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The Oxidative Properties of an Oxalate-decomposing Organism, *Pseudomonas* OD1, with Particular Reference to the Synthesis of Citrate from Glycollate

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The isolation and properties of an organism belonging to the genus *Pseudomonas* which will grow on oxalate as sole source of carbon and energy have been reported (Jayasuriya, 1955). Washed suspensions of this organism, *Pseudomonas* OD 1, were found to oxidize oxalate, and this reaction has been described. The oxidative metabolism of washed suspensions has now been studied further, particularly with respect to the oxidation of two carbon compounds and tricarboxylic acid intermediates, and the effect of sodium fluoroacetate. Of particular interest was the finding that citrate accumulates in the presence of glycollate plus sodium fluoroacetate, and this reaction has been investigated in detail. The present paper is a detailed report of these studies, preliminary accounts of which have already been given (Jayasuriya, 1954*a*, *b*).

MATERIALS AND METHODS

Organism. Pseudomonas OD1 has been described in full elsewhere (Jayasuriya, 1955). For the present studies, the organism was grown on a medium containing the following salts (mg./100 ml.): KH_2PO_4, 136; Na_2HPO_4, 213; $(NH_4)_2SO_4$, 50; $MgSO_4,7H_2O$, 20; $CaCl_2,2H_2O$, 1; $FeSO_4$, $7H_2O$, 0·5; $MnSO_4,5H_2O$, 0·25; $Na_2MoO_4,2H_2O$, 0·25. In addition, the medium contained 1% (w/v) of one of the following carbon sources: potassium oxalate, sodium DL-lactate, sodium glycollate, sodium DL-malate, sodium succinate. Unless otherwise stated, the carbon source was lactate. In each case, the pH was 7·0 and the medium was sterilized by autoclaving at 15 lb./in.^{2} for 15 min. The organism was grown in penicillin flasks for 40 hr. at 25°. The conditions of growth and the preparation of washed cells have already been described fully (Jayasuriya, 1955).

With the medium containing oxalate there was no precipitation of calcium oxalate during preparation, the medium remaining clear after autoclaving, though the possibility that some calcium oxalate might be precipitated during growth cannot be excluded. The organism can, however, utilize calcium oxalate, since clear zones are produced around colonies growing on solid medium containing this compound. In addition, washed cells prepared after growth on the liquid oxalate medium described above have a low endogenous respiration, whereas they rapidly oxidize potassium oxalate (see Table 1). This would indicate that little if any calcium oxalate remains in the washed suspensions prepared from cells grown on oxalate, or that, if present, it has little effect upon the respiration.

Reagents. All reagents used, except those mentioned below, were of A.R. grade. Triethanolamine was distilled and purified by formation of the hydrochloride. Sodium glycollate was recrystallized from acetone before use. Oxaloacetic acid was kindly provided by Professor H. A. Krebs; solutions of the sodium salt were prepared immediately before use by neutralization with 0·01 N-NaOH. 2-Oxoglutaric acid was kindly supplied by Dr R. L. Wickremasinghe. Sodium fluoroacetate was the gift of Dr B. C. Saunders of the Chemical Laboratory, University of Cambridge.

Analytical methods. Gas exchanges were measured with Warburg manometers at 25°. Where necessary, double-side-bulb cups were used and the reactions stopped by the addition of 0·2 ml. of 2 N-H_2SO_4 from the second side arm.

Rates of substrate oxidation are expressed as μl. of O_2 consumed/mg. dry wt. of cells/hr. $=Q_{O_2}$. Values for CO_2

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production were deduced after measuring the gas exchanges by the first method of Dickens & Šimer (1930) as modified by Elsden & Lewis (1953).

Glycollate was determined colorimetrically by the method of Dagley & Rodgers (1953), based on its reaction with sulphuric acid to yield formaldehyde. Citrate was determined by the colorimetric method of Weil-Malherbe & Bone (1949) as modified by Taylor (1953).

Glycine utilized by washed-cell suspensions was estimated indirectly by determining the amount of ammonia produced. This was estimated by the microdiffusion technique of Conway (1947), by adding an equal volume of 10N-NaOH to the sample in the outer chamber of the Conway unit. The ammonia distilled over into 1 ml. of 0·2N-H_2SO_4 in the inner chamber and was estimated colorimetrically by adding 1 ml. of Nessler's reagent, prepared according to Koch & McMeekin (1924), to the contents of the inner chamber. This was made up to 15 ml. with water and the intensity of the colour produced measured with the Hilger–Spekker absorptiometer with the blue–green (Ilford no. 603) filter.

