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Microbial Metabolism of C_1 and C_2 Compounds

THE INVOLVEMENT OF GLYCOLLATE IN THE METABOLISM OF ETHANOL AND OF ACETATE BY *PSEUDOMONAS* AM1

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Pseudomonas AM1 grows on ethanol with a mean generation time of about 10h. A single alcohol dehydrogenase is responsible for oxidation of both methanol and ethanol. It is proposed that the glyoxylate bypass does not operate in *Pseudomonas* AM1 during growth on ethanol. Although malate synthase is present in extracts of ethanol-grown *Pseudomonas* AM1, the activity of isocitrate lyase is negligible. Short-term incubation experiments with [^{14}C]ethanol and [^{14}C]acetate indicate that a novel pathway operates during growth of *Pseudomonas* AM1 on ethanol. Glycollate, glyoxylate and malate are probably intermediates in this pathway.

Pseudomonas AM1 is a pink bacterium that assimilates C_1 compounds and oxalate by way of the serine pathway (Large & Quayle, 1963; Blackmore & Quayle, 1970). This organism grows slowly on ethanol but cannot use glycollate, glyoxylate or glycine as a sole source of carbon. It would be reasonable to assume that ethanol is assimilated by way of the glyoxylate bypass (Kornberg, 1966). Results given in the present paper indicate, however, that ethanol is not metabolized by this pathway but by a novel route involving glycollate as an intermediate.

A study of ethanol metabolism was considered to be of particular interest because the growth responses of a mutant of *Pseudomonas* AM1, mutant PCT48 (Dunstan *et al.*, 1972), have indicated that an enzyme essential for the assimilation of ethanol is also involved in the formation of glyoxylate from C_1 compounds. For growth on these compounds glyoxylate formation from C_1 units is essential (Harder & Quayle, 1971) but this part of the serine pathway has not yet been elucidated. Preliminary reports of this work have been presented (Anthony & Dunstan, 1971; Anthony *et al.*, 1971).

Experimental

Chemicals

All chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., except the following. [U-^{14}C]Acetate (sodium salt) (specific radioactivity 56mCi/mmol), [1-^{14}C]ethanol (specific radioactivity 21mCi/mmol) and [2-^{14}C]ethanol (specific radioactivity 20.4mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [U-^{14}C]Ethanol was prepared by mixing [1-^{14}C]-ethanol and [2-^{14}C]ethanol.

DL-*erythro*-β-Hydroxyaspartic acid and DL-isocitrate (trisodium salt, allo free) were from Calbiochem Ltd., London W.1, U.K.; ATP (disodium salt) was from Boehringer Corp. (London) Ltd., London W.5, U.K.; sodium benzylpenicillin and streptomycin sulphate were from Glaxo Laboratories Ltd., Greenford, Middx., U.K.; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and phenylhydrazine hydrochloride were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; NE 250 liquid scintillator was from Nuclear Enterprises Ltd., Edinburgh, U.K.; hydroxypyruvic acid (lithium salt) was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; Ionagar no. 2 was from Oxoid Ltd., London E.C.4, U.K.; CoA from yeast (Grade 1, free acid), NADH, NADP$^+$ and NADPH were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Acetyl-CoA was prepared by the method of Srere (1969).

Bacterial strains

Pseudomonas AM1 (N.C.I.B. 9133) and *Micrococcus denitrificans* (N.C.I.B. 8944) were obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, U.K.

Escherichia coli K12 strain W3110 was a gift from Dr. J. R. Guest, Department of Microbiology, University of Sheffield, and *Pseudomonas aeruginosa* N.C.T.C. 8602 was a gift from Dr. P. H. Clarke, Department of Biochemistry, University College London.

Methods

Purification of [U-^{14}C]acetate. The [U-^{14}C]acetate obtained from The Radiochemical Centre contained small but significant amounts of radioactive impurities; it was purified before use by g.l.c. on a column of 25% diethylene glycol adipate–phosphoric acid-treated AW-DMCS Chromosorb W (100–120 mesh) at 140°C and with a nitrogen flow rate of 100ml/min. The column tended to deteriorate rapidly; a precolumn was therefore used, which was refilled before each set of purifications.

