Organic Acid Metabolism of *Penicillium chrysogenum*

1. LACTATE AND ACETATE

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The media most useful for penicillin production contain corn-steep liquor (CSL), which has a high organic acid content. Such media usually contain about 5 mg./ml. lactic acid and rather less than 0.5 mg./ml. acetic acid. It has been found beneficial to include organic acids, especially the two mentioned, in synthetic media for penicillin production (Stone & Farrell, 1946; Jarvis & Johnson, 1947; Calam & Hockenhull, 1949), and in general, media containing both acids gave better yields than those with either alone. It was considered that a study of the metabolism of these acids by *Penicillium chrysogenum* might throw light upon the biosynthesis of penicillin, and in particular upon the mode of formation of the branched-chain five-carbon skeleton of penicillamine. Dimethylpyruvic acid ($\text{CHMe}_2\text{CO}\cdot\text{CO}_2\text{H}$), a compound with a carbon chain similar to that of penicillamine, has been isolated from *Aspergillus niger* by Hida (1935) and, later, by Ramachandran & Walker (1951, 1952), but whereas our organism was found to produce keto acids, this acid was not isolated under parallel conditions. When lactate was used as starting material, α -oxoglutaric acid was isolated (Hockenhull, Wilkin & Winder, 1951). This work has been extended to cover other aspects of the metabolism of lactate and acetate so as to elucidate their general effects on the metabolic processes of *P. chrysogenum*.

EXPERIMENTAL

Organism. *Penicillium chrysogenum* Q176 var. *breviserigma* described by Foster (1949) was used throughout.

Cultural conditions. These were similar to those described earlier by Hockenhull, Walker, Wilkin & Winder (1952). A sporulating culture was grown on glycerol-molasses-peptone agar slopes for 6-8 days. The spores were removed by shaking with 0.1% (v/v) aqueous Calsolene Oil HS (Imperial Chemical Industries Ltd.) to give suspensions containing 10^7 - 10^8 spores/ml. This suspension was transferred into 20 vol. of development medium which was incubated under standard conditions for 48 hr. Samples of this vegetative inoculum (3 ml.) were used to inoculate 60 ml. of final medium contained in 250 ml. conical flasks plugged with cotton wool. The cultures were incubated on a rotary shaker at 25°.

Media

Filtered CSL was used throughout. Removal of the sediment (comprising dead bacteria, etc.) did not significantly affect fermentation performance when this was based on the total solids content. The CSL was used on a total solids weight basis throughout ('CSL solids'), although it was added to the medium as filtered liquor containing about 40% of dissolved solids.

Development of medium. 'CSL solids' (3.8%), brown sugar (2%), phenylacetic acid (0.01%), precipitated chalk (1%), all made up (w/v) in distilled water. The pH was adjusted to 6.2 with 10N-KOH (pH after steam sterilization for 20 min. at 15 lb./sq.in. was 5.7-5.9).

CSL medium. 'CSL solids' (3.8%), lactose (3%), precipitated chalk (1%), all made up (w/v) in distilled water. No adjustment of pH was made (pH after steam sterilization for 20 min. at 15 lb./sq.in. was 5.2-5.5).

Synthetic medium. (Based on that of Jarvis & Johnson, 1947.) Lactose (2.25%), glucose (0.75%), ammonium acetate (0.3%), potassium lactate (1.0%), phenylacetic acid (0.2%), KH_2PO_4 (0.3%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025%), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01%), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0004%), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (0.0004%), Na_2SO_4 (0.05%), precipitated chalk (1.0%), all made up (w/v) in distilled water and adjusted to pH 6.3 with 10N-KOH before sterilization. (pH after autoclaving 20 min. at 15 lb./sq.in. steam, 5.9-6.0.)

Preparation of mycelial suspensions. Mycelium was harvested routinely at 24 hr., when the mycelial dry weight was 15-20 mg./ml. The techniques described earlier by Hockenhull *et al.* (1952) were followed by washing and starving of the mycelium. All operations were carried out aseptically.

Analytical methods

Ketonic acids. The keto acids produced were identified by paper chromatography of either the 2:4-dinitrophenylhydrazones or the nitroquinoxalinols (Hockenhull & Floodgate, 1952). As the following α -keto acids, pyruvic, oxalacetic, oxoglutaric, oxobutyric and dimethylpyruvic, might be encountered in this type of work, the methods used were tested with mixtures of the appropriate derivatives.

The 2:4-dinitrophenylhydrazones were prepared by addition of excess of a saturated solution of 2:4-dinitrophenylhydrazine in N-HCl to filtrates containing keto acids. The derivatives were then extracted with ethyl acetate, or if a copious precipitate appeared, this was filtered off, dried, and dissolved in ethyl acetate. The filtrate was extracted three times with 0.5 vol. ethyl acetate. Further purification was effected by extracting the ethyl acetate solution three

times with 0.25 vol. 5% (w/v) anhyd. Na_2PO_4 , the aqueous solution was then acidified with H_2SO_4 and the 2:4-dinitrophenylhydrazones were extracted with ethyl acetate (three times with 0.125 vol.).