Chromatographic methods. Citrate was identified by paper-partition chromatography according to Buch, Montgomery & Porter (1952). The developing solvent was pentanol saturated with 5N formic acid, and the papers were developed by the ascending method. Acidified contents of the manometer cup were prepared for chromatography as follows. The cells were removed by centrifuging. The supernatants were made approximately 2·5N with respect to sulphuric acid and then saturated with anhydrous sodium sulphate. This solution was then extracted with an equal volume of butan-2-one. The organic layer was separated and the solvent removed on a boiling-water bath. The residue was taken up in water and samples were applied to sheets of Whatman no. 1 filter paper. After development the papers were sprayed with ammoniacal silver nitrate (equal parts of 0·1N-$AgNO_3$ and 0·1N-NH_3 soln. mixed just before use). The sprayed chromatogram was dried at room temperature away from direct sunlight. The spots that developed were observed after about 4 hr.

Citrate and glycollate were isolated by partition chromatography on Celite no. 535 according to Swim & Krampitz (1954). By developing the columns with increasing concentrations of butanol in chloroform these authors were able to separate pyruvic, fumaric, succinic, 2-oxoglutaric, malic and citric acids. The writer has found that the method can also be applied to the separation of glycollic and citric acids. The column was developed by the successive addition, in 100 ml. quantities, of chloroform followed by 5, 10, 15, 20, 25, 30, 35, 40, 50 and finally 60% (v/v) of butanol in chloroform. Glycollic acid was eluted by 20% (v/v) butanol in chloroform and citric acid was eluted by 35% (v/v) butanol in chloroform. Both were estimated by titration with 0·01N-NaOH. The identity and quantitative recovery of glycollic acid was confirmed by the colorimetric estimation according to Dagley & Rodgers (1953), and that of citric acid was confirmed by the colorimetric estimation according to Taylor (1953).

Isotopic materials. Isotopically labelled [1-^{14}C]- and [2-^{14}C]-glycine and [1-^{14}C]- and [2-^{14}C]-bromoacetic acid were obtained from the Radiochemical Centre, Amersham. Labelled sodium glycollate was prepared as described below.

Preparation of sodium [1-^{14}C]- *and* [2-^{14}C]-*glycollate.* These were prepared by the hydrolysis of [1-^{14}C]- and [2-^{14}C]-bromoacetic acid respectively, according to Euler & Fahlander (1922). The bromoacetic acid (25 mg. equivalent to 0·1 mc) was dissolved in 7 ml. of 0·1N-NaOH. To this solution was added 1 ml. of water and the solution was heated on a glycerol bath at 75–80° for 3 hr. The solution was then evaporated to less than 1 ml. on a hot plate and, after cooling to room temperature, was acidified with 10N-H_2SO_4 to pH less than 2. The glycollic acid present was isolated chromatographically on Celite as described above. After titration with 0·01N-NaOH the fractions containing the glycollic acid were pooled, and the aqueous layer was separated. The aqueous layer was evaporated to small volume on a hot plate and then dried *in vacuo* over conc. H_2SO_4. The yield of sodium glycollate was 17·9 mg. in each case (theoretical 18·0 mg.). The identity and purity of the synthetic product was established by (*a*) its chromatographic behaviour on Celite and (*b*) the colorimetric estimation carried out according to Dagley & Rodgers (1953).

Assay of ^{14}C. Radioactive carbon was measured in the form of barium carbonate, with an end-window Geiger–Müller counter, after conversion of organic compounds into CO_2 by heating with the wet-combustion mixture of Van Slyke & Folch (1940). The plating technique was introduced to the author by Dr H. J. Saz, and a full description is given by Ormerod (1956). In manometric experiments, respiratory CO_2 was collected in 0·2 ml. of CO_2-free 3N-NaOH in the centre well of the manometer cup. At the end of the experiment the alkali in the centre well was washed out with CO_2-free water and made up to 10 ml. A sample of this solution, after the addition of carrier sodium carbonate, was converted into barium carbonate as described above.

All activities have been corrected for self-absorption and background count and are expressed as counts/min. at infinite thinness.

RESULTS

Oxidative properties of Pseudomonas *OD* 1 *grown on different carbon sources*

Of the organic compounds tested as growth substrates for *Pseudomonas* OD 1 only oxalate, DL-lactate, glycollate, DL-malate, succinate and pyruvate were utilized (Jayasuriya, 1955) and the ability of washed-cell suspensions of OD 1, when grown on the first four of these substrates, to oxidize various carbon compounds was examined.

The rates of oxidation observed were relatively low compared with most other *Pseudomonas* species, even in the presence of the carbon sources used for growth. The oxidative powers of washed cells showed some response to growth in the presence of the substrate, e.g. cells grown on oxalate oxidized oxalate more rapidly than cells grown on lactate (Table 1), but there was no clear-cut instance of substrate oxidation being completely dependent upon the presence of the substance in the growth medium. In general, cells grown on malate, succinate or lactate oxidized the test compounds more readily than cells grown on oxalate or glycollate.