The radioactive sodium acetate was converted into acetic acid by the addition of 2μl of conc. HCl, then it was injected into the column. Pure acetic acid was collected in a trap cooled in liquid nitrogen, then it was dissolved in dilute NaOH (500μg/ml) and adjusted to pH7.0 before use; the yield was about 40%. This treatment separates acetate from radioactive impurities, as demonstrated by chromatography of 10μCi of purified acetate in the solvent systems described below, followed by radioautography of the chromatography papers.

Maintenance of cultures and growth media. Media were prepared as described by Anthony & Zatman (1964). Stock cultures were maintained on methylamine–agar slopes and subcultured every 2 months. The basal medium, without any carbon source, is referred to as 'salts medium'. Carbon sources used in growth media were used at a concentration of 0.2% except for methanol (0.4%), methylamine (0.4%) and sodium glyoxylate (5mM).

Growth and harvesting of cultures. Cultures were grown in shaken flasks at 30°C from a 5% inoculum of cells grown on the same carbon source. Cells were harvested by centrifugation at 10000*g* for 15min at 4°C from the mid-exponential phase of growth. The harvested cells were washed twice in 0.02M-sodium–potassium phosphate buffer, pH7.0, then resuspended in buffer and stored, when necessary, either at 2°C (for whole-cell experiments) or frozen at −15°C (for preparation of cell-free extracts).

Isolation of mutants. Mutants were isolated by a method based on that of Heptinstall & Quayle (1970). The isolation procedure was designed to select for mutants able to grow on succinate but not on methanol. The mutagen-treated cells were expressed in succinate medium and then treated with penicillin in the presence of methanol. The penicillin treatment was repeated three times, at a final concentration of 1000 units of penicillin/ml of medium.

Isolation of revertants. Initially attempts were made to isolate revertants by the method of Heptinstall & Quayle (1970). Usually only one or two possible revertants were obtained by this method, and these could not be distinguished with certainty from wild-type contaminants. The following procedure was therefore adopted for the isolation of revertants distinguishable from wild-type contaminants. The mutant was made resistant to streptomycin by plating out approx. 10^8 cells of a succinate-grown mutant culture on to succinate–agar plates containing 1mg of streptomycin/ml. After 3–5 days of incubation at 30°C one or two streptomycin-resistant colonies were observed. These were tested to ensure that they retained the growth properties of the original mutant strain. The streptomycin-resistant mutant was inoculated into 50ml of succinate medium and incubated overnight at 30°C with shaking; the cells were harvested, suspended in salts medium, and approx. 10^8 cells were spread out on methanol–agar plates containing 1mg of streptomycin/ml. The original mutant did not grow on this medium (because it is unable to grow on methanol), nor did wild-type contaminants (because they are streptomycin-sensitive). The only organisms able to grow were streptomycin-resistant revertants; these were picked off and their growth properties and enzyme activities were examined.

Measurements of oxygen uptake by whole cell suspensions. Oxygen uptake by bacterial cell suspensions was measured by using a Rank oxygen electrode. The incubation vessel contained 50μmol of 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid buffer, pH7.0, and 0.05–2.0mg dry wt. equivalent of washed cells in a total volume of 2ml. After measuring the endogenous rate of oxygen uptake, 15μmol of substrate (except for formate, when 80μmol was used) was injected and oxygen uptake was measured for 5–10min. Rates of oxygen uptake were calculated by assuming that 0.445μg-atom of oxygen is dissolved in 1ml of buffer at 30°C.

Induction of the enzymes involved in the metabolism of C_1 compounds. Some of the enzymes involved in the metabolism of C_1 compounds are inducible (Large & Quayle, 1963). For measurement of these enzymes in mutants unable to grow on C_1 compounds, succinate-grown cells were incubated overnight in salts medium containing methanol (0.4%). Preliminary experiments indicated that it is essential to use exponential-phase organisms for induction to occur. It was possible to store these organisms at 3°C for 24h before incubation in methanol medium for induction.

Preparation of sonic extracts. Washed cells were suspended in 1–2ml of 0.02M-sodium–potassium phosphate buffer, pH7.0, to a concentration of about 0.1g wet wt./ml, and were then sonicated for periods of 2–3min in a 100W MSE ultrasonic disintegrator at 20kHz, with a probe of tip diameter 9mm. The total exposure time was 10min and throughout the treatment the cells were cooled in an ice bath. The sonic extract was centrifuged at 40000*g* for 1h at 2°C and the supernatant was decanted and stored in ice. The extract was assayed immediately for enzyme activity.

