The Metabolism of Lactate and Pyruvate by *Pseudomonas* AM1

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SUMMARY

Pseudomonas AM1, which can grow on C₁ compounds, also grows well on pyruvate and lactate. Extracts of the pyruvate-grown organism catalysed the synthesis of only small amounts of phosphoenolpyruvate directly from pyruvate. Experiments tracing the incorporation of radioactivity into bacterial constituents from pyruvate or lactate, each labelled in the C-1 or C-3 position, indicated that these substrates are mainly assimilated after removal of the C-1 atom. A mutant, C5, of *Pseudomonas* AM1 was isolated by a procedure intended to select organisms lacking phosphoenolpyruvate carboxylase. Although this enzyme was present in mutant C5, growth on lactate or pyruvate was impaired and there was no growth on C₁ compounds or ethanol. Good growth on each of these substrates was restored by a supplement of glyoxylate or glycollate.

A scheme is proposed for the assimilation via malate synthase and phosphoenolpyruvate carboxykinase of the C₂ fragment produced from pyruvate and lactate.

INTRODUCTION

It has been shown that the formation of phosphoenolpyruvate from pyruvate is achieved in Enterobacteriaceae *in vivo* through the sole agency of phosphoenolpyruvate synthetase (Cooper & Kornberg, 1967; Kornberg, 1970):

The phosphoenolpyruvate can then be used for gluconeogenesis or, after carboxylation by phosphoenolpyruvate carboxylase, for the anaplerotic formation of C₄ intermediates of the tricarboxylic acid cycle (Ashworth & Kornberg, 1966; Kornberg, 1970):

Despite the presence of other enzymes capable of carboxylating pyruvate or phosphoenolpyruvate *in vitro*, behaviour of mutants lacking phosphoenolpyruvate carboxylase shows that it is this enzyme which effects the formation of C₄ dicarboxylic acids under physiological conditions. Such mutants can be isolated on the basis of their inability to grow on glucose, glycerol, lactate or pyruvate except in the presence of a supplement of an intermediate of the tricarboxylic acid cycle or an amino acid related thereto.

In *Pseudomonas* AM1 the only enzyme capable of carboxylating a C₃ compound to a C₄ compound, which has so far been detected, is phosphoenolpyruvate carboxylase (Large, Peel & Quayle, 1962). If growth of *Pseudomonas* AM1 on lactate or pyruvate entails a similar process to that of the Enterobacteriaceae then this enzyme would play a similar anaplerotic

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role in synthesis of intermediates of the tricarboxylic acid cycle. Its role during growth on C_1 compounds as sole carbon source will, however, depend on the mechanism of the net synthesis of glyoxylate from C_1 units. It has been shown (see Quayle, 1972) that during growth of *Pseudomonas* AMI on C_1 compounds, phosphoglycerate can be synthesized by a sequence of reactions, known as the serine pathway, effecting the overall transformation:

The mechanism of the net synthesis of glyoxylate from C_1 units is not known. Possible mechanisms include (a) direct condensation of two C_1 units or (b) extension of the carbon skeleton of phosphoglycerate by at least one carbon atom followed by cleavage of the molecule to glyoxylate and a residual multicarbon molecule. An obvious possibility for extension of the phosphoglycerate skeleton, prior to cleavage, is by carboxylation of derived phosphoenolpyruvate. The role of phosphoenolpyruvate carboxylase would clearly be very different in these two cases. In mechanism (a) the enzyme would mediate merely the synthesis of oxaloacetate necessary for synthesis of those amino acids derived from the tricarboxylic acid cycle, e.g. aspartate and glutamate. Lack of the enzyme would lead to a requirement during growth on C_1 compounds for small quantities of a tricarboxylic acid cycle intermediate or related amino acid. In mechanism (b) the enzyme would be directly involved in regeneration of the glyoxylate necessary for operation of the main carbon assimilation pathway. Lack of the enzyme would lead to a requirement during growth on C_1 compounds not only for small quantities of a tricarboxylic acid cycle intermediate but also for substrate quantities of glyoxylate.

The diagnostic potential of the different roles of phosphoenolpyruvate carboxylase in the metabolism of *Pseudomonas* AMI led us to attempt the isolation of mutants lacking this enzyme. Searches for such mutants were made by screening for the inability to grow on lactate unless supplemented with succinate. The results of this study show that *Pseudomonas* AMI grows on lactate or pyruvate by a different route from that used by Enterobacteriaceae and the significance of these findings with respect to C_1 and C_2 metabolism by the organism is discussed.

METHODS

Chemicals

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire. The following substrates, coenzymes and purified enzymes were purchased from Boehringer Corporation (London) Ltd, London W.5: phosphoenolpyruvic acid (tricyclohexylamine salt), pyruvic acid (sodium salt), 2-phosphoglyceric acid (trisodium salt), nicotinamide nucleotide coenzymes, ADP, ATP (disodium salt), CoA (from yeast), lactate dehydrogenase (from rabbit muscle), pyruvate kinase (from rabbit muscle), glutamate-oxaloacetate transaminase (from pig heart) and enolase (from rabbit muscle). 3-Phosphoglyceric acid (disodium salt) and thiamine pyrophosphate were from Sigma (London) Chemical Co. Ltd, London, S.W.6. Acetyl CoA was prepared by the method of Stadtman (1957). Phenylhydrazine HCl (Hopkin and Williams, Chadwell Heath, Essex) was twice recrystallized from aqueous ethanol before use.

