4. The ethereal sulphate fraction of cyanobenzene urine contains *m*- and *p*-cyanophenols, which were isolated. The formation of *m*-cyanophenol is believed to be the first reported case of *meta*-hydroxylation *in vivo*. The nature of the glucuronide fraction was not elucidated.

5. The azobenzene-4-carbonyl esters, 2:4-dinitrophenyl ethers and benzylamine salts of the cyanophenols have been synthesized and used as reference

compounds. The pK_a 's of *o*-, *m*- and *p*-cyanophenols have been measured.

6. The results have been discussed in the light of current theories concerning diol formation *in vivo* and aromatic substitution *in vitro*.

The expense of this work was in part defrayed by a grant from the Medical Research Council. We wish to thank Mr B. Spencer for the determinations of mercapturic acid quoted in this paper.

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The Oxidation of Pyruvate and Fatty Acids by *Mycobacterium ranae*

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(Received 20 September 1949)

A study of the intermediary metabolism of *Mycobacterium phlei* led Edson & Hunter (1947) to suggest that the aerobic breakdown of lactate may proceed through pyruvate to acetate by two routes, one of which involves a coupled non-enzymic oxidation

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of pyruvate. The alternative pathway via enzymic oxidation of pyruvate requires investigation, because it offers the possibility of partial assimilation at an early stage. Since added pyruvate is metabolized slowly by washed suspensions of *Mycobact. phlei*, we decided to study the oxidation of this substrate by *Mycobact. ranae* which utilizes both pyruvate and

acetate at more favourable rates (Edson & Hunter, 1943). Some related observations on the oxidation of fatty acids have been included in this paper.

METHODS

Organism. The organism used was *Mycobact. ranae* no. 2891 of the National Collection of Type Cultures, Lister Institute, London. It was cultivated at 38° on meat infusion broth, pH 7.2, containing 5% (v/v) of glycerol. On the seventh to tenth day of incubation the bacteria were harvested, washed and suspended in distilled water according to Edson & Hunter (1943).

Bacterial extracts. Extracts and solid cellular debris were prepared with the wet-crushing mill of Booth & Green (1938) or by the glass-powder grinding technique of Kalnitsky, Utter & Werkman (1945), and separated by centrifugation.

and 7.0 ml. of the clear filtrate were used for steam distillation and estimation of fatty acids by the methods of Friedemann (1938). When identification was required, Duclaux fractions were titrated and distribution constants (2 vol. distilled water/1 vol. ether) determined. The apparatus blank was 0.25 ml. of 0.01 N-NaOH.

Table 1 gives typical values obtained with the particular apparatus and specimens of fatty acids employed. The acetate was A.R. (British Drug Houses Ltd.), $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$. The serial distillate figures agree satisfactorily with those of Friedemann (1938), except in the case of acetic acid where the percentages in the fifth and sixth fractions are significantly different. The distribution constants (*K*) are uniformly higher than those given by Friedemann (1938).

Table 1. *Steam distillation of fatty acids. Duclaux fractions and distribution constants*

(Method of Friedemann (1938). Data obtained with the apparatus and specimens of fatty acid used in subsequent work.)

Fraction no. ...	1	2	3	4	5	6	Total distillate
Volume of fraction (ml.)...	25	25	25	25	50	About 40*	
	Percentage of fatty acid						Distribution constant <i>K</i> (2 vol. water/1 vol. ether)
Acetic acid	12	12	14	14	37	11	90
Propionic acid	26	23	19	16	14	2	71
<i>n</i> -Butyric acid	38	27	16	10	6	3	46
<i>n</i> -Valeric acid	—	—	—	—	—	—	32
<i>n</i> -Hexanoic acid	78	18	3	1	Nil	Nil	11

* To crystallization.

Measurement of respiration. Oxygen consumption and CO_2 production were measured at 38° by the conventional manometric methods of Warburg. Substrates (as neutral Na salts) were added from the side bulb after equilibration. The quantity of bacteria used varied from 6 to 20 mg. (dry wt.)/cup.