The nitroquinoxalinols were prepared by addition of 0.2 vol. concentrated HCl , and a calculated excess of 1:2-diamino-4-nitrobenzene as a 1% (w/v) solution in 2*N*- HCl . The mixtures were warmed to 60° and allowed to stand 16 hr. at room temperature. If there was any appreciable amount of precipitate, it was removed by filtration and examined separately after dissolving in ethyl acetate. The derivatives were transferred from the filtrate to ethyl acetate by extracting three times with 0.5 vol. of the solvent and the combined extracts were treated three times with 0.25 vol. 2*N*- NaOH . The alkaline solution of nitroquinoxalinols so obtained was acidified and the derivatives were transferred again to ethyl acetate (three extractions with 0.125 vol. solvent). The resultant solution was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure.

For the qualitative separation of the 2:4-dinitrophenylhydrazones the procedure of Cavallini, Frontali & Toschi (1949) was used with Whatman no. 4 paper. The chromatograms were developed by the ascending method with mixtures of aliphatic alcohols and dilute ammonia. The R_F values obtained are given in Table 1.

The pyruvic acid derivative gave rise to two spots, and in the early stages of this work this gave rise to some confusion. A similar phenomenon has been described by Walker, Hall & Hopton (1951), and Seligson & Shapiro (1952); it would seem probable that there are two stereoisomeric forms of pyruvic 2:4-dinitrophenylhydrazone (cf. Gordon, Wopat, Burnham & Jones, 1951). When nitroquinoxalinols were prepared from solutions giving rise to these two 'pyruvate' spots, only one derivative was obtained. A further artifact was *N*-acetyl 2:4-dinitrophenylhydrazide formed, presumably, by reaction between ethyl acetate and 2:4-dinitrophenylhydrazine. The artifact isolated after removal of the derivatives of keto acids by passage through an Al_2O_3 column. A quantity obtained from several experiments was twice recrystallized from hot aqueous ethanol as red-brown needles, m.p. 193°; the mixed melting point with authentic *N*-acetyl-2:4-dinitrophenylhydrazide (m.p. 194°) was 193–194°. (Found: C, 39.9; H, 3.6; N, 23.9. Calc. for $\text{C}_8\text{H}_8\text{N}_4\text{O}_6$: C, 40.0; H, 3.3; N, 23.3%). This artifact was reduced to a negligible amount by keeping the temperature at which the solvent was evaporated low, by keeping the time of contact with solvent as short as possible, and by removal of excess 2:4-dinitrophenylhydrazine as soon as possible.

The nitroquinoxalinols were separated by paper chromatography (Hockenhull & Floodgate, 1952). Since carrying out this work the technique has been further improved (Hockenhull, Hunter & Herbert, 1953).

Quantitative separation of 2:4-dinitrophenylhydrazones was carried out by a method based on that of Datta, Harris & Rees (1950). A typical separation was carried out as follows. Al_2O_3 (Peter Spence and Sons Ltd., Widnes, grade O) was washed with acid by stirring in 5 vol. water and adding 2*N*- H_2SO_4 until the pH became constant at 4.5 (about 30 min.). It was then drained, washed with 20 vol. water, and dried in a vacuum oven at 60°. The product (25 g.) was made into a slurry with ethyl acetate and poured into a 25 mm. chromatogram tube to give a column 120 mm. long after settling. About 100 mg. of the mixed derivatives were put on to the column in about 30 ml. ethyl acetate. Development was carried out with successive 50 ml. portions of ethyl acetate:ethanol mixtures (1:0, 4:1, 1:1, v/v). A slight broadening of the band was noted with the last. Pure ethanol caused a further slight broadening of the band, but 80% (v/v) aqueous ethanol (100 ml.) washed the α -oxobutyrate and dimethylpyruvate derivatives right down the column. On the other hand, there was so much spreading that separation did not occur. The pyruvate derivative also moved in the same solvent with marked spreading and could be completely eluted with 100 ml. 50% (v/v) aqueous ethanol. The α -oxoglutarate derivative did not move. The pyruvate isomers were not separated. In an attempt to obtain sharper separations of dimethylpyruvate, α -oxobutyrate and pyruvate, 90% (v/v) aqueous ethanol containing 2% (w/v) sodium acetate ($\text{CH}_3\text{CO}_2\text{Na}$, $3\text{H}_2\text{O}$) was employed, but, although the substances moved as compact bands, they were not separable. No suitable eluent could be found for α -oxoglutarate. This was removed, after draining the column, by extracting with 10% (w/v) Na_2PO_4 . Neutral products, including *N*-acetyl-2:4-dinitrophenylhydrazide, were washed through in the ethyl acetate. Further purification of the eluates was achieved by evaporation under reduced pressure followed by recrystallization from aqueous ethanol or ethyl acetate light petroleum (b.p. 40–60°).

The nitroquinoxalinols, if they appeared homogeneous on the paper chromatogram, were purified by recrystallization. If different components were present they were separated by chromatography on Al_2O_3 (Hockenhull & Floodgate, 1952), and the fractions were recrystallized after evaporation under reduced pressure. The dimethylpyruvate and pyruvate derivatives crystallized best from hot aqueous ethanol, that of α -oxoglutarate from ethyl acetate:light petroleum (b.p. 40–60°).

Table 1. Paper chromatography of some 2:4-dinitrophenylhydrazones of α -keto acids

(5–10 μg . derivative in ethyl acetate spotted on to 35 mm. strips of Whatman no. 4 paper, developed in 500 ml. measuring cylinder by ascending flow of various solvents. Duration of experiments, 16 hr. Distance moved by solvent front, 24–27.5 cm. All solvents, except *sec.*-butanol, saturated with 0.8*N* aqueous NH_3 .)