Sodium [$1\text{-}^{14}\text{C}$]pyruvate, sodium [$3\text{-}^{14}\text{C}$]pyruvate and sodium [^{14}C]bicarbonate were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [^{14}C]Lactate was from New England Nuclear, Boston, Massachusetts, U.S.A.

Maintenance and growth of organisms

Pseudomonas AMI (NCIB 9133) was maintained in slope culture on an inorganic salts medium (Jayasuriya, 1955) containing methylamine hydrochloride (50 mM) and agar (1.5 %, w/v). The organism was subcultured monthly and stored at 4 °C after incubation for 3 to 4 days at 30 °C. Mutant strains were maintained similarly on medium containing succinate (50 mM) in place of methylamine hydrochloride. The organisms were grown in liquid culture at 30 °C as described by Heptinstall & Quayle (1970) on carbon sources as indicated in the Results section. Growth was assessed by measuring the extinction of 650 nm. (An extinction of 0.1 corresponds to 36 µg dry wt of organism/ml over the extinction range 0 to 0.6.)

After growth of mutant strains for experimental use, samples were tested for the presence of revertants to wild-type by subculture in media containing lactate, methanol, ethanol or lactate + succinate.

Escherichia coli strain 113-3 (NCIB 8878), a cobalamin/methionine auxotroph (Davis & Mingioli, 1950), was a gift from Dr M. A. Foster, Microbiology Unit, Department of Biochemistry, University of Oxford.

Isolation of mutants

The procedure of Harder & Quayle (1971) was followed, using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for mutagenesis. The permissive growth medium for mutants contained lactate (50 mM) + succinate (2 mM). For penicillin selection following mutagenesis and expression, the medium contained lactate (50 mM) as sole carbon source. Over three cycles of selection, benzylpenicillin was used up to a final concentration of 600 units/ml of medium.

Growth responses of mutant strains

Erlenmeyer flasks (250 ml) fitted with optically matched side-arms and containing the medium under test (40 ml) were inoculated with about 10⁷ bacteria from a 24-h culture of a mutant grown on lactate + succinate. Growth was assessed by monitoring the extinction at 650 nm.

Preparation of bacteria-free extracts

Ultrasonic extracts were prepared as described by Harder & Quayle (1971). The supernatant fluids after centrifuging at 20000 g for 30 min at 0 °C were used as a source of enzymes. For assays involving the use of NADH the supernatant liquid after further centrifugation at 100000 g for 1 h at 4 °C was taken in order to avoid excessive interference by NADH oxidase activity.

Protein determination

Protein was measured by the Folin-Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Enzyme assays

All enzymes were assayed at 30 °C. Mixtures lacking a substrate or containing boiled extracts were used as controls. Spectrophotometric estimations were conducted with a Unicam SP1800 double-beam spectrophotometer (Pye Unicam Ltd, Cambridge).

Phosphoenolpyruvate carboxylyase (*orthophosphate-oxaloacetate carboxylyase* [phosphorylating] EC.4.1.1.31). The procedure was based on the method of Large *et al.* (1962). The assay

mixture contained in a final vol. of 2 ml: tris HCl, pH 8.6, 100 μ mol; MgCl_2 , 1 μ mol; 2-mercaptoethanol, 2 μ mol; NADH_2 , 3 μ mol; phosphoenolpyruvate, 2.5 μ mol; sodium [^{14}C]-bicarbonate, 20 μ mol, 2.6 μCi ; and enzymatic extract, 0 to 0.5 mg protein. After incubation for 30 min, the reaction was stopped with absolute ethanol (3 ml) and precipitated protein was removed by centrifugation (5000 g; 10 min). Samples (0.5 ml) of the supernatant fluids were transferred to aluminium planchets (diam. 25 mm), 0.1 ml formic acid (90 %, w/v) was added and the mixture dried in a stream of warm air. The non-volatile radioactive fixation products were assayed with an end-window counter (type EHM2S) coupled with a scaler (type D657; Panax Equipment Ltd, Redhill, Surrey), which had an efficiency of 8 % under the conditions used. After correction for background counts (about 10 c.p.m.) enzyme activity was calculated from the initial specific radioactivity of the sodium [^{14}C]bicarbonate.

Phosphoenolpyruvate synthetase. The initial rate of ATP-dependent removal of pyruvate was estimated by the method of Cooper & Kornberg (1969).

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC. 2.7.1.40). This enzyme was assayed spectrophotometrically according to the procedure of Bücher & Pfeleiderer (1955).

Pyruvate phosphate dikinase. Assay of the AMP- and pyrophosphate-dependent formation of pyruvate from phosphoenolpyruvate was modified from that described by Hatch & Slack (1968). The reaction mixture contained in a final vol. of 1 ml: tris HCl, pH 8.2, 50 μ mol; 2-mercaptoethanol, 5 μ mol; MgCl_2 , 5 μ mol; sodium pyrophosphate, pH 8.2, 1.2 μ mol; AMP, 3 μ mol; phosphoenolpyruvate, 2.5 μ mol; NADH_2 , 0.15 μ mol; lactate dehydrogenase, 25 μg ; and ultrasonic extract, 1 mg protein. The conversion to lactate of any pyruvate generated by the reaction mixture was followed by measuring the change in extinction at 340 nm.