In most of the subsequent experiments, lactate was used in preference to oxalate as growth substrate because (*a*) a higher yield of cells was obtained and (*b*) a wider range of respiratory substrates was attacked at a substantial rate by washed suspensions of cells grown on lactate.

Effect of fluoroacetate on oxidation by washed cells

It has been shown that animal tissues convert fluoroacetate (FAc) into fluorocitrate, which inhibits the enzyme aconitase. This action blocks the tricarboxylic acid cycle and so inhibits the oxidation of acetate and causes the accumulation of citrate (Peters, 1952; Peters, Wakelin, Rivett & Thomas, 1953). In an attempt to obtain further information on the oxidative properties of *Pseudomonas* OD 1, the effect of 0·001 M FAc on the oxygen uptake and amounts of citrate accumulating in the presence of various substrates was examined. Elliot & Kalnitsky (1950) found that pre-incubation of tissues with FAc gave more complete inhibition of oxygen uptake, and therefore in all the experiments with FAc the washed-cell suspensions were pre-incubated with the inhibitor for 30 min. before addition of the substrate. The citrate produced was determined after stopping the reaction when the rate of oxygen uptake in the presence of substrate fell to the endogenous value. The oxygen uptake in the presence of the dicarboxylic acids succinic, fumaric, malic and oxaloacetic is not much greater than in their absence, and with these the values for citrate production were obtained after 120 min. incubation and not when the oxygen uptake fell to the endogenous rate. In each experiment, a control reaction mixture containing no substrate was set up and analysed for citrate after incubation for the same time as the mixture with substrate.

The effect of 0·001 M FAc on the oxygen uptake in the presence of 10 μmoles of substrate is shown in Table 1, and the citrate accumulating in the same experiments is given in Table 2. In the absence of FAc, the amounts of citrate found in the absence of substrate are very low: the increases due to the addition of substrate are negligible and need not be considered further. In the presence of FAc there was a considerable production of citrate in the absence of added substrate in all cases.

With cells grown on lactate, the largest net accumulation of citrate was observed with 2-oxoglutarate as substrate, but perhaps of more interest were the observations with the C_2 acids. Glycollate gave rise to a relatively large amount of citrate (1·13 μmoles from 10 μmoles of substrate) and glycine gave rise to about half this quantity: with all three acids the oxygen uptake due to the substrate was strongly inhibited (about 60 %). By contrast, acetate and oxalate gave rise to virtually no citrate and the oxygen uptake in the presence of acetate was not inhibited, although that due to oxalate was inhibited somewhat. Pyruvate gave rise to less citrate than glycine or glycollate and, as with acetate, FAc had no effect on the oxygen uptake. The four C_4 acids tested gave rise to moderate amounts of citrate, in all cases less than that observed with glycine. These acids produce little increase in the oxygen uptake of the cells; consequently the degree of inhibition cannot be effectively assessed, owing to the small differences involved, though there are signs of inhibition. Glycollate does not give rise to citrate in cells grown on oxalate, but it must be borne in mind that such cells oxidize glycollate only slowly. A further distinctive feature of cells grown on oxalate is that the oxygen uptake in the presence of oxalate is unaffected by FAc, whereas with cells grown on lactate there is a substantial inhibition, indicating perhaps that a different mechanism for oxalate oxidation is operating in the latter.

With cells grown on lactate, the amount of citrate formed from 2-oxoglutarate corresponds to a 50 % conversion of the carbon of this substrate into citrate. With glycollate and glycine the citrate formed corresponded to 30 and 15 % conversion respectively. These latter observations were of considerable interest, for although much work has been done on the oxidation of acetate (and tricarboxylic acid cycle intermediates) very little is known about the metabolism of other C_2 acids. The formation of relatively large amounts of citrate from glycollate was unexpected and was therefore examined further.

Effect of fluoroacetate on the oxidation of glycollate

Fig. 1 shows in more detail the effect of FAc on the oxygen uptake of washed suspensions of cells grown on lactate in the presence of glycollate. Oxygen uptake values, corrected by subtraction of the values without glycollate, are also shown. In the absence of substrate the oxygen uptake was linear with time and values were 80 and 130 μl./hr. in the presence and in the absence of FAc respectively. From the corrected curves it is seen that the oxygen uptake due to glycollate is linear with time until complete, both in the presence and in the absence of FAc. FAc not only inhibits the rate of oxygen uptake due to glycollate but also decreases the total oxygen uptake. This decrease is about 30 % and it is perhaps significant that this is virtually the same as the percentage of glycollate carbon converted into citrate in the presence of FAc.