2:4-Dinitrophenyl- hydrazone	R_F values				
	<i>sec.</i> -Butanol: 0.8 <i>N</i> - NH_4OH (4:1, v/v)	<i>n</i> -Butanol	Commercial amy lalcohol	<i>sec.</i> -Octanol	<i>n</i> -Hexanol
α -Oxoglutaric acid	0.33	0.055	0.00	0.00	0.00
Pyruvic acid (i)	0.77	0.48	0.18	0.03	0.10
Pyruvic acid (ii)	0.85	0.61	0.41	0.18	0.31
α -Oxobutyric acid	>0.95	0.76	0.56	0.30	0.47
Dimethylpyruvic acid	>0.95	0.83	0.68	0.47	0.62

Lactic acid. This was assayed either by the method of Barker & Summerson (1941) or by that of Friedemann, Cotonio & Shaffer (1927), using the apparatus of Shinn & Nicolet (1941) but, where interference from pyruvate was likely, a modification of the ceric sulphate oxidation method of Gordon & Quastel (1939) was used. The methods agreed well. Lactic acid (90%), B.P., was used throughout.

Reducing sugars. These were determined by the method of Shaffer & Hartmann (1920) after hydrolysis in 0.8N-HCl for 30 min. at 15 lb. steam/sq.in.

Acetic and other volatile acids. These were determined by the method of Friedemann (1938) using the apparatus as modified by Hockenhull (1949). The acids were distilled in a current of steam after acidification and addition of sodium tungstate and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The distillate was decarbonated by passing CO_2 -free air, and then titrated with CO_2 -free alkali using phenol red as indicator. Determination of the partition coefficient of the volatile acids between ether and water indicated that, in the absence of phenylacetic acid or similar benzyl precursors acetic acid was the only volatile acid present in culture filtrate. Glacial acetic acid (A.R.) was used throughout.

Manometric measurements. These were carried out in a standard type Warburg apparatus (Hospital and Laboratory Supplies Ltd., London, E.C. 1), using 20 ml. cups with centre wells and single side bulbs. All observations were made at 25°. $Q_{O_2} = \mu\text{l. O}_2/\text{mg. dry weight/hr.}$

RESULTS

Lactate metabolism

Lactate utilization by growing cultures. Replicate cultures on synthetic medium were harvested daily in triplicate. The sugar and lactate contents of the media were determined, and the mycelium was used for estimation of the Q_{O_2} with lactate as the substrate (Fig. 1). Cultures grown on CSL medium were also examined (Fig. 2).

Lactate disappeared extremely slowly during the first 96 hr. from both media; this was in marked contrast with the results obtained by Jarvis & Johnson (1947), with the parent strain *P. chrysogenum* Q. 176. In CSL medium, however, there was a marked increase in rate of lactate disappearance after 96 hr. At this time, the sugar level had fallen to about 40% of the initial value. This increased rate of lactate utilization appeared to coincide roughly with a rapid rise in penicillin titre. On the other hand, in synthetic medium the rate of lactate disappearance remained extremely slow for the full 120 hr. growth period.

Examination of the Q_{O_2} (lactate) values of washed, starved mycelium from the synthetic medium, showed that the lactate disappearance in growing cultures (2 mg./ml. over 120 hr.) was far less than that calculated from the oxygen usage by washed suspensions taken at intervals over this period (about 28 mg./ml.). Assuming that the washing and starving techniques had not increased the ability of mycelium to use lactate, it appeared

Fig. 1. Disappearance of lactate in synthetic medium. Shaken cultures at 25°. 60 ml. synthetic medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. ○—○, lactic acid; □—□, reducing sugar after hydrolysis; ●—●, Q_{O_2} (lactate) values given by mycelial sample after washing and starving according to standard technique at 25°.

Fig. 2. Disappearance of lactate in CSL medium. Shaken cultures at 25°. 60 ml. CSL medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. ○—○, lactic acid; □—□, reducing sugar after hydrolysis; △—△, pH values.

that some factors slowing up the utilization of lactate were present in the medium.

Effect of glucose upon lactate utilization. An experiment was carried out using mycelium washed and starved in 0.1M potassium phosphate (pH 7.3) according to the standard technique. This was washed 3 times and suspended in 0.1M potassium phosphate (pH 5.0). Samples (20 ml.) of this suspension were measured into 250 ml. conical flasks and followed by 10 ml. of the pH 5.0 buffer containing appropriate amounts of the substrates. The flasks were shaken aerobically at 25° after withdrawal of a 5 ml. homogeneous sample for analysis. After 6 hr. the reaction mixtures were again sampled. The two sets of samples were filtered and the amounts of lactate determined (Table 2).

Table 2. *Effect of glucose on the lactate-pyruvate system*

(25 ml. suspension (about 15 mg./ml. dry weight) on 0.1 M potassium phosphate, pH 5.0 and containing added substrates neutralized with KOH placed in 250 ml. conical flasks and shaken 6 hr. at 25°. Lactate determinations at 0 and 6 hr.)