Analytical methods

(a) **Determination of pyruvate.** After the Warburg cups had been cooled in ice and the CO_2 absorbent removed, the contents were transferred to graduated centrifuge tubes, the vessels rinsed with 1 ml. of distilled water and the washings added to the centrifuge tubes. The volume was measured and the bacteria thrown down by centrifugation at 4500 rev./min.; 3.0 ml. of supernatant fluid were used for determination of pyruvic acid by the yeast carboxylase method of Westerkamp (1933), and the result corrected to give the full value for each Warburg cup.

(b) **Determination of fatty acids volatile in steam.** At the conclusion of a manometric experiment the Warburg cups were cooled in ice, the CO_2 absorbent removed and the bacterial suspension transferred to a graduated test tube by means of a pasteur pipette. The main compartment, the side bulb and the pipette were washed with small amounts of distilled water (3 ml. in all), and the washings added to the test tube; 0.5 ml. of 10% (w/v) Na tungstate solution and 0.5 ml. of 0.66 N- H_2SO_4 were added and the volume made up to 8.0 ml. with distilled water. The mixture was filtered through a Whatman no. 1 filter paper (4.25 cm. diameter)

RESULTS

Respiration of *Mycobacterium ranae*

Table 2 shows that *Mycobact. ranae* possesses a substantial endogenous respiration and that oxygen consumption is increased about four times after

Table 2. *Respiration of Mycobacterium ranae in presence of pyruvate and fatty acids*

(Washed bacterial suspension in 3.0 ml. of 0.033 M-phosphate buffer pH 7.4. 38°. Gas: air. $-\text{Q}_{\text{O}_2} = \mu\text{l. O}_2$ consumed/mg. dry wt. of bacteria/hr.)

Substrate (0.02 M)	$-\text{Q}_{\text{O}_2}$	
	Substrate absent	Substrate present
Pyruvate	8.1	34.6
Acetate	8.1	36.8
Propionate	8.5	74.5
<i>n</i> -Butyrate	8.8	79.0
<i>n</i> -Valerate	5.8	41.0
Pentane-1-carboxylate	8.4	70.0

Mean values (with s.d.) of twenty observations were as follows:

Substrate	$-\text{Q}_{\text{O}_2}$
Nil	8.7 ± 2.4
Pyruvate	34.1 ± 4.2
Acetate	35.6 ± 4.9

Table 3. *Incomplete oxidation of pyruvate and fatty acids by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033M-phosphate buffer pH 7.4. 38°. Gas: air.)

Substrate (M)	Dry wt. bacteria/cup (mg.)	Time (hr.)	O ₂ uptake (μl.)	'Extra' O ₂ uptake (μl.)	Substrate dis- appearing (mg. free acid)	Mol. 'extra' O ₂ used/mol. substrate disappearing	
						Found	Theoretical for complete combustion
Nil	10.5	5	{ 302	—	—	—	—
Pyruvate (0.02)			{ 1606	1304	3.31	1.5	2.5
Nil	7.25	4	{ 155	—	—	—	—
Acetate (0.02)			{ 1072	917	1.65	1.5	2.0
Nil	6.9	4	{ 230	—	—	—	—
Propionate (0.025)			{ 2089	1859	2.94	2.1	3.5
Nil	7.1	4	{ 225	—	—	—	—
n-Butyrate (0.02)			{ 1369	1144	2.03	2.2	5.0
Nil	7.5	4	{ 175	—	—	—	—
n-Valerate (0.017)			{ 1235	1060	1.17	4.1	6.5
Nil	8.2	3	{ 291	—	—	—	—
Pentane-1-carboxylate (0.016)			{ 2433	2142	2.19	5.1	8.0

addition of pyruvate or acetate, these substrates being oxidized at approximately equal rates. In the presence of propionate, butyrate, valerate and pentane-1-carboxylate the oxygen uptake is increased eight- to nine-fold. Bacterial suspensions which had been heated for 3 min. at 100° exhibited no oxygen uptake either in presence or absence of substrate.

Incomplete oxidation of substrates

The incompleteness of combustion of pyruvate and fatty acids is illustrated by the experiments recorded in Table 3. The ratio oxygen consumed/substrate disappearing has been calculated on the assumption that the endogenous respiration is unaffected by the metabolism of the substrate. The figures show that the oxygen uptake/mol. of substrate utilized is considerably less than that required for complete combustion. A similar incomplete oxidation of glucose and lactate was observed with *Mycobact. phlei* (Edson & Hunter, 1947). Since the relationship between pyruvate and acetate is an important feature of the metabolism of acid-fast bacteria, acetate was sought, but not found, in the suspension medium during the metabolism of pyruvate. With the exception of CO₂, no product of pyruvate catabolism is known to accumulate extracellularly. It is to be noted, however, that the combustion of acetate is more complete than that of pyruvate (75% as against 60%). The data of Table 3 suggest that a portion of each substrate is burnt to completion and the remainder assimilated.

