incorporated into this position. A comparison of the specific activities of the benzoic acid samples with those of the phenylalanine samples from which they were derived further shows that [1-^{14}C]acetate must have been incorporated into the α-carbon atom of the side chain whereas very little [2-^{14}C]-acetate can have been incorporated. Since the decarboxylation of phenylalanine with chloramine-T (Table 6) showed that neither [1-^{14}C]- nor [2-^{14}C]-acetate was incorporated into the carboxyl group, it can be assumed that this is derived from bicarbonate. This then shows that the side chain of phenylalanine has the following isotope distribution:

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

(for m, c and b see Table 12). Assuming by analogy with *E. coli* (see review by Sprinson, 1960) that the aromatic amino acids are synthesized via shikimic acid, the side chain would be derived directly from phosphoenolpyruvate and the above isotope distribution would be in accord with that found in alanine and aspartic acid in *C. thiosulphatophilum*, and lends further support to the formation of a C_3 unit (pyruvate or phosphoenolpyruvate) with this characteristic isotope distribution. The earlier experiments on acetate and bicarbonate incorporation into the side chain of tyrosine in *Rhodospirillum rubrum* are also identical with the above isotope distribution (Ehrensvard & Reio, 1953).

Further elucidation of the pathways involved in the biosynthesis of amino acids in *C. thiosulphatophilum* and in other strict anaerobes is clearly desirable. The isotope-incorporation technique coupled with the complete and unequivocal degradation of the radioactive products is a valuable indicator of novel biosynthetic pathways but studies of the intermediates in the biosynthetic processes and of the enzymes concerned demand the application of other techniques. The steps in the formation of alanine with the specific labelling pattern now established in three anaerobic bacteria, *C. thiosulphatophilum*, *Rhodospirillum rubrum* and *Clostridium kluyveri*, remain completely unknown.

Since the green sulphur bacteria are regarded as strict autotrophs these experiments, and the earlier studies with *C. limicola* by Sadler & Stanier (1960) showing that autotrophic bacteria are able to assimilate organic compounds into a large number of cell constituents, suggest that similar experiments should be carried out with the chemoautotrophs, which may also be found capable of assimilating low concentrations of some simple organic substrates such as acetate. The limitations of autotrophs may reside not in their capacity to assimilate or metabolize organic compounds (most of which they synthesize from bicarbonate and therefore have to metabolize anyhow), but in their capacity to oxidize them and so derive reducing power (presumably in the form of NADPH) for biosynthetic reactions leading to cell growth.

SUMMARY

1. Growth of *Chlorobium thiosulphatophilum* was increased by the addition of low concentrations of acetate to the basal thiosulphate medium.

2. Uptake of radioactive acetate by resting cell suspensions required the presence of bicarbonate and a source of reducing power such as hydrogen gas.

3. Acetate was incorporated into all the major cell fractions in resting cell suspensions and in growing cultures.

4. Poly-β-hydroxybutyrate was not detected in *C. thiosulphatophilum* under conditions in which it was readily isolated from *Rhodospirillum rubrum* and *Rhodomicrobium vannielii*.

5. Both [1-^{14}C]acetate and [2-^{14}C]acetate were incorporated into most of the amino acids of the cell proteins.

6. Radioactive amino acids were isolated and decarboxylated; with the exception of leucine and isoleucine, very little radioactivity was found in the carboxyl groups.

7. Alanine, aspartic acid and glutamic acid were degraded completely. The isotope distributions in alanine and aspartic acid were similar to those found in *Rhodospirillum rubrum* and *Clostridium kluyveri*.

8. Phenylalanine was partially degraded and it was established that both [1-^{14}C]acetate and [2-^{14}C]acetate were incorporated into the aromatic ring.

The authors acknowledge the skilful technical assistance of Mr R. J. Watkinson throughout these investigations. This research was supported in part by the Rockefeller Foundation.

REFERENCES

Allison, M. J. & Bryant, M. P. (1963). *Arch. Biochem. Biophys.* **101**, 269.

Allison, M. J., Bryant, M. P. & Doetsch, R. N. (1962). *J. Bact.* **83**, 523.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.

Crawford, L. V. (1958). *Biochem. J.* **68**, 221.

Cutinelli, C., Ehrensvard, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *Ark. Kemi*, **3**, 315.

Dixon, G. H., Kauffman, D. L. & Neurath, H. (1958). *J. biol. Chem.* **233**, 1373.

Duchow, E. & Douglas, H. C. (1949). *J. Bact.* **58**, 409.

Ehrensvard, G. (1955). *Annu. Rev. Biochem.* **24**, 275.

Ehrensvard, G. & Reio, L. (1953). *Ark. Kemi*, **5**, 327.