Protein determination. Protein was assayed by the

method of Lowry *et al.* (1951); procedures were those of DeMoss & Bard (1957). Crystalline bovine serum albumin (fraction V) was used as a standard.

Bacterial incorporation of [^{14}C]*ethanol and* [^{14}C]-*acetate*. Cultures of bacteria were harvested in the mid-exponential phase of growth, then they were washed and suspended in salts medium to a concentration of about 3mg dry wt./ml. A suitable volume of this suspension was incubated aerobically at 30°C in the presence of non-radioactive ethanol (4μmol/ml) or non-radioactive acetate (0.5–1.5μmol/ml). After 10min, radioactive ethanol or acetate was added to a final concentration of 8–10μCi/ml; 1ml samples were withdrawn at suitable time-intervals, immediately transferred to 3ml of boiling ethanol and left for 3min. The ethanolic suspensions were placed in a water bath at 50°C for a further 15min, then centrifuged. The supernatant was decanted and the pellet was resuspended in 1ml of boiling aq. 20% (v/v) ethanol. Insoluble material was removed by centrifugation and the combined supernatants were evaporated to dryness, either at 60°C with nitrogen bubbling through the solution for 10–12h, or at 60°C open to the atmosphere for approx. 18h.

Chromatographic analysis and radioautography. The residue obtained from the procedure described above was suspended in 0.1–0.2ml of aq. 20% (v/v) ethanol and spotted on to Whatman no. 1 chromatography paper (46cm×57cm). The radioactive compounds were separated by two-dimensional chromatography; the solvent used for the first dimension was butan-1-ol–acetic acid–water (12:3:5, by vol.). A mixture of standard amino acids (6μg each of alanine, glycine, serine, glutamic acid and aspartic acid) and carboxylic acids (30μg each of citric acid, malic acid, glycollic acid, succinic acid and fumaric acid) were run in a marker strip down one side of the paper. Standard solutions were made up in aq. 20% (v/v) ethanol at a concentration of 2mg/ml (for amino acids) or 10mg/ml (for carboxylic acids). After being dried in air the marker strip was cut off and stained in: (*a*) acridine; the paper was dipped in a solution of acridine (0.1%) in aq. 99.5% ethanol, then immediately blotted; after the paper was dried in air carboxylic acids appeared as yellow spots that fluoresced under u.v. light; (*b*) ninhydrin; after the positions of the acids stained by acridine had been marked, the same papers were dipped in a solution of ninhydrin (0.5%) in acetone; after drying, the colour was developed for a few minutes at 110°C; amino acids appeared as blue–purple spots.

The positions of the standards on the marker strip indicated the relative positions of amino acids and carboxylic acids; the paper was cut between the fastest moving amino acid (alanine) and the slowest moving carboxylic acid (citric acid). The papers were then chromatographed in the second direction, the amino acids in phenol–ammonia [200ml of water-saturated phenol (400g of phenol/100ml of water) plus 1ml of NH_3 (sp.gr. 0.88)], and the carboxylic acids in ethanol–ammonia (sp.gr. 0.92)–water (16:1:3, by vol.). In all cases the solvent was run to the end of the paper.

After the chromatograms had been dried the radioactive compounds were located by radioautography; the papers were laid in contact with X-ray film (Kodak Blue Brand BB54) in the dark for 2 weeks. Each corner of the chromatogram was marked with radioactive ink so that, when the film was developed, it could be exactly superimposed on the chromatogram.

Elution and counting of radioactive compounds. Radioactive areas of the chromatogram were cut out and eluted by cutting the paper into small pieces, which were then shaken in 2–4ml of water for 10–15min. The paper was then removed and a sample of the eluate counted for radioactivity in NE250 liquid scintillator. Less than 10% of the radioactivity remained on the paper.

All radioactivity counting was done in an Intertechnique SP60 liquid-scintillation counter programmed for automatic quench correction.

An area of chromatogram (of the size of the average spot) that did not give a darkened area on the radioautogram was cut out and counted to give an estimate of the background counts on the chromatogram. This value is referred to as 'chromatogram background'; it has not been used for purposes of correction. Correction has been made, however, for the background counts measured in vials containing scintillation fluid without a radioactive sample (about 45c.p.m.).