Anaerobic dissimilation

When pyruvate and acetate were added anaerobically to a suspension of *Mycobact. ranae*, the CO₂ production was not significantly different from the

small evolution occurring in the control without substrate (Table 4). There was a slight increase in CO₂ formation in the presence of glucose. Determination of residual pyruvate and acetate in experiments lasting 4 hr. showed that there was no significant metabolism of these substrates under anaerobic conditions. The recoveries were 95% or more of the amounts added to the bacteria.

Table 4. *Anaerobic evolution of carbon dioxide by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of bicarbonate-Ringer solution (Krebs & Henseleit, 1932); pH 7.4; 38°. Gas: 5% CO₂-95% N₂. Phosphorus in the inseal. Dry wt. of bacteria = 7 mg./cup.)

Substrate (0.02M)	CO ₂ production (μl./hr.)	Recovery of substrate (%)
Nil	21	—
Pyruvate	21	95
Acetate	29	99
Glucose	41	—

Inhibition of substrate oxidation

The effects of various reagents on the oxidation of pyruvate, acetate, butyrate and pentane-1-carboxylate are shown in Tables 5 and 6. The percentage inhibition of respiration is calculated for the oxygen uptake in excess of that due to endogenous respiration. The effects of inhibitors on the spontaneous respiration are also recorded. It will be seen that the oxidation of pyruvate and acetate is partially inhibited by azide at the concentration used; cyanide has a more pronounced effect, and 0.005M-cyanide gives almost complete inhibition in the presence of acetate. Fluoride diminishes the extra oxygen consumption by 32% in the presence of pyruvate, but has no effect when acetate is the substrate. The results with malonate are similar.

Fluoroacetate assumed importance as a selective inhibitor in studies of intermediary metabolism when Barron, Bartlett & Kalnitsky (1946), Bartlett & Barron (1947) and Kalnitsky & Barron (1947) reported that this substance inhibited acetate oxida-

tion without inhibiting the oxidation of pyruvate in animal tissues, in baker's yeast and in *Corynebacterium creatinovorans*. Since fluoroacetate depresses the oxygen consumption of *Mycobact. ranae* to the same degree (approximately 30% in a 2 hr.

Table 5. *Inhibition of the oxidation of fatty acids by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033 M-phosphate buffer pH 7.4. 38°. Gas: air. Inhibitor in contact with the bacteria from the beginning of the experiment; substrate added after equilibration. When HCN was used, the CO₂ absorbent was a mixture of KCN and KOH (Krebs, 1935).)

			Oxygen uptake (μl.)		Inhibition (%)
Inhibitor	Dry wt. of bacteria/cup (mg.)	Time (hr.)	Substrate absent	Substrate present Acetate (0.02 M)	
Nil	10.4	2	{ 175	591	—
Sodium azide (0.002 M)			{ 179	445	36
HCN (0.005 M)			{ 105	121	96
Sodium fluoroacetate (0.005 M)			{ 178	438	38
Nil	7.2	2	{ 118	533	—
Sodium fluoride (0.005 M)			{ 120	542	-2
Sodium malonate (0.01 M)			{ 126	583	-9
Nil	14.0	4	{ 454	2061	—
Hydroxylamine-HCl (neutralized) (0.02 M)			{ 415	574	90
Nil	7.5	2	{ 151	593	—
Sodium arsenite (0.004 M)			{ 128	127	100
Nil	6.7	2	{ 142	596	—
Sodium iodoacetate (0.002 M)			{ 130	152	95
Sodium iodoacetate (0.001 M)			{ 144	155	98
<i>n</i> -Butyrate (0.02 M)					
Nil	6.7	2	{ 142	758	—
Sodium iodoacetate (0.002 M)			{ 130	156	96
Pentane-1-carboxylate (0.016 M)					
Nil	8.4	2	{ 128	1110	—
Sodium arsenite (0.004 M)			{ 95	189	90
Nil	10.0	7	{ 407	2839	—
Sodium iodoacetate (0.002 M)			{ 408	759	86

Table 6. *Inhibition of the oxidation of pyruvate by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033 M-phosphate buffer pH 7.4. 38°. Gas: air. Inhibitor in contact with the bacteria from the commencement of the experiment; substrate added after equilibration. Final conc. pyruvate = 0.02 M.)