Phosphoenolpyruvate carboxykinase (ATP: oxaloacetate carboxylyase [transphosphorylating], EC. 4.1.1.32). This enzyme was assayed by a modification of the method of Herbert & Guest (1971). The reaction mixture contained in a final vol. of 2 ml: potassium phosphate buffer, pH 7.0, 100 μ mol; phosphoenolpyruvate, 10 μ mol; ADP, 10 μ mol; MnCl_2 , 10 μ mol; potassium L-glutamate, 20 μ mol; sodium [^{14}C]bicarbonate, 30 μ mol, 6.6 μCi ; glutamate-oxaloacetate transaminase, 7.2 units; and enzymatic extract, 0 to 2.0 mg protein. At 3 min intervals, samples (0.1 ml) were pipetted into 0.9 ml formic acid (0.1 M) in scintillation vials and left overnight at room temperature to remove dissolved $^{14}\text{CO}_2$. Water was added to a final vol. of 0.6 ml followed by 6 ml of liquid scintillator (xylene, 667 ml; Triton X-100, 333 ml; 2, 5-diphenyloxazole, 5 g; 1,4-bis-(5-phenyloxazol-2-yl) benzene, 0.5 g). The radioactivity was measured in a Nuclear-Chicago scintillation counter (Model 6801). Losses of phosphoenolpyruvate carboxykinase activity were reduced by the inclusion of glutathione (1 mM) in buffer solutions (Utter, 1961). Controls lacking phosphoenolpyruvate or ADP were run with all experiments. These assay conditions were selected to minimize interference by phosphoenolpyruvate carboxylase. The latter reaction is Mg^{2+} ion-dependent with an optimum pH of 8.5 and is competitively inhibited by phosphate ions (Large *et al.* 1962). Fixation of CO_2 in the absence of ADP never exceeded 25 % of the total fixation and was deducted to give the activities quoted. It was possible to reduce this to 10 % of total fixation by passage of extracts through G-25 Sephadex, but in these systems phosphoenolpyruvate carboxykinase activity was reduced to approximately 50 % of the levels recorded without this treatment.

Pyruvate carboxylase (pyruvate: carbon dioxide ligase [ADP], EC. 6.4.1.1). The procedure described above for the estimation of phosphoenolpyruvate carboxylase was used, except that the reaction mixture contained ATP (2 μ mol) and phosphoenolpyruvate was replaced by sodium pyruvate (5 μ mol).

Malate dehydrogenase (decarboxylating) (L-malate: NAD oxidoreductase [decarboxylating], EC. 1.1.1.39). The ATP-independent fixation of $^{14}\text{CO}_2$ observed in the assay for pyruvate carboxylase was taken as a measure of malate dehydrogenase (decarboxylating) activity.

Phosphoglycerate mutase (2,3-diphospho-D-glycerate: 2-phospho-D-glycerate phosphotransferase, EC. 2.7.5.3). The reaction mixture contained in a final vol. of 3 ml: tris HCl, pH 7.6, 250 μmol ; KCl, 300 μmol ; MgSO_4 , 75 μmol ; NADH_2 , 0.75 μmol ; ADP, 4 μmol ; lactate dehydrogenase, 100 μg ; pyruvate kinase, 40 μg ; phosphopyruvate hydratase, 100 μg ; 3-phosphoglycerate, 50 μmol ; and ultrasonic extract, 0 to 3 mg protein. Enzymatic activity was calculated from the decrease in extinction at 340 nm following the addition of 3-phosphoglycerate.

Phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase, EC. 4.2.1.11). The formation of phosphoenolpyruvate from 2-phosphoglycerate was coupled to the oxidation of NADH_2 in the following reaction mixture (3 ml final vol.): tris HCl, pH 7.6, 250 μmol ; KCl, 300 μmol ; MgSO_4 , 75 μmol ; NADH_2 , 0.75 μmol ; ADP, 4 μmol ; lactate dehydrogenase, 100 μg ; pyruvate kinase, 40 μg ; 2-phosphoglycerate, 5 μmol ; and ultrasonic extract, 0 to 3 mg protein. The reaction was followed at 340 nm.

Malate synthase (L-malate glyoxylate-lyase [CoA-acetylating], EC. 4.1.3.2). This enzyme was assayed by the method of Dixon & Kornberg (1962).

Pyruvate dehydrogenase (pyruvate: lipoate oxidoreductase [acceptor acylating], EC. 1.2.4.1). The reduction of NAD^+ was estimated at 340 nm in the following reaction mixture (1 ml final vol.): tris HCl, pH 8.5, 135 μmol ; CoA, 88 nmol; L-cysteine HCl, 3.6 μmol ; thiamine pyrophosphate, 240 nmol; NAD, 1.5 μmol ; sodium pyruvate, 10 μmol ; and ultrasonic extract, 0.01 to 0.5 mg protein.