Expt.	Substrates added			Utilization of lactic acid (mg./ml.)	Average (mg./ml.)
	Lactic acid (4 mg./ml.)	Pyruvic acid (4mg./l.)	Glucose (40 mg./ml.)		
1	+	-	-	1.54 2.02 2.02 1.92	1.87
1	+	-	+	0.54 0.31 0.11 0.33	0.32
2	+	-	-	0.17* 0.70 1.01	0.63
2	+	+	-	0.75 0.89 0.12	0.59
2	+	-	+	0.05 0.37 0.25	0.22
2	-	+	-	0.00 0.01 0.02	0.01
2	-	+	+	-0.26 -0.20 -0.32	-0.26
2	-	-	+	-0.05 -0.07 +0.15	0.01

* Note low value for lactic acid utilization.

Although, in absence of glucose, there was sometimes a very slow utilization of lactate (one result marked * in Table 2), it is clear that in general glucose suppressed lactate utilization, while it did not give rise to lactate on its own. Glucose, however, caused the reduction of pyruvate to lactate. Washed, starved mycelium would not oxidize lactate anaerobically with either methylene blue, resazurin or potassium ferricyanide (Quastel & Wheatley, 1938) as acceptors. All subsequent respirometric studies were carried out aerobically (in air).

Effect of hydrogen-ion concentration on oxygen uptake. Washed, starved suspensions were prepared according to the standard technique. Starving was carried out in 0.1 M potassium phosphate at pH 7.3 and after washing re-suspended in buffer at the pH to be investigated. The Q_{O_2} (lactate) values were determined over 30 min. in Warburg manometers. Fig. 3 shows the relation of activity to pH.

Effect of substrate concentration on oxygen uptake. Washed, starved suspensions at pH 7.3 were used.

Fig. 3. Effect of hydrogen-ion concentration upon Q_{O_2} (lactate) values. Warburg flasks. Main compartment: 2 ml. washed, starved suspension (30 mg. dry wt.) in 0.1 M potassium phosphate at appropriate pH. Side bulb: 0.3 ml. 0.1 M potassium lactate at appropriate pH. In centre well: 0.2 ml. 10 N-KOH on filter paper, 25°. Air. ○—○, Q_{O_2} (lactate) corrected for endogenous respiration; □—□, Q_{O_2} (endogenous) controls.

Their Q_{O_2} (lactate) were measured in Warburg manometers against various concentrations of lactate over 30 min. The curve obtained (Fig. 4) was somewhat shallower than the typical Michaelis curve. This appeared to be due to further metabolism of pyruvate and was probably further complicated by exhaustion of substrate which made it difficult to estimate accurately the initial velocity at the lower concentration. The lowest concentration of lactate used (0.0001 M) gave a total oxygen consumption of 2.8 moles/mole lactate. Complete oxidation to carbon dioxide required 3 moles.

The same experiment was repeated in presence of arsenite. Owing to the inhibition of the oxidation of pyruvate by arsenite, the amount of oxygen consumed was reduced to one-sixth, which was below the limit of accuracy of the manometric method. Accordingly, the amount of keto acid formed in 20 min. was measured using the method of Friedemann & Haugen (1943). When the amount of keto acid produced was plotted against substrate concentration, a Michaelis-type curve ($K_m = 2 \times 10^{-4}\text{ M}$) was obtained (Fig. 4). It was confirmed by paper chromatography that the substance measured was pyruvate. It must be emphasized that the Michaelis constant is merely a convenient symbol, and does not have the same meaning for whole cells as that defined originally for isolated enzymes.

Effect of inhibitors on oxygen uptake. The oxygen uptake of suspensions of *P. chrysogenum* at pH 7.3 prepared in the usual way was studied in presence of various inhibitors. Arsenite (0.01 M) and iodoacetate (0.001 M) showed considerable inhibition. On the other hand, 2:4-dinitrophenol had no effect on the oxygen uptake in the presence of substrate, although it increased the endogenous respiration (Table 3). Malonate (0.01 M) inhibited lactate oxidation at neither pH 5.2 nor 7.2. Streptomycin (0.001 M) and methylene blue (0.001 M) were also found to have no effect. Arsenite (0.01 M) and iodoacetate (0.001 M) were found to inhibit oxidation of pyruvate.

Products obtained by aerobic metabolism of lactate. Keto acids accumulated when washed, starved mycelium, suspended in 0.1 M potassium phosphate, pH 7.0–7.5, was incubated with lactate aerobically

Fig. 4. Effect of substrate concentration upon Q_{O_2} (lactate). Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (about 30 mg.) in 0.1 M potassium phosphate buffer pH 7.3. Side bulb: 1 ml. potassium lactate at appropriate concentration (10^{-5} – 10^{-3} M). In centre well: 0.2 ml. 10 N-KOH on filter paper, 25° . Air. ' K_m ' = $2\text{ M} \times 10^{-4}$. All figures were corrected for endogenous respiration or production of pyruvate. The latter correction was negligible.

Table 3. *Effect of inhibitors on Q_{O_2} (lactate) and Q_{O_2} (pyruvate) values*

(Warburg manometers. In main vessel, washed, starved mycelial suspension (25–30 mg. dry wt.) in 0.1 M potassium phosphate, pH 7.3, 0.3 ml. 0.1 M potassium lactate or potassium pyruvate, 0.3 ml. 0.1 M potassium phosphate. In side bulb, 0.3 ml. 0.1 M potassium phosphate containing inhibitor. In centre well, 0.1 ml. 10 N-KOH on filter paper. Respiration measured over 20 min. at 25° in air.)