Inhibitor	Time (hr.)	Oxygen uptake (μl.)		Inhibition (%)
		Substrate absent	Substrate present	
Nil	2	{ 106	425	—
Sodium azide (0.002 M)		{ 105	341	26
HCN (0.001 M)		{ 80	212	59
Nil	1	{ 80	319	—
Sodium fluoride (0.005 M)		{ 82	245	32
Nil	1	{ 88	470	—
Sodium malonate (0.01 M)		{ 91	393	21
Nil	2	{ 201	837	—
Sodium fluoroacetate (0.005 M)		{ 221	671	29
Nil	4	{ 454	1939	—
Hydroxylamine-HCl (neutralized; 0.01 M)		{ 415	2022	-8
Nil	1	{ 76	303	—
Sodium arsenite (0.005 M)		{ 64	85	91
Nil	1	{ 78	317	—
Sodium iodoacetate (0.002 M)		{ 69	159	63

experiment) whether the substrate undergoing oxidation be acetate or pyruvate, it is unlikely to be a useful agent in attempts to dissociate their metabolism. Moreover, the inhibitory action decreases with time. Inhibitions of the order of 50% have been observed in the first hour, only to diminish to negligible effects after 3 or 4 hr. Progressive diminution of the specific inhibitory action of fluoroacetate has been reported by Kalnitsky & Barron (1947) and by Black & Hutchens (1948) in studies of baker's yeast.

As with *Mycobact. phlei* (Edson & Hunter, 1947), arsenite inhibits the oxidation of both pyruvate and acetate completely or almost completely. It has the same effect on the oxidation of pentane-1-carboxylate (Table 5).

Two of the reagents investigated exhibit selective properties which are potentially useful. Hydroxylamine, which does not affect oxygen uptake significantly in the presence of pyruvate, causes almost full inhibition of acetate oxidation. Equally noticeable is the influence of iodoacetate, which reduces extra oxygen uptake by 95–100% in the presence of acetate, but only by 63% in the presence of pyruvate. Iodoacetate has a similar profound effect on the oxidation of other fatty acids, 96% inhibition in the case of butyrate and 86% in that of pentane-1-carboxylate.

Dissociation of the metabolism of pyruvate and acetate. The time course of the respiration of *Mycobact. ranae* in presence of pyruvate and acetate is illustrated in Fig. 1, which represents an experiment lasting 7 hr. The endogenous respiration diminished slowly from the commencement, whereas oxygen consumption was maintained for 3 hr. at a constant rate in the presence of both substrates, but fell off subsequently. Iodoacetate produced a slight depression of the endogenous respiration. Addition of acetate did not affect the oxygen consumption of cells poisoned with iodoacetate; whereas the addition of pyruvate was followed by an extra oxygen uptake amounting to 16% of that which occurred in control organisms without iodoacetate. This experiment shows that the selective action of iodoacetate on the metabolism of *Mycobact. ranae* is such that it can be employed to dissociate the oxidation of acetate from that of pyruvate. If the reaction, $\text{pyruvate} \rightarrow \text{acetate} + \text{CO}_2$, is the sole, or the chief, reaction persisting in cells poisoned with iodoacetate, the residual metabolism should be capable of producing measurable quantities of acetic acid in experiments of 5–7 hr. duration.

When a small initial concentration (0.005M) of pyruvate was used in an experiment without inhibitor, the extra oxygen uptake was rapid during the first hour, but declined to the endogenous level in 180 min. By this time the whole of the pyruvate had disappeared from the suspension medium

(nitroprusside reaction negative), showing that the organism is able to utilize pyruvate completely although this substrate is not completely burnt (Table 3).