Identification of radioactive compounds. The identity of the radioactive compounds was confirmed by cochromatography with authentic standards in the original solvents (separately in one dimension) and in a third solvent. A suitable portion of the radioactive sample was evaporated to dryness together with an authentic sample (50μg of carboxylic acid, 10μg of amino acid). The residue was then dissolved in 0.05ml of aq. 20% (v/v) ethanol and spotted on to Whatman no. 1 chromatography paper. For the third solvent amino acids were subjected to chromatography in butan-1-ol–ethanol–water (2:2:1, by vol.) and carboxylic acids were chromatographed in ether–acetic acid–water (15:3:1, by vol.). After being dried the chromatograms were stained as described above. The stained areas were cut out and counted by placing the paper in 10ml of NE250 liquid scintillator. The rest of the paper was cut into small strips and counted in a similar way to ensure that only one radioactive area was present on the chromatogram.

Enzyme assays. Unless otherwise stated, enzymes were assayed spectrophotometrically in a Unicam SP.500 spectrophotometer at room temperature;

10mm light-path silica cells were used. The reference cell contained water.

Methanol dehydrogenase. The enzyme was assayed at 30°C by using the oxygen electrode. The assay mixture contained 120 μmol of sodium pyrophosphate buffer, pH 9.0, 45 μmol of NH_4Cl, 2 μmol of phenazine methosulphate, 8 μmol of substrate (methanol or ethanol) and various amounts of extract in a total volume of 2 ml. The reaction was started by the addition of phenazine methosulphate. Correction was made for the oxygen uptake that occurs at this pH in the absence of enzyme and for the endogenous oxygen uptake by the extract in the absence of substrate. Specific activities measured by this method were similar to those obtained by the method of Anthony (1971).

Hydroxypyruvate reductase (EC 1.1.1.29). This was assayed by a method based on that of Large & Quayle (1963). The reaction mixture contained 30 μmol of sodium acetate–acetic acid buffer, pH 4.5, 0.1 μmol of NADH, various amounts of extract and 1 μmol of lithium hydroxypyruvate (added to start the reaction) in a total volume of 1 ml. The E_{340} was measured and correction was made for endogenous NADH oxidation.

Other enzymes. The following enzymes were measured by published methods: citrate synthase, EC 4.1.3.7 (Srere, 1969); isocitrate dehydrogenase, EC 1.1.1.42 (Ochoa, 1955*a*); malate dehydrogenase, EC 1.1.1.37 (Ochoa, 1955*b*); malate synthase, EC 4.1.3.2 (Dixon & Kornberg, 1959); isocitrate lyase, EC 4.1.3.1 (Kornberg, 1965; McFadden, 1969); *erythro*-β-hydroxyaspartate dehydratase, EC 4.3.1.– (Kornberg & Morris, 1962); glyoxylate carboligase, EC 4.1.1.– (Kornberg & Gotto, 1961); serine–glyoxylate aminotransferase (Blackmore & Quayle, 1970).

Results

A mean generation time of about 10h was measured for growth of *Pseudomonas* AM1 on ethanol. To account for this growth rate the minimum specific activities of enzymes for the assimilation of this compound must be of the order of 1.6 μmol of substrate utilized/h per mg of protein. This calculation is based on the assumptions (*a*) that 50% of the cell dry weight is carbon and (*b*) that 50% of the cell dry weight is protein (Luria, 1960). The specific activities of enzymes possibly involved in growth on ethanol are presented below. Failure to measure enzyme activities greater than the calculated minimum specific activity does not eliminate the pathway under consideration, but it is a strong indication that ethanol is metabolized predominantly by an alternative route. Although growth of *Pseudomonas* AM1 on acetate does occur, it is very slow; in this work on the metabolism of C_2 compounds, therefore, organisms were always grown on ethanol.