Bacterial incorporation of [^{14}C]lactate

Lactate-grown *Pseudomonas* AMI was washed once with growth medium lacking a carbon source and resuspended in fresh lactate growth medium to give 100 mg wet wt/ml. Samples (2.25 ml) of the suspension were placed in each of two conical centrifuge tubes (15 ml capacity) and these were stirred rapidly at room temperature with magnetic stirrers. After 15 min of incubation, 0.45 ml of each suspension was removed and pipetted into separate tubes containing 5 ml absolute ethanol, 0.05 ml sodium [$3\text{-}^{14}\text{C}$]lactate (17 mM; 100 $\mu\text{Ci/ml}$) was then added to one tube and 0.05 ml sodium [$1\text{-}^{14}\text{C}$]lactate (19 mM; 100 $\mu\text{Ci/ml}$) to the other. These tubes served as controls. To the remaining suspensions 0.2 ml [$3\text{-}^{14}\text{C}$]lactate or [$1\text{-}^{14}\text{C}$]lactate solution was added respectively. Samples (0.5 ml) were removed at 30-second intervals and pipetted into separate tubes containing absolute ethanol (5 ml). All samples were then treated in similar fashion. The ethanolic bacterial suspensions were centrifuged and the resulting precipitates extracted with 1 ml of cold aq. 20% (v/v) ethanol. The combined supernatant fluids were evaporated to dryness in a stream of air at 50 °C. The residues were dissolved in 0.5 ml of 0.01 M-HCl and applied to columns (25 mm $\times$ 5 mm diam.) of Dowex 50 (H^+ form). The columns were washed with 10 ml of water. The combined effluent and washings contained neutral and anionic compounds. The columns were then eluted with 4 ml of 2 M- NH_4OH . The eluates which contained amino acids were evaporated to dryness and each dissolved in 0.1 ml of water. The neutral/anionic fractions and the amino acid fractions were analysed by ion exchange chromatography and thin-layer chromatography (t.l.c.) as described below.

Chromatography and identification of labelled compounds. The neutral/anionic fractions obtained from the Dowex-50 columns were analysed by a modification of the method of von Korff (1969). The fractions were applied to columns (1.5 $\times$ 17 cm) of Dowex-I

Fig. 1. Influence of succinate on the growth of mutant *c5* on (a) C_3 compounds and (b) C_1 and C_2 compounds. For details of growth tests, see Methods section. Carbon sources for mutant *c5*: $\circ$, pyruvate (30 mM); $\bullet$, pyruvate, (30 mM) + succinate (0.5 mM); $\triangle$, lactate (50 mM); $\blacktriangle$, lactate (50 mM) + succinate (0.5 mM); $\blacksquare$, ethanol (25 mM) + succinate (0.5 mM); $\blacktriangledown$, methanol (0.5%, v/v) + succinate (0.5 mM); $\diamond$, succinate (0.5 mM). Carbon sources for wild-type *Pseudomonas* AM1: $\square$, pyruvate (30 mM); $\blacksquare$, lactate (50 mM); $\times$, methanol (0.5%, v/v).

(200 to 400 mesh; $\times 8$; Cl^- form) and eluted with 1.5 mM-HCl at a rate of 35 ml/h. Fractions of 5 ml were collected. Lactate was eluted between fractions 30 and 40, followed closely by succinate. A linear gradient was formed from 400 ml of 1.5 mM-HCl and 400 ml of 3.8 mM-HCl and applied to the column. The radioactivity of the fractions was measured by adding 0.5 ml samples to 10 ml of Bray's solution (Bray, 1960) and assayed in a liquid scintillation counter. The identity of the organic acids obtained from the Dowex-1 column was checked by t.l.c. on thin-layer microcrystalline cellulose plates using the upper phase of the solvent system: ethyl acetate + formic acid (90%, w/v) + water (60:5:35, by vol.).

Amino acids were analysed by two-dimensional t.l.c. on microcrystalline cellulose plates using the solvent system of Kung & Wagner (1970). The radioactive amino acids were located by autoradiography and assayed directly with a windowless gas-flow counter.

RESULTS

Isolation and nutritional properties of mutants

From a screening procedure designed to select mutants unable to grow on lactate (50 mM) unless provided with a supplement of succinate (2 mM), a group of auxotrophs was obtained which all possessed very similar nutritional properties. They were able to grow on succinate or malate as main carbon source but were apparently unable to grow on lactate or pyruvate unless supplemented with a small amount (2 mM) of succinate, malate, aspartate or citrate. They were not able to grow appreciably on methanol or ethanol, even when supplemented with 2 mM-succinate or malate. One mutant, *c5*, was investigated in detail.

The further studies of mutant *c5* showed that it could indeed grow on lactate or pyruvate as sole carbon source but only slowly as compared with wild-type *Pseudomonas* AM1 (Fig. 1a). This effect was particularly marked with pyruvate as carbon source on which the wild-type organism grew well within 24 h at 30 °C, whereas growth of the mutant under the

Table 1. Activities of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in bacteria-free extracts of wild-type and mutant *Pseudomonas* AM1

The enzymes were assayed as described in the Methods section.

