proteins. Nevertheless, the high extractions here reported have been obtained under conditions less likely to cause grave alteration to the proteins than any so far reported in the literature.

8. Approximate calculations based on the extraction give the average protein content of tobacco leaves to be 15 % of the dry matter, of which 95 % is extractable.

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Oxidations in Acetobacter

By J. TOSIC, *Department of Biochemistry, University of Sheffield*

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The question of the intermediary stages of the bacterial oxidation of acetic acid is one of the major unsolved problems in the intermediary metabolism of bacteria. Quastel & Webley (1941) showed that aneurin and co-carboxylase can increase the rate of oxidation of acetic acid in strain number 4759* of the National Collection of Type Cultures, but other evidence indicated that pyruvate is not an intermediate in acetate oxidation.

In *Acetobacter*, acetic acid is an end-product under some conditions, especially when ethanol is the substrate (Brown, 1886), but it is readily oxidized under other conditions (Tosic, 1942). This observation was studied in detail, as it was thought that an organism in which incomplete oxidations occur might be specially suited for work on intermediary metabolism.

* This organism is listed as a '*Propionibacterium*', but it was shown by Krebs & Eggleston (1941) that the organism does not possess the generic characteristics of *Propionibacterium*.

EXPERIMENTAL

Material and methods

The organism. The original strain of *Acetobacter turbidans* (Cosbie, Tosic & Walker, 1942) was used in most experiments.

The cells were grown on a basal medium to which suitable organic substrates, such as glucose, glycerol, ethanol, and sodium lactate, were added. The basal medium contained: 5·0 g. 'Difco' yeast extract, 2·0 g. $(NH_4)_2SO_4$, 2·0 g. KH_2PO_4, 0·2 g. $Na_2HPO_4.2H_2O$, 2·0 g. $MgCl_2.6H_2O$ in 1000 ml. tap water. Larger quantities of the organism were grown in Roux bottles containing 100 ml. basal medium, substrates and 2% agar, at the optimum temperature for growth (25°). The inoculum per 100 ml. medium was a 2 ml. portion of a 24 hr. old test-tube culture grown in the basal medium. After 40 hr. incubation (unless otherwise stated) cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. The concentration of the 'stock suspension' was determined by drying 1 ml.

at 100° and weighing. Stock suspensions usually contained about 20 mg. cells (dry matter)/1 ml., and were stored at about 2° (see below).

Yields of cells. The effects of substrates and of the duration of the incubation on yields are summarized in Table 1.

Table 1. *Effect of the addition of substrates to the basal medium and of the period of incubation on the yield of bacteria*

(The data refer to one Roux bottle containing 100 ml. medium.)

Substrate added to basal medium	Yield of cells (mg. dry matter) after incubation at 25° for 24 hr.	40 hr.	48 hr.
None	5	7	8
1% glucose	27	40	46
1% glycerol	22	48	67
1% ethanol	40	76	80
1% Na lactate	75	110	130
0·5% Na lactate + 0·5% ethanol	130	150	160

Addition of ethanol, glucose, glycerol or Na-lactate greatly increased the yield of cells; the highest yield was obtained when the medium contained ethanol and lactate together. During growth, the former substrate yields acetic acid and the latter $NaHCO_3$, and as a result the medium retains a hydrogen ion concentration compatible with rapid growth longer than other substrates. An analogous 'buffer' effect has been recorded by Grey (1924) for *Esch. coli*.

Manometric procedure. The oxidative activity of cell suspensions was measured in the usual way in Warburg manometers at 25° in the presence of 0·1 M-phosphate buffer at pH 6·8. Conical cups (about 20 ml. capacity) with a centre well were used; substrates were added from the side arm.

Oxidative activity of cell-suspensions. The rate of oxidative metabolism of freshly harvested cells varied according to the age of the culture, the chemical nature of the medium on which the cells were grown and on the substrate of oxidation. The data in Table 2 indicate that the older

Table 2. *The influence of age of culture of* A. turbidans *on* Q_{O_2}

(Q_{O_2} in 0·1 M-phosphate buffer at pH 6·8 in the presence of ethanol; 25°.)