*Oxidation of ethanol by Pseudomonas AM*1

Anthony & Zatman (1965) have presented evidence that the phenazine methosulphate-linked alcohol dehydrogenase of *Pseudomonas* sp. M27 oxidizes ethanol and methanol at similar rates. The alcohol dehydrogenases purified from *Pseudomonas* AM1 and *Pseudomonas* sp. M27 are identical (Anthony, 1971). No typical NAD-linked ethanol dehydrogenase could be detected in extracts of ethanol-grown *Pseudomonas* AM1. Mutant PCT29, isolated during the present work, is similar to mutant M15A (isolated by Heptinstall & Quayle, 1970) and grows on succinate, methylamine, formate and oxalate, but not on methanol or ethanol. For all further investigations this mutant was grown on succinate and induced with methanol as described in the Experimental section. The rates of oxidation of methanol and ethanol by whole cells of the mutant were less than 3% of rates for the wild type. The specific activities of the alcohol dehydrogenase in extracts of the mutant were also about 3% of those for the wild type when either methanol or ethanol was substrate (specific activity with either methanol or ethanol as substrate in extracts of methanol-grown wild-type organisms: 32.5 μmol of O_2 used/h per mg of protein). Other substrates (formaldehyde, formate, methylamine and succinate) were oxidized at wild-type rates by whole cells of the mutant. The specific activities of key enzymes of the serine pathway (serine–glyoxylate aminotransferase and hydroxypyruvate reductase) were similar to those measured in extracts of wild-type organisms. Revertants of mutant PCT29, able to grow on methanol and on ethanol, had regained alcohol dehydrogenase activity. These results show that the alcohol dehydrogenase responsible for methanol oxidation is also essential for the oxidation of ethanol in *Pseudomonas* AM1. Ability to oxidize both alcohols is also lost in a mutant unable to synthesize soluble cytochrome *c* (Anthony & Dunstan, 1971).

The product of ethanol oxidation is probably free acetaldehyde, which is oxidized at the same rate as formaldehyde by the aldehyde dehydrogenase described by Johnson & Quayle (1964).

Acetyl-CoA, formed directly or indirectly from acetaldehyde, is presumably oxidized by the enzymes of the tricarboxylic acid cycle. Citrate synthase (specific activity 1.8 μmol/h per mg of protein), malate dehydrogenase (194.0 μmol/h per mg of protein) and isocitrate dehydrogenase (10.0 μmol/h per mg of protein) are present in extracts of ethanol-grown organisms and their activities are probably sufficient to support the slow growth of *Pseudomonas* AM1 on ethanol.

Enzymes of the glyoxylate bypass, the β-hydroxyaspartate pathway and the glycerate pathway

Extracts of ethanol-grown *Pseudomonas* AM1 contained malate synthase in sufficient activity to account for the growth rate on this substrate (specific activity 1.9 μmol of CoA formed/h per mg of protein).

Isocitrate lyase was assayed by the continuous assay of Kornberg (1965) and by the discontinuous assay of McFadden (1969). Only trace amounts of material capable of forming a phenylhydrazone were found when isocitrate, *cis*-aconitate or citrate was substrate. Assays were done at four pH values between 4.7 and 9.0 both with sonic extracts (assayed before and after dialysis) and with extracts prepared by grinding whole cells with alumina at 2°C. Assay methods were tested by using extracts of acetate-grown and of ethanol-grown *Ps. aeruginosa*; the isocitrate lyase in these extracts was not inhibited when *Pseudomonas* AM1 extracts were also included in the incubation mixture. The highest specific activity measured in the *Pseudomonas* AM1 extracts under any of these assay conditions was 0.26 μmol/h per mg of protein. This is insufficient to account for the observed rate of growth of *Pseudomonas* AM1 on ethanol and is about 1% of the specific activity measured in acetate-grown *Ps. aeruginosa* and about 6% of the specific activity measured in ethanol-grown *Ps. aeruginosa*.

erythro-β-Hydroxyaspartate dehydratase and glyoxylate carboligase were not present in sonic extracts of ethanol-grown *Pseudomonas* AM1. When assayed in extracts of *M. denitrificans* and *E. coli* respectively these enzymes were not inhibited by the inclusion of *Pseudomonas* AM1 extracts in the reaction mixtures.

Although malate synthase may be involved in the pathway for ethanol assimilation, the negligible amounts of isocitrate lyase, β-hydroxyaspartate dehydratase and glyoxylate carboligase preclude the operation of the three known routes for the aerobic assimilation of C_2 compounds.

Metabolism of radioactive C_2 compounds by ethanol-grown Pseudomonas AM1

The enzymic studies described above suggest that none of the known pathways for the assimilation of C_2 compounds is operating during growth of *Pseudomonas* AM1 on ethanol. The metabolism of C_2 compounds by *Pseudomonas* AM1 was therefore investigated by using short-term labelling experiments with [U-^{14}C]ethanol and with [U-^{14}C]acetate. In this type of experiment the incorporation of a constant or decreasing proportion (with time) of the total radioactivity into a given compound indicates that the added substrate is rapidly metabolized through this compound. Such a compound is therefore either an early intermediate in the metabolism of the substrate or is in rapid equilibrium with an early intermediate (Kornberg, 1958).