		Concen- tration (M)	Q_{O_2} values				Inhibi- tion (%)
Substrate	Inhibitor		Control	With inhibitor	With substrate	With substrate and inhibitor	
Potassium lactate	Sodium arsenite	0.01	2.95	2.15	11.80	3.55	85
			2.95	2.10	11.40	3.25	
Potassium lactate	Sodium arsenite	0.01	2.05	1.25	3.80	1.30	95
Potassium lactate	2:4-Dinitro- phenol	0.001	2.05	2.40	3.80	4.25	0
				2.30		4.00	
Potassium lactate	Potassium iodoacetate	0.001	2.15	1.25	2.95	1.15	100
			1.85	1.15	2.85	1.10	
Potassium pyruvate	Sodium arsenite	0.01	3.50	1.60	4.80	2.00	65
			3.25	1.50	4.55	1.95	
Potassium pyruvate	Potassium iodoacetate	0.001	2.70	2.10	4.25	2.15	100
			2.85	2.20	4.20	2.15	

for 10–24 hr. in the presence of either 0.01M sodium arsenite or 0.001M 2:4-dinitrophenol. For an extended period, during which most of the reported results were obtained, the yields of keto acids accounted for 20–40 % of the lactate carbon supplied. However, after an interval of some months, when it was desired to check and extend certain observations, great difficulty was encountered in getting yields even as high as 5–10 %, although the procedure was exactly the same. Finally, for no apparent reason, yields of the order of 20 % were again encountered. This variation, which was not susceptible to our direct control, might have been due to such phenomena as differences in the nitrogen retention of the mycelium, or the leaching of essential substances during starvation, or even to the previous mycological history of the organism.

With all treatments, both pyruvate and α -oxoglutarate were obtained in varying ratios from 0 to 100 %. Again these were not predictable.

The accumulation of α -oxoglutarate in presence of arsenite is in apparent contradiction to the results quoted in the substrate concentration experiment (Fig. 4) though here conditions were very different (incubation time 24 hr. as against 30 min.; substrate concentration 0.11M as against 0.01M).

Metabolic products obtained at pH 5.0. The isolation and characterization of the 2:4-dinitrophenylhydrazone of α -oxoglutaric acid under these conditions have been described already (Hockenhull *et al.* 1951). Similar experiments yielded pyruvic acid almost exclusively. This was characterized as both the 2:4-dinitrophenylhydrazone and as the 3-methylnitroquinoxalin-2-ol. Washed, starved suspensions from 60 ml. of broth prepared under the standard conditions and re-suspended in 60 ml. 0.1M potassium phosphate of pH 5.0 (about 15 mg./ml. dry weight) were shaken at 25° in 250 ml. flasks for 24 hr. Reaction mixtures from three such flasks were filtered and treated with about 1 g. 1:2-diamino-4-nitrobenzene; the product which was mainly the derivative of pyruvate was purified as described above, and recrystallized from hot aqueous ethanol to yield about 300 mg., m.p. 254–255° (authentic 3-methylnitroquinoxalin-2-ol, 255–6°, mixed m.p. 254–255° (uncorr.)). (Found: C, 52.1; H, 3.3; N, 20.9. Calc. for $C_9H_7N_3O_3$: C, 52.7; H, 3.4; N, 20.5 %.)

Metabolic products obtained in presence of arsenite. A washed, starved, mycelial suspension in 0.1M potassium phosphate of pH 7.4 (15 mg./ml. dry weight) was prepared from an equal volume of 24 hr. culture on synthetic medium. To this was added 10 mg./ml. lactic acid as the potassium salt, and sodium arsenite to a final concentration of 0.01M. The suspensions were shaken in 250 ml. flasks, 60 ml. in each, for 24 hr. at 25°, and then filtered. From 280 ml. of filtrate (five flasks), 400 mg. of

crude 2:4-dinitrophenylhydrazone were isolated. This was almost wholly the derivative of α -oxoglutarate. Its characterization is described elsewhere (Hockenhull *et al.* 1951).

Other experiments yielded pyruvate equivalent to 30–40 % of the lactate carbon supplied.

Metabolic products obtained in presence of 2:4-dinitrophenol. An experiment similar to the above but in which 0.001M dinitrophenol was used instead of arsenite yielded about 1.0 g. of 2:4-dinitrophenylhydrazones from eight flasks. The mixed 2:4-dinitrophenylhydrazones were separated on an alumina column and the α -oxoglutarate derivative was isolated. Recrystallization of the product gave three fractions weighing 142, 359 and 202 mg. respectively. These melted between 212 and 215°. After being pooled and recrystallizing from ethyl acetate:light petroleum, a pure product m.p. 218–219°, giving no depression of melting point with authentic material (m.p. 219°), was obtained.

In a similar experiment 800 mg. of impure dinitrophenylhydrazones consisting largely (about 80 %) of the derivative of pyruvate were obtained. After purification by chromatography on alumina and recrystallization of the 450 mg. of derivative in the eluate, a product, m.p. 217–218° (mixed m.p. 218° with authentic material), was isolated.

Acetate metabolism

Acetate utilization by growing cultures. Replicate cultures on synthetic medium were harvested daily in triplicate and the amounts of acetate determined. It was found that the acetate had almost completely disappeared by the time that the sugar level had fallen to some 50 % of its initial value (Fig. 5). Similar behaviour was shown in CSL medium as recorded in a previous paper (Hockenhull *et al.* 1952, Fig. 1).

Fig. 5. Disappearance of acetate in synthetic medium. Shaken cultures at 25°. 60 ml. synthetic medium/250 ml. flask. Triplicate flasks removed at intervals for analysis. O—O, Acetic acid; □—□, reducing sugars after hydrolysis.