Additions to basal medium	Q_{O_2} at different ages of culture 24 hr.	40 hr.	48 hr.	72 hr.
1% glucose	84	*100*	54	5
1% glycerol	99	*154*	146	93
1% ethanol	314	*332*	196	141
1% Na lactate	87	*91*	72	5
0·5% ethanol + 0·5% Na lactate	*210*	201	180	120

cultures contained less active cells than the younger ones. They also show that some enzymes (e.g. those concerned with the oxidation of ethanol) were partially adaptive. Similar results were obtained when glucose, glycerol or lactate was the substrate of oxidation. As the cells grown on the medium containing lactate and ethanol together gave a high Q_{O_2} (as well as a high yield of cells) this medium was used throughout for further experiments, with a period of incubation of 40 hr.

The oxidative activity of cell-suspensions showed little loss on storage up to 12 days at about 2°. After 8 days' storage the fall of Q_{O_2} amounted to 10, 14 and 15% when tested on glucose, ethanol and lactate as substrates respectively.

Cells suspended in phosphate buffer alone, serving as controls, gave negligible if any oxygen uptake.

RESULTS

Oxidation of various substances. It will be seen from Table 3 that the number of substrates oxidized in *A. turbidans* is limited. The organism oxidized *n*-propanol but not *iso*-propanol; of all the sugars tested only glucose was attacked. Neither glycollic nor oxalic acid, which might be expected to be intermediates in the oxidation of acetic acid, was oxidized. The oxidation of ethanol, *n*-propanol, formate, lactate and pyruvate was very rapid, whilst that of the other oxidizable substances (Table 3) was relatively slow. All substrates, except ethanol, propanol and formate, showed a lag period during which the rate of oxidation gradually increased (Fig. 1). The high rate of formate oxidation suggests the possibility that this substance might be an intermediate in acetate oxidation.

Further experiments in which the oxygen uptake was measured until it ceased showed that the total oxygen consumption was 90–95 % of that required for complete oxidation in the case of formate, acetate, pyruvate and lactate. When ethanol was the substrate one molecule of oxygen was rapidly absorbed. This was followed by the slower uptake of two further molecules of oxygen as required for complete oxidation. Propanol absorbed one molecule of oxygen. In the case of glucose half a molecule of oxygen was rapidly absorbed. This was followed by the very slow uptake of about 80 % of that necessary for complete oxidation.

The lag period in the oxidation of acetate. The factors influencing the oxidation of acetic acid have been studied and attempts have been made to elucidate the cause of the lag period. At pH 5·9, 6·8 and 7·8 there was no difference in the length of the lag period or in the rate of oxidation of acetate (Table 4). The lag period was little affected by the concentration of acetate. Table 5 shows that the lag period was shortened and the initial Q_{O_2} raised by an increase of the density of cell suspensions. Storage of cell suspensions at about 2° prolonged the lag period as shown in Table 6.

Effect of various substances on the lag period in acetate oxidation. A relatively small quantity (1 μmol.) when added to a larger amount of acetate (5 μmol.) had a pronounced effect on both the reduction of the lag period and the rate of acetate

Table 3. *Oxidation of various substrates in* A. turbidans

(Main compartment: 1 ml. cell suspension; 1 ml. 0·1 M-phosphate buffer, pH 6·8; 0·9 ml. H_2O. Side arm: 0·1 ml. substrate (the acids added in form of neutral Na-salts). Centre cup: 0·2 ml. 2 N-NaOH; air; 25°.)

Substance tested	Added amount of: Substrate (μmol.)	Cell (mg.)	μl. O_2 absorbed after (min.): 10	20	40	60
Formate	20	4·0	90	142	170	178
Acetate	5	5·0	0	4	16	27
Glycollate	5	4·0	0	0	0	0*
Propionate	5	5·0	0	0	0	0*
dl-Lactate	5	3·7	48	132	237	254
Pyruvate	5	3·7	15	43	153	223
n-Butyrate	5	5·0	0	0	0	0*
Oxalate	5	4·0	0	0	0	0*
Malonate	5	4·0	0	0	0	0
Succinate	5	5·0	7	26	76	122
dl-Malate	5	3·7	0	0	0	5
l-Malate	1	5·0	0	0	0	6
Fumarate	1	5·0	5	14	48	67
α-Ketoglutarate	5	3·7	0	0	28	41
Citrate	5	4·0	0	0	0	0*
*l-iso*Citrate	5	4·0	0	0	0	0*
Methanol	5	5·0	0	0	0	0*
Ethanol	5	1·3	40	61	87	114†
n-Propanol	5	1·3	59	91	99	100†
*iso*Propanol	5	5·0	0	0	0	0
Glycerol	5	5·0	7	34	112	197
α-Glycerophosphate	5	5·0	0	0	0	0
β-Glycerophosphate	5	5·0	0	0	0	0
d-Sorbitol	5	5·0	0	0	0	0*
d-Mannitol	5	4·0	0	0	0	0*
Dulcitol	5	4·0	0	0	0	0*
i-Inositol	5	4·0	0	0	0	0*
Formaldehyde	1	5·0	2	7	10	17
d-Glucose	5	4·0	31	65	118	155
Galactose	5	4·0	0	0	0	0*
Mannose	5	4·0	0	0	0	0
Fructose	5	4·0	0	0	0	0
Maltose	5	4·0	0	0	0	0*
Lactose	5	4·0	0	0	0	0*
Sucrose	5	4·0	0	0	0	0*