Metabolism of [^{14}C]ethanol. Whole cells of ethanol-grown *Pseudomonas* AM1 were incubated for short times with [U-^{14}C]ethanol as described in the

Table 1. *Distribution of ^{14}C among the non-volatile labelled components of the ethanol-soluble fractions of ethanol-grown Pseudomonas AM1 incubated with [U-^{14}C]ethanol*

Washed organisms (21 mg dry wt.) were preincubated aerobically in 7 ml of salts medium with 30 μmol of ethanol at 30°C for 10 min. [U-^{14}C]Ethanol (2.5 μmol; 50 μCi of ^{14}C) was added at zero time and 1 ml samples were removed at suitable time-intervals into 3 ml of boiling ethanol. The precipitates were extracted with 1 ml of aq. 20% (v/v) ethanol and the combined extracts analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and the radioactive compounds were eluted, counted and identified as described in the Experimental section. Correction was made for quenching and for the background counts of vials (about 45 c.p.m.). The chromatogram background count was 40–90 c.p.m.; it is given for reference but was not used for correction purposes. A blank sample containing boiled cells and radioactive substrate was treated in an identical manner with the experimental samples; no radioactive compounds were detected in the blank sample after chromatography and radioautography.

Time (s)	Compound ...	Radioactivity in compound (c.p.m.)			
		Glutamate	Compound 'B'	Others	Total
3		228	292	0	520
6		2275	608	0	2883
12.5		2357	527	0	2884
19		4354	535	0	4889
30		6921	811	842	8574
50		16049	1137	1010	18196
109		27921	1129	1877	30927

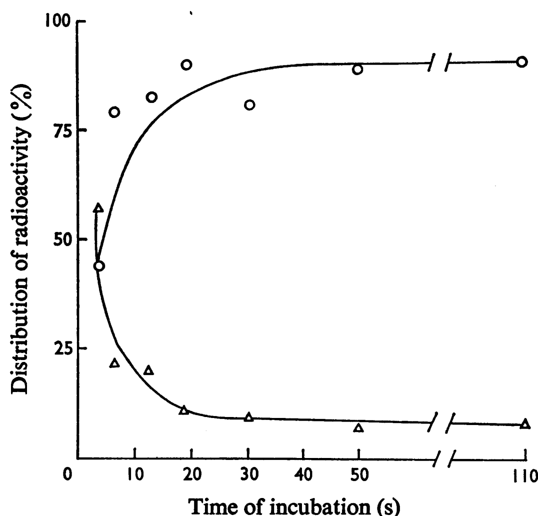


Fig. 1. *Variation with time of the percentage distribution of radioactivity incorporated from [U-^{14}C]ethanol into non-volatile components of the ethanol-soluble fraction of ethanol-grown Pseudomonas AM*1

For experimental details see Table 1 and the text. o, Glutamate; △, compound 'B'.

Experimental section. The radioactive ethanol-soluble intermediates were extracted, counted for radioactivity and identified, and the results are shown in Table 1. The total amount of radioactivity incorporated into the cells increased in an approximately linear manner during the experiment. At early times radioactivity was found in only two compounds, glutamate and an unknown compound designated 'B'. The percentage of the total radioactivity found in glutamate increased with time (Fig. 1), showing that it is not an early intermediate. By contrast, the percentage of the total radioactivity found in compound 'B' showed a pronounced negative slope, indicating that this unidentified compound, or a substance in rapid equilibrium with it, may be an early intermediate in the assimilation of ethanol by *Pseudomonas* AM1. It is assumed that glutamate is formed by transamination of the tricarboxylic acid cycle intermediate, 2-oxoglutarate, and detectable amounts of radioactivity might therefore have been expected in citrate and other intermediates of the cycle.