Effect of hydrogen-ion concentration on oxygen uptake. Washed, starved suspensions were prepared according to the standard techniques. Starving was carried out at pH 7.3 with subsequent washing and re-suspension in buffer at the pH value to be investigated. The Q_{O_2} values were determined during 30 min. in Warburg manometers. Fig. 6 shows the relation of activity to pH. The optimum lay between pH 6 and 7.

Effect of substrate concentration on oxygen uptake. Washed, starved suspensions prepared according to the standard technique at pH 7.0 throughout were used. The Q_{O_2} values were determined in Warburg manometers over 15 min. with different levels of

potassium acetate. A curve, resembling in form the typical Michaelis curve for a single enzyme, was obtained. The concentration at which half maximum activity was shown was higher than might be expected (0.0012 M) (Fig. 7).

Effect of inhibitors on oxygen uptake. Marked inhibition occurred in presence of arsenite, iodoacetate or sodium azide (Table 4); on the other hand, malonate (0.01 M), streptomycin (0.001 M) and methylene blue (0.001 M) were ineffective as inhibitors. Calcium ions (0.1 M), added to trap oxalate, did not affect the amount of oxygen taken up in presence of acetate. This implied that acetate was not oxidized to carbon dioxide by way of oxalate.

Fig. 6. Effect of hydrogen-ion concentration upon Q_{O_2} (acetate) values. Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (30 mg. dry wt.) in 0.1 M potassium phosphate buffer at appropriate pH, 0.7 ml. water. 0.3 ml. 0.1 M potassium acetate at appropriate pH. In centre well: 0.2 ml. 10 N-KOH on filter paper. ○—○ and □—□ represent two similar experiments both at 25° in air.

Fig. 7. Effect of substrate concentration upon Q_{O_2} (acetate) values. Warburg flasks. Main compartment: 2 ml. washed, starved mycelial suspension (30 mg. dry wt.) in 0.1 M potassium phosphate buffer pH 7.3. Side bulb: 1 ml. potassium acetate, pH 7.3, at appropriate concentration (10^{-5} – 10^{-2} M). In centre well: 0.2 ml. 10 N-KOH on filter paper, 25°. Air. ' K_m ' = $1.2 \text{ M} \times 10^{-3}$.

Table 4 *Effect of inhibitors on Q_{O_2} (acetate) values*

(Warburg manometers. In main vessel, washed, starved mycelial suspension (25–30 mg. dry wt.) in 0.1 M potassium phosphate, pH 7.3, 0.3 ml. 0.1 M potassium acetate, 0.3 ml. 0.1 M potassium phosphate. In side bulb, 0.3 ml. 0.1 M potassium phosphate containing inhibitor. In centre well, 0.1 ml. 10 N-KOH on filter paper. Oxygen uptake measured over 20 min. at 25° in air.)

Expt.	Inhibitor	Concentration (M)	Q_{O_2} (acetate) values				Inhibition (%)
			Control	With inhibitor	With substrate	With substrate and inhibitor	
1	Sodium arsenite	0.01	{2.5 2.40}	{1.10 1.10}	{6.30 6.00 6.10}	{1.10 1.10}	100
2	Potassium iodoacetate	0.001	{2.60 2.55}	{2.40 2.45}	{6.00 5.80}	{2.40 2.50}	100
3	Potassium iodoacetate	{0.01 0.001 0.0001 0.00001}	1.45	{1.80 1.50 1.50 1.40}	2.55	{1.75 1.70 1.70 2.85}	100 80 80 -30
4	NaN ₃	0.003	{2.55 2.30}	{2.75 2.55}	{5.30 5.40}	{3.60 3.55}	70

Products obtained by aerobic metabolism of acetate and other acids related to the tricarboxylic acid cycle. Attempts were next made to demonstrate the formation of ketonic acids from acetate using the three sets of conditions under which these compounds were produced from lactate. Very small amounts of keto acids were produced from acetate, although parallel experiments with lactate as the substrate gave high yields.

Similar experiments were carried out with certain of the intermediates of the tricarboxylic acid cycle and related compounds. Portions of mycelial suspensions at pH 5.0 were incubated with lactate, acetate, malate, fumarate, succinate, glutamate and citrate, respectively. The amount of substrate (neutralized with potassium hydroxide) was adjusted to give 4 g. carbon/l. (equivalent to 1%, w/v, lactic acid). Incubation was carried out in 60 ml. flasks as described above. The keto acids were converted into 2:4-dinitrophenylhydrazones, which

Table 5. *Production of ketonic products from various organic acids*

(24 hr. incubation at 25° with shaking of 250 ml. conical flask containing 60 ml. mycelial suspension (25–30 mg./ml. dry wt.) in 0.1M potassium phosphate, pH 5.0, together with organic acid neutralized with 10N-KOH at a carbon level corresponding to 1% lactic acid.)

Substrate	Yield of 2:4-dinitrophenylhydrazones equivalent to 12 g. original C. (g.)	Approx. proportions of constituents	
		α -Oxo-glutarate (%)	Pyruvate (%)
Lactate	18.8	30	70
No addition	Negligible	.	.
Acetate	2.3	0	100
Malate	6.6	40	60
Fumarate	9.9	70	30
Succinate	5.3	100	0
Glutamate	1.6	100	0
Citrate	Negligible	.	.