* No O_2 uptake after 3 hr.

† O_2 uptake values lower than theoretically expected, owing to evaporation of the substrates from the side arm during equilibration before mixing.

Table 4. *Effect of* pH *on acetate oxidation in* A. turbidans

(Main compartment: 1 ml. cell suspension (5 mg. dry-cell matter). 1 ml. 0·1 M-phosphate buffer; H_2O to 3 ml. vol. Side arm: 0·1 ml. 0·05 M-acetate; centre cup: 0·2 ml. 2 N-NaOH; air; 25°.)

Time (min.)	μl. oxygen uptake: pH 5·9	pH 6·8	pH 7·8
10	0	1	1
20	2	5	5
30	6	9	9
60	20	21	22

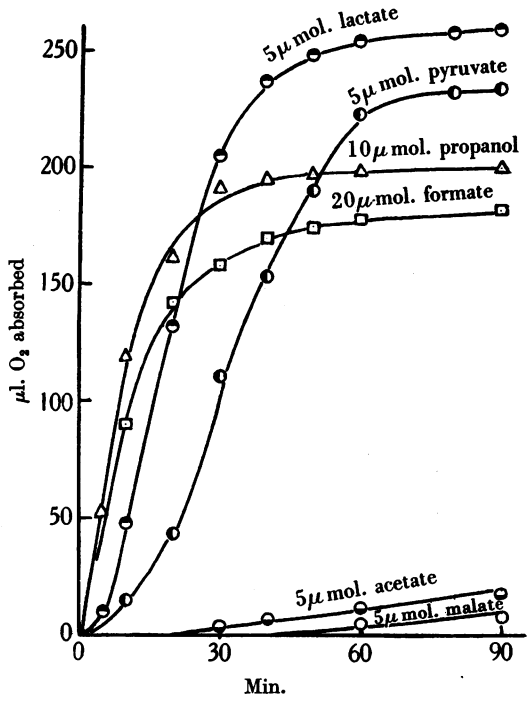

Fig. 1. Lag period in oxidation of some substrates in *A. turbidans*. Contents of cups: 1 ml. 0·1 M-phosphate buffer, pH 6·8; 1 ml. fresh cell suspension (=4 mg. dry weight in case of acetate, formate and propanol; in other cases = 3·7 mg.); 0·2 ml. substrate solution; 0·8 ml. water; air; temp. 25°.

Table 5. *Effect of the amount of cell matter on acetate oxidation in* A. turbidans

(Condition as in Table 4, but pH 6·8. The quantity of cells stated was suspended in 3 ml.)

Time (min.)	μl. O_2 absorbed by: 1 mg.	5 mg.	10 mg.
10	0	1	2
20	1	5	12
30	1	9	20
60	3	21	53

Table 6. *Effect of storage of cell suspensions on the lag period in acetate oxidation in* A. turbidans

(Conditions as in Table 4, but pH 6·8.)

Storage at 2° (days)	μl. O_2 absorbed after: 10 min.	20 min.	30 min.	60 min.
2	0	7	16	52
5	0	6	12	37
12	0	0	0	6

oxidation. It can be seen from curves in Fig. 2 that the O_2 uptake due to acetate and lactate, when added together, is very much more rapid than the O_2 uptake of the suspensions to which the substrates are added separately. After 30 min., for example, the cell suspension with acetate alone absorbed no measurable amount of oxygen, but when 1 μmol. of lactate was present 216 μl. O_2 were taken up. The complete oxidation of the added lactate required 67·2 μl. O_2. The excess—149 μl.—accounts for the complete oxidation of 67 % of the added acetate. A similar accelerating effect was obtained when 0·1 μmol. of lactate was added together with 5 μmol. of acetate.