Elsden, S. R. & Gibson, Q. H. (1954). *Biochem. J.* **58**, 154.

Englesberg, E. & Levy, J. B. (1955). *J. Bact.* **69**, 418.

Gibson, J. & Hoare, D. S. (1963). *J. gen. Microbiol.* **31**, xxii.

Gilvarg, C. & Bloch, K. (1952). *J. biol. Chem.* **199**, 689.

Hausman, W. (1952). *J. Amer. chem. Soc.* **74**, 3181.

Hoare, D. S. (1963). *Biochem. J.* **87**, 284.

Jungwirth, C. & Umbarger, H. E. (1962). *Fed. Proc.* **21**, 10.

Kemble, A. R. & McPherson, H. T. (1954). *Biochem. J.* **56**, 548.

Knight, M. (1962). *Biochem. J.* **84**, 170.

Koeppe, R. E. & Hill, R. J. (1955). *J. biol. Chem.* **216**, 813.

Krebs, H. A. & Bellamy, D. (1960). *Biochem. J.* **75**, 523.

Larsen, H. (1952). *J. Bact.* **64**, 187.

Larsen, H. (1953). *K. norske vidensk. Selsk. Skr.* **1**, 153.

Law, J. H. & Slepecky, R. A. (1961). *J. Bact.* **82**, 33.

Mechsner, K. (1957). *Arch. Mikrobiol.* **26**, 32.

Mosbach, E. H., Phares, E. F. & Carson, S. F. (1951). *Arch. Biochem. Biophys.* **33**, 179.

Perkins, H. R. (1963). *Bact. Rev.* **27**, 18.

Richmond, V. & Hartley, B. S. (1959). *Nature, Lond.*, **184**, 1869.

Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1955). *Publ. Carnegie Instn*, no. 607.

Sadler, W. R. & Stanier, R. Y. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1328.

Sakami, W. (1955*a*). *Handbook of Isotope Tracer Methods*, p. 1. Cleveland, Ohio: Western Reserve University.

Sakami, W. (1955*b*). *Handbook of Isotope Tracer Methods*, p. 63. Cleveland, Ohio: Western Reserve University.

Schlegel, H. (1962). *Arch. Mikrobiol.* **42**, 110.

Schlegel, H. & Gottschalk, G. (1962). *Angew. Chem.* **74**, 342.

Seki, T. (1958). *J. Biochem., Tokyo*, **45**, 855.

Shaposhnikov, V. V., Kondratieva, E. N. & Fedorov, V. D. (1960). *Nature, Lond.*, **187**, 167.

Smillie, R. M., Rigopoulos, N. & Kelly, H. (1962). *Biochim. biophys. Acta*, **56**, 612.

Sprinson, D. B. (1960). *Advanc. Carbohyd. Chem.* **15**, 235.

Stanier, R. Y., Doudoroff, M., Kunisawa, R. & Contopoulou, R. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1246.

Strassman, M. & Ceci, L. N. (1962). *Fed. Proc.* **21**, 10.

Strassman, M., Locke, L. A., Thomas, A. J. & Weinhouse, S. (1955). *Science*, **121**, 303.

Tomlinson, N. (1954*a*). *J. biol. Chem.* **209**, 585.

Tomlinson, N. (1954*b*). *J. biol. Chem.* **209**, 605.

Umbarger, H. E. & Davis, B. D. (1962). In *The Bacteria*, vol. 3, p. 167. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.

Van Slyke, D. D., Plazin, J. & Weissiger, J. R. (1951). *J. biol. Chem.* **191**, 299.

Yemm, E. W. & Cocking, E. C. (1954). *Analyst*, **80**, 209.

Biochem. J. (1964) **91**, 559

Metabolism of Nucleic Acids during Liver Maturation in the Neonatal Rat

BY I. T. OLIVER

Department of Biochemistry, University of Western Australia

AND W. F. C. BLUMER

Department of Anatomy, University of Western Australia, Nedlands, Western Australia

(*Received* 1 *November* 1963)

In a previous paper (Oliver, Ballard, Shield & Bentley, 1962) several biochemical aspects of normal liver growth in postnatal rats were reported. Changes observed in the concentration of liver nucleic acids during the first 25 days after birth suggested that rapid cell division preceded a later phase of cytoplasmic growth. Since these results appeared to be the reverse of the situation that occurs in regenerating adult rat liver (Hecht & Potter, 1956), observations have now been extended by a study of the rates of incorporation of labelled precursor ([^{14}C]orotic acid) into liver nucleic acids *in vivo*. In addition, it has been necessary to attempt a definition of the cell types that contribute to changes in nucleic acids, since the rat liver is known to be haematopoietic in early