Growth substrate	Specific activity ($\mu\text{mol CO}_2/\text{min/mg}$ of protein)			
	Phosphoenolpyruvate carboxylase		Phosphoenolpyruvate carboxykinase	
	Wild-type	Mutant c5	Wild-type	Mutant c5
Methanol (0.5 %, v/v)	0.13	0.088*	0.022	—
Methanol (0.5 %, v/v) + succinate (1 mM)	0.087	—	—	—
Methylamine (50 mM)	0.092	—	0.018	—
Ethanol (0.1 %, v/v)	0.018	—	—	—
Lactate (50 mM)	0.020	—	0.027	—
Lactate (50 mM) + succinate (1 mM)	0.022	0.018	—	—
DL-Malate (0.1 M)	—	—	0.028	0.018

* Organism first cultured in lactate (50 mM) + succinate (1 mM) and then resuspended and shaken at 30 °C for 12 h in medium containing methanol (0.5 %, v/v).

same conditions was barely visible after this time. Repeated subculture showed that this slow growth on C_3 compounds was not due to appearance of revertant strains. The addition of 0.5 mM-succinate to the growth medium considerably increased the rate of growth on lactate or pyruvate. The slow rate of growth on lactate, coupled with the stimulation of growth by a supplement of succinate, probably explains how these mutants were selected by the enrichment procedure.

Mutant c5 was unable to grow on methanol or ethanol and addition of a supplement of 0.5 mM-succinate to media containing these alcohols resulted in little more growth than did this amount of succinate alone (Fig. 1b).

Carboxylation of phosphoenolpyruvate

The purpose of the mutant isolation procedure was to obtain mutants lacking phosphoenolpyruvate carboxylase. The growth properties of mutants, such as c5, were suggestive of a dysfunction in this enzyme. However, extracts of mutant c5 grown on lactate supplemented with succinate contained phosphoenolpyruvate carboxylase at a specific activity comparable with that in the wild-type organism cultured in the same medium (Table 1). In wild-type *Pseudomonas* AM1 the specific activity of this enzyme varied with the growth substrate, being fivefold higher during growth on methanol or methylamine than on lactate or ethanol. Although mutant c5 was unable to grow on methanol, it was able to synthesize phosphoenolpyruvate carboxylase in response to exogenous methanol to the same extent as the wild-type organism. Hence mutant c5 appeared to have no lesion in synthesis of phosphoenolpyruvate carboxylase. Bacteria-free extracts of the malate-grown mutant also contained phosphoenolpyruvate carboxykinase at a specific activity of 60 % that of wild-type (Table 1).

Activities of enzymes involved in C_3 and C_4 metabolism

A further study (Table 2) of the enzymatic content of wild-type *Pseudomonas* AM1 cast doubt on the validity of the initial premise upon which the mutant isolation procedure was based, viz. that anaplerotic formation of C_4 intermediates of the tricarboxylic acid cycle is

Table 2. *Activities of enzymes associated with the metabolism of C₃ compounds by wild-type Pseudomonas AM1*

Enzymes were assayed in crude ultrasonic extracts as described in the Methods section. Activities have been corrected for product formed in the presence of boiled extracts.

Enzyme	Growth substrate		
	Pyruvate	Methanol	Succinate
	Specific activity (μmol of product formed/min/mg of protein)		
Phosphoenolpyruvate synthetase	0.00083	0.001	0.001
Pyruvate kinase	0.017	0.027	0.026
Pyruvate phosphate dikinase	N.D.	—	—
Pyruvate carboxylase	N.D.	N.D.	N.D.
Malate dehydrogenase (decarboxylating)	N.D.	—	—
Phosphoglycerate mutase	0.13	0.040	0.11
Phosphopyruvate hydratase	0.38	0.29	0.31
Pyruvate dehydrogenase	0.16	0.040	—
Malate synthase	0.055	0.081	0.074

N.D., not detected.

accomplished by the concerted operation of reactions (1) and (2). It is generally recognized that the formation of pyruvate from phosphoenolpyruvate, catalysed by pyruvate kinase:

is essentially irreversible under physiological conditions (see Cooper & Kornberg, 1967; Kornberg, 1970). Hence, although *Pseudomonas* AM1 contains pyruvate kinase, this enzyme would not be expected significantly to catalyse reaction (4) in the reverse direction. As the specific activity of this enzyme is lower during growth on pyruvate than on methanol or succinate this also argues against any special involvement during growth on pyruvate. Three routes are known which in other tissues can overcome the energy barrier associated with reversal of reaction (4): (a) the phosphoenolpyruvate synthetase reaction—reaction (1); (b) phosphorylation of pyruvate by means of pyruvate phosphate dikinase found in propionic acid bacteria (Evans & Wood, 1971) and certain tropical grasses (Hatch & Slack, 1968):

(c) carboxylation of pyruvate itself to malate or oxaloacetate, followed by formation of phosphoenolpyruvate from oxaloacetate by way of phosphoenolpyruvate carboxykinase:

The rate of ATP-dependent removal of pyruvate, which is used as a measure of the activity of phosphoenolpyruvate synthase (Cooper & Kornberg, 1969), was extremely low in extracts of *Pseudomonas* AM1 and indeed no higher during growth on pyruvate as compared with methanol or succinate. The specific activity of 0.83 nmol/min/mg of protein may be compared with 1.7 μmol /min/mg of protein found in lactate-grown *Escherichia coli* strain B (Cooper & Kornberg, 1969). No activity of pyruvate phosphate dikinase could be detected. Although phosphoenolpyruvate carboxykinase was found in extracts of *Pseudomonas* AM1 (Table 1) no activity of ATP-dependent pyruvate carboxylase or malate dehydrogenase (decarboxylating) could be detected (Table 2). Thus no enzymatic evidence could be found for appreciable overall conversion of pyruvate to phosphoenolpyruvate by routes (a), (b) or (c) in extracts of *Pseudomonas* AM1 even when grown on pyruvate. Nevertheless, phospho-

pyruvate hydratase and phosphoglycerate mutase, which can convert phosphoenolpyruvate to other gluconeogenic substrates, were present in extracts (Table 2).