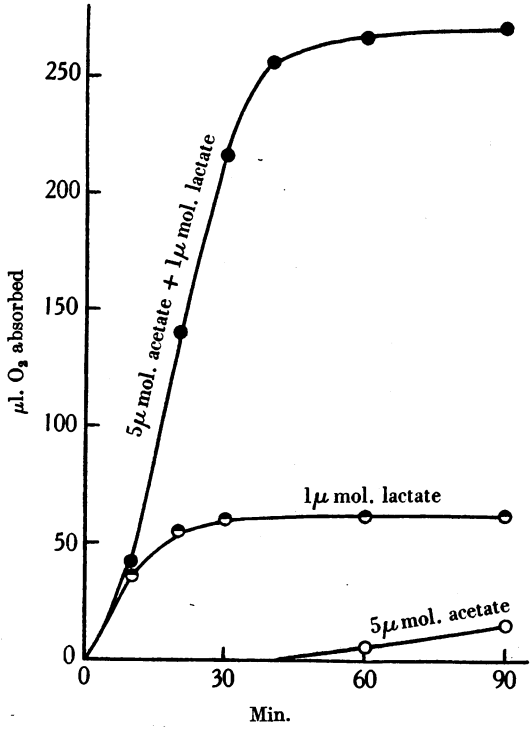

Fig. 2. Effect of lactate on acetate oxidation in *A. turbidans*. Contents of cups: 1 ml. cell suspension (5 mg. dry weight) stored for 11 days at about 2°; 1 ml. 0·1 M-phosphate buffer, pH 6·8; 0·2 ml. substrate solution; made up to 3 ml. with water; air; temp. 25°.

Similar effects on acetate oxidation were observed when lactate was replaced by pyruvate, succinate, *l*- or *dl*-malate, fumarate, α-ketoglutarate, glycerol, glucose, formate or formaldehyde. Representative curves showing the effect of α-ketoglutarate and formate on acetate oxidation are given in Figs. 3 and 4 respectively. Experiments with washed suspensions of another species, *Acetobacter mobile*, confirm the enhancing effects of lactate and other substrates on acetate oxidation.

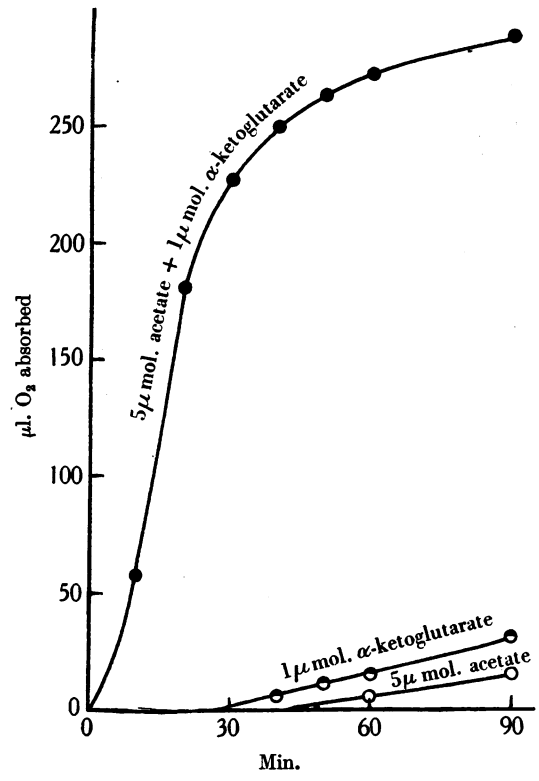

Fig. 3. Effect of α-ketoglutarate on acetate oxidation in *A. turbidans*. Conditions as given in Fig. 2.

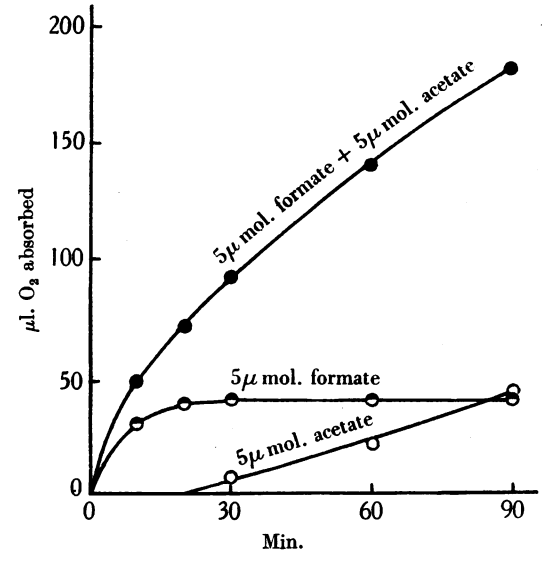

Fig. 4. Effect of formate on acetate oxidation in *A. turbidans*. Conditions as given in Fig. 2.