Table 1. *Effect of fluoroacetate on the oxidation of various substrates*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02 M FAc; first side bulb, substrate (10 μmoles); second side bulb, 0·2 ml. of 2 N-H_2SO_4; centre well, 0·2 ml. of 10% (w/v) KOH. Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air. The Q_{O_2} values are initial rates. Where the magnitude of the inhibition is difficult to assess, because of the small differences in measurements involved, no figure is given.

	Q_{O_2}						
	No FAc			With FAc			
Substrate	Endogenous (*a*)	With substrate (*b*)	Net (*c*) = (*b* − *a*)	Endogenous (*d*)	With substrate (*e*)	Net (*f*) = (*d* − *e*)	Percentage inhibition 100 (*c* − *f*)/*c*
Cells grown on lactate							
Formate	10	17	7	7	13	6	14
Oxalate	4	27	23	2	16	14	39
Acetate	9	27	18	5	22	17	6
Glycollate	5	42	37	3	18	15	60
Glycine	4	11	7	2	5	3	56
Pyruvate	10	40	30	7	36	29	3
Succinate	9	12	3	5	5·5	0·5	—
Fumarate	9	16	7	5	10	5	—
DL-Malate	5	12	7	3	8	5	—
Oxaloacetate	7	9	2	4	5	1	—
2-Oxoglutarate	11	21	10	8	12	4	60
Cells grown on oxalate							
Oxalate	3	40	37	2	39	37	0
Glycollate	3	7	4	2	4	2	—

Table 2. *Citrate production from various substrates in the presence of fluoroacetate*

The data are from the corresponding experiments of Table 1.

	Citrate formed (μmoles)				
	No FAc		With FAc		
Substrate	Endogenous	With substrate	Endogenous (*a*)	With substrate (*b*)	Net (*b* − *a*)
Cells grown on lactate					
Formate	0·03	0·04	1·15	1·15	0
Oxalate	0·04	0·04	0·59	0·64	0·05
Acetate	0	0·01	0·50	0·50	0
Glycollate	0·04	0·08	0·59	1·72	1·13
Glycine	0·02	0·02	0·64	1·18	0·54
Pyruvate	0·03	0·05	1·15	1·59	0·44
Succinate	0·07	0·07	0·84	1·07	0·23*
Fumarate	0·07	0·09	0·84	1·22	0·38*
DL-Malate	0·04	0·05	0·59	1·05	0·46*
Oxaloacetate	0·05	0·07	0·74	1·06	0·32*
2-Oxoglutarate	0·02	0·02	0·91	4·87	3·96
Cells grown on oxalate					
Oxalate	0·04	0·07	0·17	0·17	0
Glycollate	0·04	0·09	0·17	0·18	0·01

* Citrate was determined after 120 min. incubation with substrate. In all other cases citrate was determined when the rate of O_2 uptake fell to the endogenous value.

The effect of different concentrations of FAc on citrate formation from glycollate was next examined. From the results obtained (Table 3), 0·001 M seems to be the optimum concentration for maximal citrate production. Higher concentrations of FAc suppress endogenous citrate formation as well as net citrate production due to glycollate. On the other hand, with a lower concentration of FAc (0·0001 M) endogenous citrate formation is almost the same, but net citrate production is much less. In all subsequent experiments, FAc was used at a final concentration of 0·001 M.

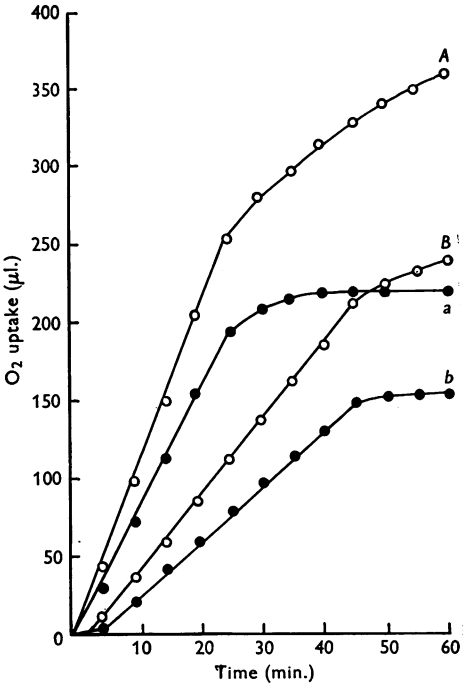

Fig. 1. Progress curves for the oxidation of glycollate by washed-cell suspensions. Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02M FAc (where indicated); side bulb, 0·1 ml. of 0·1M sodium glycollate; centre well, 0·2 ml. of 10% (w/v) KOH. Total volume of contents, 2·2 ml.; temp. 25°; gas phase, air. *A*, FAc absent; *B*, FAc present; *a*, *b*, as for *A*, *B*, but values corrected by subtraction of corresponding blank values in absence of glycollate.