Fig. 1. Effect of iodoacetate on the oxygen consumption of *Mycobact. ranae* in presence of pyruvate and acetate. Washed suspension of organisms in 3.0 ml. of 0.033M-phosphate buffer pH 7.4. 38°. Gas: air. 21.0 mg. dry wt. of bacteria/Warburg cup. Pyruvate and acetate, each 0.02M. Iodoacetate, 0.002M. No substrate, ○; iodoacetate, ●; acetate, □; pyruvate, △; acetate + iodoacetate, ×; pyruvate + iodoacetate, △. The curves representing the effects of iodoacetate alone and of iodoacetate + acetate are almost identical.

Formation of acetate from pyruvate. If *Mycobact. ranae* oxidizes pyruvate to acetate, the reaction is likely to proceed according to the over-all equation

The results of a typical experiment designed to test this hypothesis are set out in Table 7, iodoacetate being employed to inhibit acetate oxidation selectively. In the absence of inhibitor pyruvate and acetate are oxidized vigorously, and the disappear-

ance of acetate reaches the expected order of magnitude. Acetate does not accumulate as a result of endogenous respiration or of pyruvate metabolism. The inhibitor, however, depresses oxygen consumption in the presence of pyruvate, and simultaneously causes the accumulation of significant amounts of steam-volatile acid. The oxygen uptake and the recovery figures for acetic acid show that the metabolism of acetate is abolished. It will be seen that a small quantity of steam-volatile acid accumulates in a suspension to which inhibitor alone has been added (vessel 6).

The steam-volatile acid formed from pyruvate and accumulating in vessel 3 was subjected to analysis by the methods of Friedemann (1938). The Duclaux constants and the distribution constant (Table 7) indicate that it was a specimen of acetic acid which did not contain significant amounts of other steam-volatile acids (cf. Table 1).

Controls. The possibility of errors arising in the steam distillations was controlled by investigation of the reagents, separately and combined, and by conducting blanks with suspensions of the organisms, both viable and heated (100°). Neither pyruvate nor iodoacetate yields acid volatile in steam from solutions put through the whole procedure used in this work, even when the concentration is several times greater than that used in experiments. Suspensions of *Mycobact. ranae* shaken at 38° for 7 hr. and subsequently clarified by tungstic acid precipitation, yield a trace of steam-volatile acid equal in titre to 0.1-0.2 ml. of 0.01 N-NaOH. This blank, which is constant over a density range of 6-25 mg. of bacteria (dry wt.) per 4 ml. of medium, was deducted in titrations of steam-volatile acid.

When these controls are taken into account, it appears justifiable to conclude that the trace of steam-volatile acid found in vessel 6 (Table 7) was a true metabolic product formed endogenously under the influence of the inhibitor. There was insufficient of this acid to permit identification by fractional distillation, but the lanthanum-iodine colour test (Table 8) indicates that it was probably acetic acid.

Table 7. Formation of acetate from pyruvate in the presence of iodoacetate by *Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033 M-phosphate buffer pH 7.4. 38°. Gas: air. Time: 7 hr. Dry wt. of bacteria/cup 21.0 mg.)

Vessel no.	Substrate (0.02 M)	Iodoacetate (0.002 M)	O ₂ uptake (μl.)	Acetic acid added (mg.)	Volatile acid found reckoned as CH ₃ COOH (mg.)
1	Nil	Absent	800	Nil	0.00
2	Pyruvate	Absent	2701	Nil	0.00
3	Pyruvate	Present	1122	Nil	1.45
4	Acetate	Absent	3166	4.60	0.72
5	Acetate	Present	814	4.60	4.70
6	Nil	Present	816	Nil	0.20

Constants of the volatile acid formed from pyruvate in vessel 3

Duclaux fractions		Distribution constant <i>K</i> (2 vol. water/1 vol. ether)
Fraction no.	Percentage of total fatty acid	
1	14	90
2	12	
3	12	
4	13	
5	38.5	93
6	10.5	

Table 8. Formation of acetate from pyruvate by *Mycobacterium ranae*

(4.9 g. of washed bacteria suspended in 30 ml. distilled water and 10 ml. of the suspension heated at 100° for 5 min. Experiment conducted in 50 ml. stoppered conical flasks shaken in a thermostat at 38°. Time: 6 hr. Gas: air. The contents of each vessel were deproteinized and cleared of cells by addition of 1.0 ml. of 7% (w/v) metaphosphoric acid followed by filtration. Steam distillates were used for the La-iodine reaction (see Edson & Hunter, 1947).)