Metabolism of [^{14}*C*]*acetate*. Ethanol is probably assimilated by way of acetate (or acetyl-CoA). For this reason the metabolism of C_2 compounds by ethanol-grown *Pseudomonas* AM1 was further investigated by using [U-^{14}C]acetate in experiments similar to that described above. The results are shown in Table 2 and Fig. 2. The total radioactivity

Table 2. *Distribution of ^{14}C among the non-volatile labelled components of the ethanol-soluble fraction of ethanol-grown Pseudomonas AM*1 *incubated with* [*U-^{14}C*]*acetate*

Washed organisms (21 mg dry wt.) were preincubated aerobically in 7 ml of salts medium with 10 μmol of acetate at 30°C for 10 min. [U-^{14}C]Acetate (4.2 μmol; 50 μCi of ^{14}C) was added at zero time and 1 ml samples were removed at suitable time-intervals into 3 ml of boiling ethanol. Treatment of these samples was as described in Table 1.

Time (s)	Compound ...	Radioactivity in compound (c.p.m.)							
		Glutamate	Compound 'B'	Aspartate	Malate	Glycollate	'Succinate plus fumarate'	Citrate	Others	Total
4		786	140	528	736	262	178	0	0	2630
8		1857	316	652	932	369	608	100	0	4834
12		8364	1732	2130	2084	1344	1826	618	1178	19276
15		13364	1200	1336	3510	1800	2400	1296	4189	29095
21		22420	3260	2090	4696	3530	2888	2480	9354	50718
29		33650	3275	2110	3640	3540	3954	1078	12455	63702
50		95300	*	3456	9232	13332	10576	3240	15224	150360

* Compound 'B' was not observable as a distinct spot at this time.

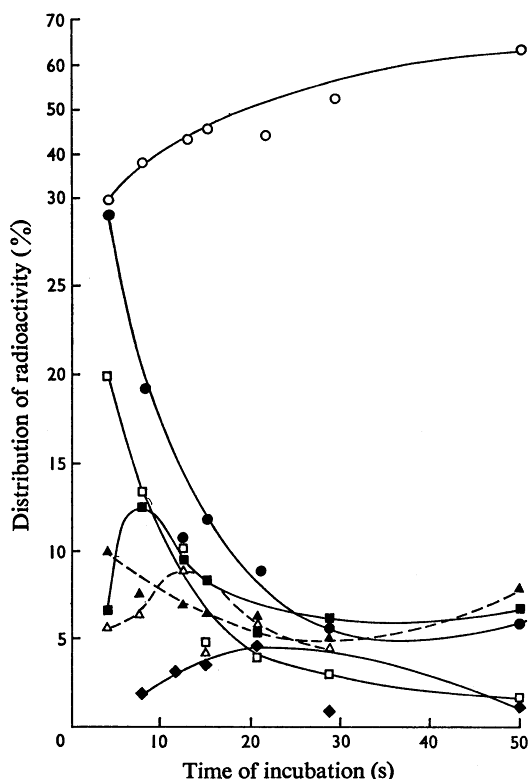


Fig. 2. *Variation with time of the percentage distribution of radioactivity incorporated from [U-^{14}C]acetate into non-volatile components of the ethanol-soluble fraction of ethanol-grown Pseudomonas AM*1

For experimental details see Table 2 and the text. o, Glutamate; △, compound 'B'; □, aspartate; ●, malate; ▲, glycollate; ■, 'succinate plus fumarate'; ◆, citrate.

incorporated into the cells (and into any given compound) increased in an approximately linear manner with time. The main conclusions to be drawn from the results shown in Fig. 2 are presented below.

1. The proportion of radioactivity in glutamate increased with time, indicating that glutamate is unlikely to be an early intermediate in the assimilation of acetate. After 50s of incubation 66% of the total label incorporated into the cells was found in glutamate, suggesting that there is a large glutamate pool in ethanol-grown *Pseudomonas* AM1.

2. The proportion of radioactivity in malate and aspartate decreased with time, indicating that malate is probably an early intermediate in acetate assimilation. Aspartate was presumably formed from malate by way of oxaloacetate.

3. The proportion of the total radioactivity in 'succinate plus fumarate' (which were not distinguished) increased at first and then decreased.

4. Before 12s the amount of radioactivity in citrate was not significantly greater than the background value. After this time there was a small proportion of radioactivity in citrate that increased slightly and then decreased with time.

5. Radioactivity was present from the earliest time in glycollate. The proportion of the total radioactivity in this compound decreased slightly over the first 15s, indicating that glycollate is probably an early intermediate in acetate assimilation. The high percentage of the total radioactivity found in glycollate after 50s (compared with the percentage at 29s) was not observed in later similar experiments.