Such findings pointed to the operation of a novel route for synthesis of phosphoenolpyruvate (or gluconeogenic intermediates) and intermediates of the tricarboxylic acid cycle during growth of the organism on lactate or pyruvate. Recent work by Anthony and his colleagues (Anthony & Dunstan, 1972; Dunstan & Anthony, 1972; Dunstan, Anthony & Drabble, 1972*a, b*) suggests that *Pseudomonas* AMI may possess a means of oxidizing acetate to glyoxylate, which would then enable the organism to grow on ethanol, malonate or β -hydroxybutyrate by the reactions shown in Scheme 1*a*. It is considered that each of these substrates is catabolized to acetate, or a derivative, which is then oxidized to glyoxylate. One molecule each of acetyl CoA and glyoxylate are in turn condensed to malate via malate synthase:

Evidence for the acetate to glyoxylate transformation has been obtained from studies with intact bacteria and mutants but not, so far, with bacterial extracts.

If such a transformation can be effected by *Pseudomonas* AMI, then it clearly might offer an explanation for growth on pyruvate as the organism possesses both malate synthase and an active pyruvate dehydrogenase converting pyruvate to acetyl CoA (Table 2). These reactions could then provide for synthesis of malate from pyruvate (Scheme 1*a*), and the action of malate dehydrogenase and phosphoenolpyruvate carboxykinase – reaction (6) could result in formation of phosphoenolpyruvate. Extracts of *Pseudomonas* AMI contain both malate dehydrogenase (Large, Peel & Quayle, 1963) and phosphoenolpyruvate carboxykinase (Table 1).

Evidence for operation of such a pathway (hereafter termed the malate synthase pathway) was then looked for in isotopic studies with intact bacteria.

Incorporation of [^{14}C]lactate into bacterial constituents

The incorporation of [$1\text{-}^{14}\text{C}$]lactate and [$3\text{-}^{14}\text{C}$]lactate into *Pseudomonas* AMI growing on lactate was investigated. It was not possible to use paper chromatography and autoradiography to follow directly the time course of incorporation of tracer into metabolites owing to masking of the autoradiograms with residual radioactivity from unchanged lactate. Instead the samples were first separated into a neutral plus anionic fraction and a cationic fraction by ion exchange chromatography before being further analysed by ion exchange chromatography, t.l.c. and paper chromatography (see Methods section).

Two intermediates of the tricarboxylic acid cycle, malate and succinate, became labelled during incubation of *Pseudomonas* AMI with [$3\text{-}^{14}\text{C}$]lactate (Table 3). A number of other compounds, both neutral and anionic, became labelled but these were not identified. No radioactive malate or succinate was detected during the similar incubation with [$1\text{-}^{14}\text{C}$]lactate. This indicates that the C-1 atom of lactate is lost during conversion of lactate into C_4 dicarboxylic acids whereas the C-3 atom is retained. Such behaviour would be consistent with formation of malate by the malate synthase pathway but would not be consistent with formation of malate or oxaloacetate by carboxylation of pyruvate or phosphoenolpyruvate derived directly from pyruvate.

A variety of amino acids became labelled after incubation of *Pseudomonas* AMI with [$3\text{-}^{14}\text{C}$]lactate (Table 4). The most rapidly labelled were glutamate, alanine and glycine. Incubation with [$1\text{-}^{14}\text{C}$]lactate resulted in much less label appearing in amino acids, with the exception of alanine which was rapidly labelled in both cases. Equal labelling of alanine from either [$1\text{-}^{14}\text{C}$]lactate or [$3\text{-}^{14}\text{C}$]lactate is to be expected since alanine is probably formed

Table 3. *Incorporation of [3-¹⁴C]lactate and [1-¹⁴C]lactate into malate and succinate by Pseudomonas AMI growing on lactate*

[3-¹⁴C]Lactate or [1-¹⁴C]lactate (0.85 μ mol or 0.95 μ mol respectively; 5 μ Ci) was added at zero time to 2.25 ml of a suspension of bacteria (100 mg wet wt/ml) in lactate growth medium at room temperature. Samples (0.5 ml) were removed at intervals into 5 ml of absolute ethanol and centrifuged, the pellets were extracted with 1 ml of aq. 20% (v/v) ethanol. The neutral plus anionic fractions were analysed by ion exchange chromatography and t.l.c. as described in the Methods section.

Labelled substrate	Time of incubation (s)	Radioactivity (d.p.m.)	
		Succinate	Malate
[3- ¹⁴ C]Lactate	30	N.D.	N.D.
	60	500	750
	90	3100	1250
	120	6000	2500
[1- ¹⁴ C]Lactate	30	N.D.	N.D.
	60		
	90		
	120		

N.D., not detected.