Quastel & Webley (1942) recorded somewhat similar observations; they showed that strain no. 4759 of National Collection of Type Cultures, after incubation with hexosediphosphate, pyruvate, fumarate, glycerol or fructose, oxidized acetate at an increased rate.

The oxidation of substances, other than acetate, which showed a pronounced lag period (e.g. succinate, fumarate, α-ketoglutarate, glucose) was not increased by the addition of lactate. The substances in Table 3, which were not oxidized when added alone, remained unaffected in the presence of lactate. Neither ethanol nor propanol, nor any of the non-oxidizable substances (Table 3), when added together with acetate accelerated its oxidation.

The fact that so many different substances, including such a simple compound as formate, have a similar accelerating effect, makes it unlikely that the carbon skeleton of these substrates provides directly an intermediate which is essential for the oxidation of acetate. It is more probable that the process of oxidation of the second substrate leads to the formation of a substance essential for acetate oxidation.

Effect of H_2O_2 *on acetate oxidation.* It seemed possible that H_2O_2 might be formed in small quantities in the course of oxidation of the second substrate, and induce acetate oxidation through a peroxidase. Different concentrations of H_2O_2 (equivalent to 5–100 μl. O_2) were added together with acetate in order to test this hypothesis, but it was found that the addition did not accelerate the oxidation of acetate. The fact that some substances reduce the lag period of acetate oxidation without undergoing, in the early stages, a measurable degree of oxidation (e.g. α-ketoglutarate, Fig. 3) is also contrary to the peroxidase hypothesis.

Effect of vitamin B complex components on acetate oxidations. An alternative explanation of the lag period would be the hypothesis that for the oxidation of acetate a specific catalyst is required, which may be formed under the influence of one of the substances reducing the lag period. To replace the effect of these substrates, components of the vitamin B complex, which are known to be catalysts in bacterial oxidation of acetate (see especially Quastel & Webley, 1942) were added to acetate. The substances and the quantities added to 4 ml. suspensions were: aneurin (10 μg.), co-carboxylase (10 μg.), riboflavin (10 μg.), pyridoxin (10 μg.), nicotinic acid and its amide (20 μg.), biotin (10 μg.), pantothenate (10 μg.), inositol (20 μg.) and *p*-aminobenzoic acid (20 μg.). In view of the finding of Quastel & Webley (1942) that K^+ and Mg^{++} increase oxidation of acetate in the presence of vitamin B_1 in strain no. 4759, the tests were conducted in the presence and absence of these ions. None of the components of the vitamin B complex accelerated the oxidation of acetate, either singly or in various combinations, one of which included all the substances. The addition of K^+ or Mg^{++} did not accelerate oxidation of acetate in these experiments to any appreciable extent.

Effect of adenosinetriphosphate (ATP) on acetate oxidation. It was found (Table 7) that addition of ATP increased the rate of acetate oxidation in *A. turbidans*, but the accelerating effect was not as great as that of simpler organic substances, e.g. lactate. The preliminary incubation of vitamin B-deficient cells of strain no. 4759 was shown by Quastel & Webley (1942), to cause a small but

Table 7. *Effect of adenosinetriphosphate (ATP) on acetate oxidation in* A. turbidans

(Conditions as in Table 4, but pH 6·8. ATP added as Na salt.)

	μl. O_2 absorbed with substrate			
Time (min.)	ATP (1 mg.)	Acetate 5 μmol.	Acetate 5 μmol. +1 mg. ATP	Acetate 5 μmol. +0·1 mg. ATP
10	0	1	2	1
20	0	3	10	7
30	0	7	18	12
60	0	20	40	29
120	0	55	95	73

Table 8. *Effect of ATP on acetate oxidation in* A. turbidans *in presence and absence of pyruvate*

(Conditions as in Table 4, but pH 6·8, and the cell suspension was stored at about 2° for 10 days before use.)