Table 6. Q_{O_2} values for members of the tricarboxylic acid cycle

(Warburg manometers. In main vessel: washed, starved mycelial suspension (about 30 mg. dry wt.) in 2.7 ml. 0.1M potassium phosphate, pH 7.3. In side bulb, substrate pH 7.3 at 0.1M. In centre well, 0.1 ml. 10N-KOH on filter paper. Oxygen uptake measured over 20 min. at 25° in air.)

Substrate	Q_{O_2} (total) (uncorrected)	Q_{O_2} corrected for endogenous respiration
Acetate	6.10	3.40
Lactate	3.80	1.10
Succinate	5.55, 5.50	2.85, 2.80
Fumarate	4.65, 4.70	1.90, 2.00
Malate	6.20, 6.70	3.50, 3.40
Citrate	3.70, 3.40	0.95, 0.65
None	2.75, 2.70*	0, 0

* Endogenous respiration.

were then treated as described on p. 74. The final solution in ethyl acetate was dried over anhydrous sodium sulphate and then evaporated to dryness and the product was weighed. Table 5 gives the weights of total 2:4-dinitrophenylhydrazones produced from the various substrates. A rough quantitative estimate of the relative amounts of pyruvate and α -oxoglutarate was made by paper chromatography. The estimate was made by visual comparison with standard mixtures, and the precision was about $\pm 10\%$. The experiment quoted (Table 5) was typical, but in view of the variability sometimes encountered, for example with lactate, caution must be exercised in the interpretation of these results. When glucose or lactose at comparable levels were used as substrate there was no appreciable accumulation of keto acids.

The oxygen consumption of washed, starved mycelium in the presence of acetate, lactate, fumarate, malate and citrate was measured, and the results, expressed as Q_{O_2} , are given in Table 6. The Q_{O_2} was smallest in the presence of citrate, which might imply that the conversion of this compound into α -oxoglutarate was slow. It must, however, be emphasized that the Q_{O_2} values varied from preparation to preparation.

Effect of incubation with acetate and lactate on the dry weight of the mycelium. Washed, starved mycelium suspended in phosphate buffer of pH 7.4 was incubated for 24 hr. with either acetate or lactate, and the dry weights of representative samples of mycelium were determined at the beginning and end of the experiment. The results are shown in Table 7. It will be seen that, in the absence of substrate, the dry weight fell over the 24 hr. period, and that addition of acetate increased this loss of weight. In contrast, incubation with lactate prevented any loss of weight. Inclusion of either cyanide, semicarbazide, 2:4-dinitrophenol or arsenite in the incubation mixture had little effect, although there was a slight loss of weight in the presence of the last two when lactate was the substrate.

Identification of tricarboxylic acid-cycle intermediates in the mycelium. Mycelium grown on 1 vol. CSL medium was harvested at 48 hr. It was spun off, washed 3 times in 0.1M potassium phosphate buffer, pH 7.3, re-suspended in 0.5 vol. of this buffer, and 0.5 vol. of a simplified double-strength medium pH 6.5 (lactose 4%, ammonium acetate 0.75%, precipitated chalk 1%, KH_2PO_4 0.1%, all w/v) was added aseptically. This suspension was incubated under standard conditions (60 ml./250 ml. conical flask) at 25° for 24 or 66 hr.

At the end of the chosen period the mycelium was filtered off and extracted with hot 50% (v/v) aqueous ethanol (5 ml./flask). The extract so obtained was studied by descending chromatography on Whatman no. 4 paper with the following solvent mixtures: *n*-butanol:formic acid:water (4:1:5, v/v), mesityl oxide:formic acid:water (1:1:2, v/v), and isopropanol:tert.-butanol:benzyl alcohol:formic acid:

Table 7. *Effect of lactate or acetate upon mycelial weight*

(250 ml. shake flasks containing about 15 mg./ml. mycelium harvested at 24 hr. suspended in 0.1 M potassium phosphate, pH 7.3, containing either 1% acetic acid or 1% lactic acid as K salts, together with additional trapping agents or inhibitors as indicated. Incubated 24 hr. with shaking at 25°.)

Addition	Mycelial dry weight (g./flask)			
	Initial (0 hr.)	Control	Final (24 hr.)	
			Acetic acid 1%	Lactic acid 1%
None	0.88	0.65	0.47 (72)*	0.82 (126)
None	0.93	0.73	0.52 (71)	0.80 (110)
0.01 M-KCN	0.88	0.65	0.49 (76)	0.92 (142)
0.01 M Semicarbazide	0.88	0.60	0.45 (75)	0.83 (138)
0.001 M 2:4-Dinitrophenol	0.88	0.67	0.51 (76)	0.72 (108)
0.01 M Sodium arsenite	0.93	0.74	0.51 (69)	0.75 (102)

* Figures in parentheses represent weight as percentage of final control.

water (10:10:30:2:10, v/v) and the aqueous layers were discarded after equilibration. The techniques were similar to those described by Bryant & Overell (1951), and Stark, Goodman & Owens (1951). They suffered from the disadvantage that lactate and succinate were not sharply separable.

The following acids were identified after 24 hr. incubation: glutamic acid (0.13 mg.), citric acid (0.10 mg.), malic acid (0.80 mg.), lactic or succinic acid (0.05 mg. as lactate), and fumaric acid (0.03 mg.). The quantities given were rough estimates ($\pm 25\%$) of the total quantities of acid produced in the felt from 60 ml. of original broth. Qualitatively similar results were obtained after 66 hr. incubation. The extract gave a positive reaction with sulphuric acid and ethanolic guaiacol, which suggested the presence of lactate.