Table 4. *Incorporation of [3-¹⁴C]lactate and [1-¹⁴C]lactate into amino acids by Pseudomonas AMI growing on lactate*

The cationic fractions obtained from the experiments described in Table 3 were analysed by ion exchange chromatography and t.l.c. as described in the Methods section.

Labelled substrate	Time of incubation (s)	Radioactivity (c.p.m.)									
		Glu	Ala	Asp	Gln	Cys	Ser	Tyr	Met	Val	Gly
[3- ¹⁴ C]Lactate	30	400	368	—	—	—	—	—	—	—	60
	60	1176	654	98	70	64	62	60	156	—	62
	90	3192	1606	158	100	84	96	72	58	118	96
	120	4840	2522	320	152	90	132	74	144	208	132
[1- ¹⁴ C]Lactate	30	—	530	—	—	—	—	—	—	—	—
	60	—	730	—	—	92	—	—	—	—	—
	90	80	1920	—	—	88	66	—	—	—	66
	120	90	2266	92	—	102	104	64	86	—	104

by amination of pyruvate derived from lactate. Aspartate is probably formed from amination of oxaloacetate and hence the lack of labelling of aspartate from [1-¹⁴C]lactate again indicates that the C-1 atom of lactate is lost during conversion of lactate into C₄ dicarboxylic acids.

Specific activity of cell material of Pseudomonas AMI and Escherichia coli grown on [1-¹⁴C]-pyruvate and [3-¹⁴C]pyruvate

Operation of the proposed malate synthase pathway during growth of *Pseudomonas* AMI on lactate or pyruvate leads to formation of C₄ dicarboxylic acids which are unlabelled from [1-¹⁴C]lactate or [1-¹⁴C]pyruvate and labelled from [3-¹⁴C]pyruvate. In the absence of an enzyme which catalyses phosphorylation of pyruvate *in vivo*, phosphoenolpyruvate is probably made from oxaloacetate by means of reaction (6), and this again would be unlabelled from [1-¹⁴C]lactate or [1-¹⁴C]pyruvate and labelled from [3-¹⁴C]lactate or [3-¹⁴C]pyruvate. Hence all constituents derived from the tricarboxylic acid cycle and from phosphoenolpyruvate would be either unlabelled or labelled depending on whether the organisms were

Table 5. Incorporation of radioactivity from [1-¹⁴C]pyruvate and [3-¹⁴C]pyruvate into growing cultures of *Pseudomonas* AM1 and *Escherichia coli*

Cultures (250 ml) were shaken at 30 °C in Erlenmeyer flasks (1000 ml) fitted with side-arms for assessment of growth by extinction measurements at 650 nm. The medium contained [1-¹⁴C]pyruvate or [3-¹⁴C]pyruvate (1.25 µCi) and unlabelled pyruvate was added to a final concentration of 30 mM. In addition, the medium for *E. coli* contained 0.1 mM L-methionine. The inoculum was 1 ml of a culture growing exponentially on pyruvate. A stream of moist, sterile air containing CO₂ (2 %, v/v) was passed over the media throughout growth. Samples containing 0.25 to 0.65 mg dry wt of organism were withdrawn at intervals and bacteria collected on a membrane filter previously washed with unlabelled pyruvate (20 mM). The bacteria were then washed with distilled water (2 × 5 ml), 2 ml of formic acid (5 %, v/v) to remove dissolved [¹⁴C]bicarbonate, and finally with distilled water (5 ml). The filters were transferred to scintillation vials and covered with 5 ml of scintillation fluid for estimation of radioactivity. The specific radioactivities given are the constant, maximum values obtained from samples taken between six and eight generations of growth on the labelled substrate.

Organism	Labelled substrate	Specific activity (d.p.m./µg dry wt of bacteria)	Relative specific radioactivity
<i>Pseudomonas</i> AM1	[3- ¹⁴ C]Pyruvate	11.06	5.47
	[1- ¹⁴ C]Pyruvate	2.02	1
<i>Escherichia coli</i>	[3- ¹⁴ C]Pyruvate	7.33	2.66
	[1- ¹⁴ C]Pyruvate	2.76	1

grown on [1-¹⁴C]- or [3-¹⁴C]lactate/pyruvate. This situation would contrast with that predicted for an organism such as *Escherichia coli*, which by virtue of phosphoenolpyruvate synthetase, would synthesize labelled phosphoenolpyruvate and labelled C₄ dicarboxylic acids during growth on lactate/pyruvate labelled in either C-1 or C-3. Hence the ratio of specific activity of cell material synthesized from [3-¹⁴C]pyruvate to that synthesized from [1-¹⁴C]pyruvate should be considerably higher for an organism growing on pyruvate/lactate by the proposed malate synthase pathway than for an organism, such as *E. coli*, growing on the same substrate by the phosphoenolpyruvate synthetase pathway.

This prediction was verified in an experiment in which *Pseudomonas* AM1 and *Escherichia coli* were each grown on [3-¹⁴C]pyruvate and [1-¹⁴C]pyruvate. Air, containing carbon dioxide (2 %, v/v), was continually flushed through the growth flasks so as to reduce incorporation of ¹⁴CO₂ derived from oxidation of the substrates. It can be seen (Table 5) that the ratio of the constant maximum specific radioactivity attained by *Pseudomonas* AM1 on [3-¹⁴C]pyruvate to that attained on [1-¹⁴C]pyruvate was 5.47. This can be compared with a similar ratio of 2.66 obtained for *E. coli* in a similar experiment.