Table 3. *Effect of concentration of fluoroacetate on citrate formation from glycollate*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05M triethanolamine buffer, pH 8·0, FAc (to give final concentration as indicated below); first side bulb, 10 µmoles of sodium glycollate in 0·2 ml.; second side bulb, 0·2 ml. of 2N-H_2SO_4; centre well, 0·2 ml. of 10% (w/v) KOH. Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air.

Concn. of fluoroacetate (M)	Citrate formed (µmoles) Endogenous (*a*)	With glycollate (*b*)	Net (*b* − *a*)
0·02	0·80	1·41	0·61
0·01	0·80	1·60	0·80
0·002	0·99	1·79	0·80
0·001	1·11	2·05	0·94
0·0001	1·19	1·30	0·11

Isolation and characterization of citrate

Citrate was determined by the colorimetric method of Weil-Malherbe & Bone (1949) as modified by Taylor (1953). The method is highly specific but it seemed important to confirm that the material giving the colour reaction was in fact citrate.

The supernatants obtained from incubation mixtures both with and without added glycollate were examined by paper-partition chromatography (see Materials and Methods), and in both cases a spot was obtained in the position corresponding to citrate. In both cases also, partition chromatography of the supernatants on Celite columns gave a substance having the same mobility as citrate. The amount of this substance eluted from the column prepared from incubation mixtures to which glycollate was added was much greater than that eluted from the column derived from the incubation mixture containing no glycollate.

Further evidence that the substance accumulating is citrate was provided by the isolation of the quinidine derivative from large-scale experiments, in which higher proportions of glycollate to cells were used. The method of isolation used was based on that described by Weinhouse, Medes & Floyd (1946). Conical flasks (100 ml. capacity) fitted with a side bulb, centre well and a ground-glass neck carrying a tap, were employed. The main compartment contained 10 ml. of cell suspension (15 mg. dry wt./ml.), 5·0 ml. of 0·05M triethanolamine–HCl buffer, pH 8·0, and 1 ml. of 0·02M FAc. The centre well contained 0·5 ml. of 20% (w/v) KOH and the side arm 4·0 ml. of 0·1M sodium glycollate (400 µmoles). The volume of the reaction mixture was made up to 20 ml. with water. The flasks were incubated, with shaking, at 25°. A control experiment was also carried out in which the glycollate solution was replaced by water. After equilibration, the contents of the side arm were tipped in. The O_2 consumption was followed manometrically in parallel experiments carried out with conventional Warburg manometers holding a total volume of 2 ml. of reaction mixture containing one-tenth of the above quantities.

When the rate of O_2 consumption in the manometer cups with added glycollate had fallen to the value in the cups with cells alone, 2·0 ml. of 2N-H_2SO_4 was added to each of the larger vessels to stop the reaction. The acidified contents were centrifuged to remove cells and to each of the supernatants 8·0 ml. of 10N-H_2SO_4 was added to make the solution approximately 2·5N with respect to sulphuric acid. This solution was saturated with anhydrous sodium sulphate and filtered. The filtrate was extracted with an equal volume (30 ml.) of butan-2-one. The organic layer was separated and the solvent removed on a boiling-water bath. The residue was dissolved in 5 ml. of water. At this stage the solution was tested for citrate by paper chromatography, and colorimetrically. The solution was then brought to the boil and 250 mg. of basic quinidine added to the hot solution. This was cooled rapidly to room temperature under the tap, filtered with suction and without washing on a Büchner funnel, and then allowed to stand at room temperature.

After about 48 hr., crystals formed in the supernatant derived from the incubation mixture to which glycollate was added. In form the crystals were characteristic rosettes

typical of quinidine citrate. They were filtered off and dried *in vacuo* over anhydrous calcium chloride. The m.p. of this material was 131–138°, and the m.p. of an authentic sample of quinidine citrate prepared from analytical reagent citric acid was 132–138°. The m.p. of the mixture was 130–137°. The wide range in the melting points of these quinidine derivatives, which has been observed by others (Weinhouse *et al.* 1946), is probably due to the adsorption of some of the basic quinidine on the quinidine citrate. The yield of quinidine citrate was 42 mg. (80% calculated on the colorimetric estimation of the citrate produced from incubation mixtures, in the large-scale experiments, to which glycollate had been added).

The quinidine derivative could be isolated only from vessels to which glycollate was added. In the control experiment in which glycollate was omitted the concentration of citrate was presumably too low to permit the isolation of the quinidine salt. The amount of citrate isolated as the quinidine derivative (28 μmoles) from vessels to which glycollate was added was greater than the amount formed endogenously (15 μmoles) as estimated colorimetrically.