Fermentations carried out using radioactive acetate. In an experiment designed for the production of penicillin containing radioactive carbon (to be described later), a medium (Calam & Hockenhull, 1949) was made up to contain $^{14}\text{CH}_3\text{COOH}$ (0.33 $\mu\text{C}/\text{mg.}$). After the growth of *P. chrysogenum* under standard cultural conditions for 6 days at 25°, the specific activity of the acetate fell from 0.33 to 0.0046 $\mu\text{C}/\text{mg.}$ This large dilution indicated that a considerable amount of acetate was produced by the mould. During the course of the experiment the acetate concentration fell from 2.5 to 0.20 mg./ml.

DISCUSSION

Glucose has been found to suppress lactate utilization by washed, starved mycelium and *P. chrysogenum*, and when glucose was added to such preparations in the presence of pyruvate, lactate was produced. It is interesting that this effect was observed under the highly aerated conditions obtained in shake-flask cultures.

Lactate is oxidized to pyruvate, and this step does not appear to be carried out unless oxygen is

supplied. Electron acceptors such as ferricyanide and methylene blue were ineffective. α -Oxoglutarate has been shown to be formed from lactate.

The most likely catabolic pathways open to pyruvate (and hence to lactate) are (a) oxidation to 'active acetate' and carbon dioxide, and (b) carboxylation by a Wood-Werkman type of reaction (Wood & Werkman, 1938), or one involving the malic enzyme of Ochoa, Mehler & Kornberg (1948). Further oxidation of 'active acetate' is known to proceed, at least in animal tissues, by the tricarboxylic acid cycle. This requires to prime it a supply of C_4 -dicarboxylic acids, a small amount of these acids being all that is required for continuous operation of the cycle. If, however, there is, at the same time, a synthesis of C_4 -acids from part of the pyruvate, it is not unreasonable to expect an accumulation of components of the cycle such as α -oxoglutarate. On the other hand, unless acetate itself can give rise to C_4 acids, no accumulation of α -oxoglutarate is to be expected, for, if there were, this would result in the locking up of the small basal amount of C_4 acids, with the result that the cycle would come to a stop. This clearly does not happen with acetate which is metabolized rapidly. It is possible that there is some wastage of C_4 acids, as they participate in the cyclic oxidation of acetate. If these are replenished from the mycelial contents, the increased mycelial wastage in presence of acetate might in some part be explained.

There is no reason to suppose that, in *P. chrysogenum*, acetate is condensed to a C_4 compound by a process such as that depicted by Ajl (1951) in his Fig. 2. This deficiency may be a specific property of the organism, or it may be due to lack of activation energy, for it has been reported by Foster *et al.* (1949, 1950) that the synthesis of fumarate from acetate is only 10–15% of that from a similar molar concentration of ethanol. It is improbable that any significant amount of acetate is oxidized directly by way of oxalic acid.

Attention is drawn to differences in the relationships between substrate concentration and enzyme activity for the two substrates, especially with respect to the acetate curve. While it is not in order to base any reasoning about mechanisms upon this type of evidence, there is nothing in the data to contradict the above hypotheses.

The high degree of dilution of radioactive acetate by the mould points to the internal production of acetate and its presence as a normal participant in mould metabolism. Acetate has been detected in cultures of *Aspergillus niger* (Chughtai, Pearce & Walker, 1947), and *A. nidulans* (Hockenhull, 1950), when glucose was the sole carbon source.

SUMMARY

1. Lactate and acetate are oxidized by growing cultures and washed, starved mycelial suspensions of *Penicillium chrysogenum*.

2. Mycelial suspensions oxidize lactate only in the presence of oxygen, and the optimum pH is in the range 4.0 to 6.0. In the presence of arsenite, half maximum velocity of lactate oxidation occurs at a substrate concentration of 2×10^{-4} M.

3. The first product of lactate oxidation is pyruvate. This is reduced back to lactate in presence of glucose. Lactate is not formed from glucose alone.

4. The Q_{O_2} (lactate) was inhibited by both arsenite (0.01 M) and iodoacetate (0.001 M); these compounds also inhibited the Q_{O_2} (pyruvate). Malonate, streptomycin, methylene blue or 2:4-dinitrophenol had no effect on the Q_{O_2} (lactate).

5. Prolonged aerobic incubation of mycelium with lactate under specified conditions leads to the accumulation of considerable amounts of pyruvate and α -oxoglutarate. Acetate, citrate and glutamate under the same conditions give very low yields of ketonic products. Succinate, malate and fumarate give intermediate amounts of keto acids, all much less than lactate. All these acids increase oxygen uptake by mould mycelium.

6. Acetate utilization, unlike that of lactate, is rapid in presence of glucose.

7. Acetate oxidation by mycelium is maximal between pH 6 and 7. The rate of oxidation of acetate is half maximum at an acetate concentration of 1.2×10^{-3} M.

8. The Q_{O_2} (acetate) is depressed by arsenite (0.01 M), iodoacetate (0.001 M), and azide (0.003 M). Malonate, streptomycin and methylene blue do not inhibit.

9. Acetate causes mycelium to 'waste' on prolonged incubation, whereas lactate tends to conserve mycelial dry matter.

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