Growth response of mutant C5 to glyoxylate and glycollate

Mutant C5 was able to grow on a C₃ compound alone, though appreciable growth only occurred several hours later than in a similar control culture of the wild-type organism (Fig. 1a). Small amounts of succinate or other tricarboxylic acid cycle intermediates considerably stimulated growth of the mutant, especially with pyruvate as the major carbon source. In view of the evidence that during growth on a C₃ compound, the carbon source is metabolically linked to C₄ dicarboxylic acids via C₂ intermediates, the ability of glyoxylate or glycollate to replace succinate in enhancing the growth of mutant C5 on a C₃ substrate was investigated. The stimulatory effects of small quantities of glyoxylate or glycollate were indeed comparable to those of succinate (Fig. 2a).

Mutant C5 possesses a metabolic lesion preventing growth on a C₁ compound (Fig. 1b),

Fig. 2. Influence of glyoxylate and glycollate on the growth of mutant c5 on (a) C_3 compounds and (b) methanol. For details of growth tests, see Methods section. Carbon sources: ●, lactate (50 mM); ■, lactate (50 mM) + glyoxylate (0.5 mM); △, pyruvate (30 mM); ○, pyruvate (30 mM) + glyoxylate (0.5 mM); ▽, methanol (0.5%, v/v), ▼, methanol (0.5%, v/v) + glyoxylate (10 mM); □, methanol (0.5%, v/v) + glycollate (10 mM).

and glycollate and glyoxylate were tested as supplements for growth on methanol as carbon source. Although small amounts of the C_2 supplements were rather ineffective, increasing their concentration to 10 mM permitted good growth (Fig. 2b). Neither glycollate nor glyoxylate alone (10 mM) supported measurable growth of either mutant c5 or wild-type *Pseudomonas* AM1.

DISCUSSION

The enzymatic content of *Pseudomonas* AM1 and the results of the isotopic experiments point to a route for metabolism of lactate or pyruvate which differs fundamentally from that operating in Enterobacteriaceae. This novel route (Scheme 1a) would place lactate and pyruvate alongside ethanol, malonate and hydroxybutyrate as substrates catabolized to C_2 units, from which acetyl CoA and glyoxylate are condensed by means of malate synthase to lead to the anaplerotic formation of C_4 intermediates of the tricarboxylic acid cycle (Anthony & Dunstan, 1972). The precise nature of the interconversions of C_2 units to give acetyl CoA and glyoxylate are not known, but glycollate may be involved.

It is now clear why the original mutant isolation procedure did not lead to isolation of mutants lacking in phosphoenolpyruvate carboxylase. There would not appear to be an important role for this enzyme during growth of *Pseudomonas* AM1 on C_3 compounds by Scheme 1 (a). This is borne out by the depressed specific activity of phosphoenolpyruvate carboxylase in the lactate-grown organism (Table 1). The actual lesion present in mutant c5 is not known, but its response to glycollate, glyoxylate or succinate suggests that it is impaired in its ability to interconvert C_2 units. This is further supported by the fact that it is unable to grow on ethanol.

Mutant c5 is unable to grow on methanol unless supplemented with glycollate or glyoxylate. It thus provides a further example of a mutant indicating a connexion between the pathways of assimilation of C_1 and C_2 compounds perhaps by way of glyoxylate or a com-

Scheme 1. Possible involvement of C₂ compounds in growth of *Pseudomonas* AMI on (a) compounds catabolized via acetate and (b) C₁ compounds. The boxes enclose interconversions of C₂ units at different oxidation levels between acetate and glyoxylate.

mon C₂ precursor of glyoxylate. The other examples are PCT48 (Dunstan *et al.* 1972*b*) and G5 (Salem & Quayle, 1971), both of which are unable to grow on either methanol or ethanol unless supplemented with glycollate or glyoxylate. Despite their similarity to mutant C5, both PCT48 and G5 were selected for quite differently, viz. inability to grow on methanol but ability to grow on succinate (PCT48); inability to grow on methanol unless supplemented with glyoxylate (G5).

Scheme 1(b) expresses two ways in which glyoxylate might be synthesized in net fashion from C₁ units, either by direct C₁ + C₁ condensation or by cleavage of a C₄ compound into two C₂ compounds both of which being converted into glyoxylate. Recent work by Salem, Large & Quayle (1972) supports the latter mechanism rather than the former. The possible connexion between metabolism of C₁ compounds and compounds catabolized via acetate might then lie in the interconversion of C₂ intermediary metabolites between the oxidation levels of acetate and glyoxylate. Lesions in enzymes involved in these interconversions would result in impaired ability to grow on both C₁ compounds and compounds catabolized via acetate.

The initial purpose of the work described in this paper, to isolate mutants of *Pseudomonas* AMI which lack phosphoenolpyruvate carboxylase, remains unfulfilled. These mutants would still be valuable for reasons outlined in the Introduction, but in view of the singular metabolism of C₃ substrates by this organism it is clear that a different isolation procedure will have to be used. It may be necessary to find such mutants amongst those which are unable to grow on C₁ compounds but able to grow on C₄, C₃ or C₂ compounds.

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