Evidence for the conversion of glycollate into citrate

Since some citrate was formed on incubating the cells alone with FAc it was essential to establish that the extra citrate accumulating in the presence of glycollate was derived from the glycollate and was not due to an indirect effect of glycollate on the endogenous metabolism. The following experiments were carried out with this object in view.

Effect of cell concentration. The effect of varying the concentration of cells on citrate formation in the presence of a fixed amount of glycollate was investigated. In this experiment the reactions were stopped when the rate of oxygen uptake in the presence of glycollate fell to the endogenous value. Since this occurred at different times with the different cell concentrations it was necessary to carry out a control without glycollate for each concentration of cells used. The results (Table 4) show that the amount of citrate formed due to the added glycollate is independent of the concentration of cells and hence independent of the amount of endogenous material and metabolism.

Effect of glycollate concentration. The complementary experiment of determining citrate production in the presence of a fixed amount of cells and varying amounts of glycollate was also carried out. As with the previous experiment, the reactions were stopped in the individual incubation mixtures when the rate of oxygen consumption in the presence of glycollate fell to the endogenous rate. Again the reactions at each concentration of glycollate took a different time for completion and control experiments without glycollate were analysed at each of these times. Table 5 shows that the ratio of the net citrate produced to the glycollate added is constant. Therefore in the presence of a fixed amount of cells, citrate production is directly proportional to the quantity of glycollate added. Any citrate production due to a stimulation of the endogenous metabolism by glycollate would cause a departure from the linear relationship observed between citrate formation and glycollate added.

Table 4. *Effect of concentration of cells on citrate formation from glycollate*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (dry wt. as indicated below), 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02 M FAc; first side bulb, 5 μmoles of sodium glycollate in 0·1 ml.; second side bulb, 0·2 ml. of 2 N-H_2SO_4; centre well, 0·2 ml. of 10% (w/v) KOH. Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air.

	Citrate formed (μmoles)		
Cells (mg. dry wt.)	Endogenous (*a*)	With glycollate (*b*)	Net (*b* − *a*)
5	0·21	0·74	0·53
10	0·46	1·02	0·56
15	0·69	1·22	0·53
30	1·36	1·94	0·58
60	1·84	2·42	0·58

Table 5. *Effect of concentration of glycollate on citrate formation*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02 M FAc; first side bulb, glycollate as indicated below; second side bulb, 0·2 ml. of 2 N-H_2SO_4; centre well, 0·2 ml. of 10% (w/v) KOH. Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air.

		Citrate formed (μmoles)			
Glycollate added (μmoles)	Duration of experiment (min.)	Endogenous	With glycollate	Net	Net citrate/added glycollate
2·5	40	0·47	0·73	0·26	0·104
5·0	60	0·57	1·10	0·53	0·106
10·0	110	0·73	1·77	1·04	0·104
20·0	200	1·12	3·25	2·13	0·107
40·0	270	1·29	5·42	4·13	0·103

Table 6. *Progress of citrate formation and its relationship to oxygen uptake*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02 M FAc; first side bulb, 0·1 ml. of 0·05 M sodium glycollate; second side bulb 0·2 ml. of 2 N-H_2SO_4; centre well, 0·2 ml. of 10 % (w/v) KOH. Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air.

Time (min.)	10	20	30	45	65
Oxygen uptake (μmoles)					
(*a*) Cells alone	0·63	1·25	1·92	2·23	3·35
(*b*) Cells + 5 μmoles of glycollate	1·92	5·18	7·37	9·15	11·16
(*c*) Net (*b* − *a*)	1·29	3·93	5·45	6·92	7·81
Citrate formed (μmoles)					
(*d*) Cells alone	0·54	0·56	0·57	0·57	0·61
(*e*) Cells + 5 μmoles of glycollate	0·64	0·80	0·90	0·98	1·07
(*f*) Net (*e* − *d*)	0·10	0·24	0·33	0·41	0·46
Quotient (*c*)/(*f*)	12·9	16·3	16·5	16·9	17·0

Table 7. *Carbon balance for synthesis of citrate from glycollate*

Manometer cups contained: main compartment, 1·0 ml. of cell suspension (15 mg. dry wt./ml.), 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, 0·1 ml. of 0·02 M FAc; first side bulb, 0·2 ml. of 2 N-NaOH (CO_2-free); second side bulb, 0·4 ml. of 4 N-H_2SO_4; Keilin tube, 0·1 ml. of 0·05 M sodium glycollate. Total volume of contents, 2·6 ml.; temp. 25°; gas phase, air.

	Substance (μmoles)	Carbon content (μatoms)
Glycollate added	5·0	10·0
Glycollate recovered	0	0
Glycollate utilized	5·0	10·0
CO_2 produced	6·52	6·52
Citrate produced	0·55	3·30
Total carbon recovered	—	9·82

Progress of citrate formation. The progress of citrate formation with time was also followed. It can be seen from Table 6 that the ratio of the net oxygen consumption to the net citrate production is almost constant for any given interval of time, except in the 10 min. experiment in which the experimental errors may be unavoidably large. These results indicate that citrate formation follows the same course as the oxygen consumption during the oxidation of the added glycollate.