6. An unknown radioactive compound, which had similar chromatographic properties to the compound 'B' obtained in the previous experiment, was present from the earliest times; the proportion of the total radioactivity in this compound increased slightly and then decreased with time.

The experiment described above was repeated a number of times, with sampling usually at only 10 or 15 and 60s. The radioactive compounds formed from [U-^{14}C]acetate were always the same as those listed in Table 2, but there was some variation in the relative amounts of label in these compounds. For example, malate often had a smaller proportion of the radioactivity, aspartate having a relatively higher proportion. Compound 'B' was always radioactive from the earliest times, and the percentage of the total radioactivity in 'B' usually decreased between 10 and 60s. This is similar to the result obtained with [U-^{14}C]ethanol. In experiments where the final proportion of radioactivity in carboxylic acids was small, compound 'B' usually contained a relatively higher proportion of the total counts.

In summary, the results given in Table 2 and Fig. 2 indicate that malate, glycollate and compound 'B' are early intermediates in the metabolism of [^{14}C]-acetate. Radioactivity was rapidly incorporated into these compounds and the proportion of the total radioactivity incorporated decreased slightly with time (sometimes after an initial increase in compound 'B').

Characterization of compound 'B'

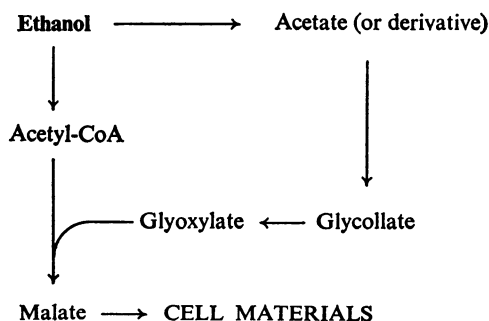
Some preliminary attempts were made to characterize compound 'B'; the R_F values of this compound were 0.22–0.26 in butan-1-ol–acetic acid–water and 0.00–0.03 in phenol–ammonia. It was not possible to extract compound 'B' into ethyl acetate, chloroform or ether from neutral, acid or alkaline solutions. The chromatographic properties of compound 'B' indicate that it is not a common amino acid, a common carboxylic acid (oxalic acid, succinic acid, glutaric acid, fumaric acid, glycollic

acid, lactic acid, malic acid, citric acid, pyruvic acid, oxalic acid or 2-oxoglutaric acid), a common sugar (glucose, sucrose, maltose, lactose, ribose, trehalose) or phosphoglycollic acid.

Discussion

The results of experiments with [U-^{14}C]acetate indicate that malate, glycollate and compound 'B' are early intermediates in the metabolism of acetate by *Pseudomonas* AM1. Radioactive glycollate has not been detected in this type of experiment with organisms in which the glyoxylate bypass is operating, although it could be formed by reduction of glyoxylate (this being derived in turn from acetate by way of isocitrate). It is unlikely, however, that the radioactive glycollate detected during the metabolism of [^{14}C]acetate by *Pseudomonas* AM1 was formed by this route, because only trace amounts of isocitrate lyase are present in these organisms. It is more probable that a novel pathway is involved in the formation of malate (and thus cell material) during growth of *Pseudomonas* AM1 on C_2 compounds, and that glycollate is an early intermediate in this pathway.

A scheme for the assimilation of ethanol and acetate by way of glycollate and malate is proposed (Scheme 1). It involves the direct conversion (hydroxylation) of acetate (or a derivative) into glycollate, followed by the oxidation of this compound to glyoxylate. This could then condense with acetyl-CoA to give malate. The scheme explains the early incorporation of radioactivity into malate and glycollate observed when ethanol-grown organisms were incubated with [^{14}C]acetate. Radioactivity was also incorporated into compound 'B' at early times, but it is impossible to speculate on the role of this unidentified substance in the metabolism of C_2 compounds at this stage. It has not as yet been possible to demonstrate conversion of acetate into glycollate in cell-free extracts of *Pseudomonas* AM1. Further evidence for the operation of the proposed pathway is presented in the following paper (Dunstan *et al.* 1972).



Scheme 1. *Postulated scheme for the assimilation of ethanol and of acetate by Pseudomonas AM*1

For details see the text.

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