	O_2 uptake (μl.)						
Time (min.)	5 μmol. acetate + 1 μmol. pyruvate + 1 mg. ATP	5 μmol. acetate + 1 mg. ATP	5 μmol. acetate + 1 μmol. pyruvate	1 μmol. pyruvate + 1 mg. ATP	5 μmol. acetate	1 μmol. pyruvate	1 mg. ATP
10	22	0	22	12	0	6	0
20	106	6	97	33	0	26	0
30	156	13	145	35	1	30	0
60	242	32	224	38	10	35	0
120	266*	65	262*	38*	29	36*	0

* No further O_2 uptake occurred.

definite acceleration of acetate oxidation. The effect of ATP on acetate oxidation in our organism was also tested in the presence of vitamin B_1 and other members of the vitamin B complex, but it was not greater than that obtained in the presence of ATP alone. The oxygen uptake on the addition of acetate together with a little pyruvate was slightly increased by the further addition of ATP (Table 8); thus the effect of ATP appears to be additive in *A. turbidans*.

DISCUSSION

Whilst it has not been possible to elucidate the intermediary stages of acetate oxidation in *Acetobacter*, some factors influencing the oxidation of acetate have come to light:

(1) The presence of small quantities of certain simple organic substances (lactate, pyruvate, glycerol, fumarate, succinate, malate, α-ketoglutarate, glucose, formate, formaldehyde) increases the rate of acetate oxidation and diminishes, or almost abolishes, the lag period in *Acetobacter*. The effective substances have the common property of undergoing oxidation, though at very different rates, in the organism, but not every substance which is oxidizable has an accelerating effect; ethanol and propanol are rapidly oxidized yet they do not affect acetate oxidation.

(2) ATP (but not any of the members of the vitamin B complex tested either alone or in various combinations) also increases the rate of acetate oxidation and reduces the lag period, but the effect is smaller than that of any of the above-named substances. ATP caused a slight additional increase in the rate of oxidation of the pyruvate-acetate mixture.

Effects resembling those described in this paper were found by Quastel & Webley (1942) in strain no. 4759, as already quoted.

To explain the enhancing effects of relatively simple substances we may assume that: (*a*) the carbon skeleton of the added substrates provides directly an intermediate which is essential for the oxidation of acetate, or (*b*) that the added compounds (or their breakdown product) give rise in the presence of acetate to the formation of an intermediate necessary for the oxidation, or (*c*) that the simple substances cause the activation of one of the enzymes essential for acetate oxidation. The fact that so many different substances, of which some are oxidized at a very low rate, have a similar enhancing effect makes it unlikely that explanation (*a*) is the correct one. Assumption (*b*) is more probable, although the fact that some substances reduce the lag period of acetate oxidation without, in the early stages, being themselves measurably oxidized (e.g. α-ketoglutarate) is not in agreement with this hypothesis. Furthermore, the increased oxidation is produced even by very small quantities of the second substrate (e.g. 0·1 μmol. lactate). It is more likely, therefore, that the effect is of the type suggested under (*c*), so that the last assumption may prove more useful as a working hypothesis.

SUMMARY

1. Conditions have been described for the preparation of washed cell suspensions of *A. turbidans* suitable for metabolic experiments. The best yield of highly active cells was obtained when the medium contained 0·5 % ethanol and 0·5 % lactate as substrates. The acid produced from ethanol is neutralized by bicarbonate formed from lactate and thus a pH compatible with rapid growth is maintained for a relatively long period.

2. The oxidation of various substances by a washed cell suspension was examined. Ethanol, propanol, lactate, pyruvate, glycerol, glucose, formate and a number of other substances (Table 3) were found to be oxidized. Methanol, *iso*propanol, glycollate, propionate and butyrate are among the substances which were not oxidized. In the case of formate, formaldehyde, lactate and pyruvate the oxidation was almost complete. Ethanol was rapidly oxidized to acetic acid; acetate was slowly oxidized almost to completion. Propanol was oxidized to propionic acid. The oxidation of glucose was also incomplete.

3. Formate, ethanol and propanol were oxidized without a lag period. Other substances show lag periods of various lengths, e.g. with acetate as substrate, 10–60 min. The duration of the lag period increased with the time of storage of cells and dilution of the suspension.

4. The lag period of acetate oxidation was greatly reduced by the addition of small quantities of lactate, pyruvate, glycerol, succinate, malate, fumarate, α-ketoglutarate, glucose, formate and formaldehyde. The same substances increased the rate of acetate oxidation. The lag period was also somewhat reduced by adenosinetriphosphate.

5. The mechanism of the effects of various substances on the lag period and on the rate of oxidation of acetate is discussed.

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