Carbon balance. Carbon-recovery experiments (Table 7) show that 98 % of the carbon of the glycollate utilized could be accounted for as respiratory carbon dioxide and citrate produced. Approximately one-third of the carbon of glycollate is converted into citrate.

The results of the preceding experiments therefore suggest that the added glycollate acts as the sole source of carbon for the synthesis of the extra citrate produced in its presence, and eliminate the possibility that the increased citrate production is caused by a stimulation of endogenous citrate formation.

Isotope balance. Attempts were next made to confirm these results by using isotopically labelled [1-^{14}C]- and [2-^{14}C]-glycollate as substrate. The citrate formed was isolated chromatographically on Celite and its activity determined by quantitative oxidation to carbon dioxide.

The incubation of washed-cell suspensions of OD 1 in the presence of glycine and FAc also results in the accumulation of citrate (Table 2). As glycine is structurally very similar to glycollic acid, and as isotopically labelled [1-^{14}C]- and [2-^{14}C]-glycine were available, a few preliminary experiments were also done with [1-^{14}C]- and [2-^{14}C]-glycine as substrate.

Table 8 shows that some of the activity of both [1-^{14}C]- and [2-^{14}C]-glycollate as well as [1-^{14}C]- and [2-^{14}C]-glycine is incorporated into citrate. This confirms that the carbon of the added glycollate (or glycine) is converted into citrate. It is evident that with both glycollate and glycine the initial activity of the substrate can be accounted for almost entirely in the respiratory carbon dioxide and citrate produced. These results are therefore in complete agreement with carbon-recovery experiments (Table 7).

From Table 8 it can also be seen that with either [1-^{14}C]glycollate or [1-^{14}C]glycine as substrate, less than 10 % of the initial activity of the substrate is incorporated into citrate, and the rest of the activity was recovered in the respiratory carbon dioxide. On the other hand, with either [2-^{14}C]glycollate or [2-^{14}C]glycine, about 45 % of the initial activity of the substrate was incorporated into citrate, and the remaining activity was recovered in the respiratory CO_2.

DISCUSSION

The principal finding reported in this paper is the formation of citrate from glycollate by washed suspensions of *Pseudomonas* OD 1 in the presence of fluoroacetate. Under these conditions, the citrate

Table 8. *Isotope balance for synthesis of citrate from labelled glycollate and glycine*

Manometer cups contained: main compartment, 0·5 ml. of 0·05 M triethanolamine buffer, pH 8·0, isotopically labelled substrate as indicated below; first side bulb, 0·1 ml. of 0·02 M FAc, 0·5 ml. of cell suspension (30 mg. dry wt./ml.); second side bulb, 0·2 ml. of 2 N-H_2SO_4; centre well, 0·2 ml. of 3 N-NaOH (CO_2-free). Total volume of contents, 2·4 ml.; temp. 25°; gas phase, air. All activities are expressed as counts/min. at infinite thinness.

	Substrate			
	5 μmoles of glycine		10 μmoles of glycollate	
Substrate	[1-^{14}C]-	[2-^{14}C]-	[1-^{14}C]-	[2-^{14}C]-
Initial activity	13 240	16 180	1 417 000	1 232 000
Final activity	0	0	0	0
Activity recovered in				
(*a*) Respiratory CO_2	12 980	9 280	1 277 000	632 000
(*b*) Citrate formed	480	6 560	94 000	566 000
Total recovery	13 460	15 840	1 371 000	1 198 000

which accumulates in excess of that appearing in the absence of substrate appears to be formed entirely from glycollate. This conclusion is based on the following evidence: (1) In the presence of a fixed amount of glycollate, the amount of extra citrate formed is independent of the amount of cells present. (2) In the presence of a fixed amount of cells, the extra citrate produced is directly proportional to the amount of glycollate added. (3) Citrate formation parallels the oxygen consumption due to glycollate. Oxygen consumption is linear, and if it be taken as a measure of the amount of glycollate metabolized then it follows that citrate appearance parallels the disappearance of glycollate. (4) The carbon of the glycollate utilized can be accounted for entirely by the extra citrate and carbon dioxide produced; 33 % of the carbon appears in citrate and the remainder in the carbon dioxide. (5) The isotope experiments indicate that 46 % of the C-2 atom of glycollate and about 7 % of the C-1 atom are incorporated into citrate. This corresponds to a conversion of 27 % of the total carbon of the glycollate into citrate, which corresponds reasonably well with the 33 % conversion indicated by the carbon-recovery experiment.

The action of fluoroacetate in this organism will now be briefly considered. Whilst it is known that animal tissues convert fluoroacetate into fluorocitrate, which inhibits aconitase (Peters, 1952; Peters *et al.* 1953), so blocking the tricarboxylic acid cycle, it is by no means certain that this is the sole effect of fluoroacetate on metabolic processes. Data on the effect of this inhibitor on whole cells must be interpreted with appropriate caution. In the present work the inhibition of glycollate oxidation is accompanied by an accumulation of citrate, suggesting that the inhibition is in fact due to inhibition of aconitase. There are, however, certain features of the effects of fluoroacetate upon this organism which cannot be readily explained. These are: (1) fluoroacetate does not inhibit the oxygen uptake due to acetate or pyruvate; (2) no citrate accumulates from acetate in the presence of fluoroacetate; (3) 2-oxoglutarate is by far the best substrate for citrate formation in the presence of fluoroacetate; (4) fluoroacetate has no effect upon the oxidation of oxalate by cells grown on oxalate, but an appreciable inhibition is observed with cells grown on lactate, although citrate does not accumulate in either case.

In animal tissues and in some micro-organisms, there is abundant evidence that citrate is formed from acetyl-coenzyme A and oxaloacetate (Ochoa, 1954). The recent work of Campbell, Smith & Eagles (1953) and of Saz (1954) on *Pseudomonas aeruginosa* and *P. fluorescens* and of Olson (1954) on *Penicillium chrysogenum* suggest that citrate may also be synthesized by a condensation of glyoxylate with succinate. A third reaction possibly involved in citrate synthesis is the reductive carboxylation of 2-oxoglutarate (Ochoa, 1948), and with regard to this possibility it may be significant that 2-oxoglutarate gives the highest yield of citrate in *Pseudomonas* OD 1. This present knowledge, however, concerns only the final stage in citrate synthesis; little is known of the synthesis of C_4 acids or of 2-oxoglutarate, or of the pathways whereby citrate is synthesized entirely from C_2 substrates in general and glycollate in particular. The formation of citrate from glycollate by *Pseudomonas* OD 1 is a new reaction which appears worthy of further study. The isotope experiments reported in this paper already show one interesting feature of this process, namely, that there is a preferential incorporation of the C-2 atom of glycollate or glycine into citrate. Further experiments on the mechanism of citrate synthesis by this organism will be published in a subsequent paper.

SUMMARY

1. The oxidizing abilities of washed suspensions of cells of *Pseudomonas* OD1 grown on oxalate, glycollate, lactate, malate and succinate have been examined. Q_{O_2} values are low compared with those for other species of *Pseudomonas*. The oxidizing powers show some response to growth substrate, but there is no clear-cut case of adaptive oxidation of any of the test substrates (mainly C_2 acids and tricarboxylic acid cycle intermediates). In general, cells grown on lactate displayed the best oxidizing ability.

2. With cells grown on lactate, 0·001M fluoroacetate strongly inhibits the oxygen uptake due to glycine, glycollate or 2-oxoglutarate, but has little effect on that due to acetate or pyruvate. Under the same conditions, the amount of citrate accumulating is increased by glycollate, glycine, pyruvate, succinate, fumarate, malate, oxaloacetate and 2-oxoglutarate, but not by acetate or oxalate.

3. The highest amounts of extra citrate were produced from 2-oxoglutarate, glycollate and glycine, representing about a 50, 30 and 15% conversion, respectively, of substrate carbon into citrate.

4. Glycollate appears to act as the sole source of carbon for the extra citrate synthesized in its presence. The glycollate carbon not converted into citrate appears as carbon dioxide.

5. The two carbon atoms of glycollate do not contribute equally to the synthesis of citrate; there is a preferential incorporation of the C-2 atom.

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Carbohydrate Metabolism in Citric Acid Fermentation

5. PURIFICATION AND PROPERTIES OF *ZWISCHENFERMENT* FROM *ASPERGILLUS NIGER**

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Zwischenferment, which catalyses the dehydrogenation of glucose 6-phosphate by triphosphopyridine nucleotide, was discovered by Warburg & Christian (1931). The high specificity of the enzyme makes it valuable for the estimation of glucose 6-phosphate, triphosphopyridine nucleotide, fructose 6-phosphate, glucose 1-phosphate, hexokinase, phosphoglucomutase and phosphoglucoisomerase. *Zwischenferment* from yeast has been purified about 500-fold by Negelein & Gerischer (1936). A simple procedure for the preparation of yeast *Zwischenferment* suitable for the estimation of glucose 6-phosphate and triphosphopyridine nucleotide has been described by Kornberg (1950). But the specific activity

* Part 4: Jagannathan, Kartar Singh & Damodaran (1956).