Flask no.	...	...	...	1	2	3	4	5	6
Volume (ml.)									
Bacterial suspension	4.0	4.0	4.0	4.0	0	4.0	0	4.0	0
Heated bacterial suspension	0	0	0	0	0	0	0	4.0	4.0
Phosphate buffer (pH 7.4; 0.1 M)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Pyruvate (0.2 M)	0	0.8	0	0.8	0	0.8	0	0.8	0.8
Sodium iodoacetate (0.05 M)	0	0	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Distilled water	1.5	0.7	0.8	0	0.8	0	0.8	0	0
Total volume	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Lanthanum-iodine test	-	-	Trace	+++	-	-	-	-	-

Experiments similar to those reported in Table 7 were conducted on a larger scale for the purpose of applying the lanthanum-iodine colour test for the identification of acetic acid (see Table 8). The steam distillates from suspensions without substrate and from those to which pyruvate alone was added gave no colour reaction, whereas an intense blue coloration was obtained from distillates containing the products of metabolism of pyruvate in the presence of iodoacetate. The colour obtained from distillates representing endogenous metabolism under the influence of iodoacetate was a faint purplish blue. Heat-inactivated organisms used as controls gave negative reactions. Controls with pyruvate and iodoacetate solutions were also negative.

In view of the data which are presented in Tables 7 and 8 it may be concluded that the steam-volatile acid formed during the oxidation of pyruvate is indeed acetic, and not propionic, acid.

Molecular relationships in the metabolism of pyruvate

By measuring simultaneously pyruvate disappearance, oxygen consumption and acetate formation, it is possible to obtain some further insight into the metabolism of pyruvate. Warburg vessels were set up in duplicate for determination of pyruvic and acetic acids, the density of suspended

organisms and other conditions being uniform. The results are displayed in Table 9. In absence of iodoacetate the disappearance of 37.6 $\mu\text{mol.}$ of pyruvate corresponds to an extra oxygen uptake of 58.2 $\mu\text{mol.}$, giving a ratio O_2 uptake/pyruvate used = 1.55. When iodoacetate is present the pyruvate disappearance is reduced to 9.5 $\mu\text{mol.}$ and the extra oxygen consumption to 6.0 $\mu\text{mol.}$ Under these conditions the ratio, O_2 uptake/pyruvate used = 0.63. It will be noticed that in the presence of iodoacetate the steam-volatile acid formed endogenously, assumed not without reason to be acetic acid, is almost half that produced when pyruvate is present.

Again these figures illustrate that the unhindered oxidation of pyruvate is incomplete, the theoretical oxygen/pyruvate ratio for complete combustion being 2.5. When acetate oxidation is suppressed by the selective inhibitor, the oxygen/pyruvate ratio is slightly greater than the theoretical 0.5, although the deviation is close to the limit of experimental error. If the inhibition of acetate oxidation is not quite complete, the ratio will move in the direction found. That pyruvate disappearance (9.5 $\mu\text{mol.}$) is not equal to accumulation of acetate (8.3 $\mu\text{mol.}$) is also consistent with such an explanation.

Table 9. *Relationship between oxygen consumption, pyruvate disappearance and acetate formation by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033M-phosphate buffer pH 7.4. 38°. Gas: air. Time: 5 hr. Dry wt. of bacteria/cup 10.5 mg.)

Substrate	Iodoacetate (0.002M)	O_2 uptake ($\mu\text{mol.}$)	'Extra' O_2 consumed ($\mu\text{mol.}$)	Pyruvate added ($\mu\text{mol.}$)	Pyruvate disappearing ($\mu\text{mol.}$)	Acetic acid formed ($\mu\text{mol.}$)	'Extra' acetic acid ($\mu\text{mol.}$)	$\frac{\mu\text{mol. 'Extra' O}_2}{\mu\text{mol. Pyruvate disappearing}}$
Nil	Absent	13.5	—	—	—	0.00	—	—
Nil	Present	13.2	—	—	—	8.0	—	—
Pyruvate	Absent	71.7	58.2	47.3	37.6	0.00	—	1.55
Pyruvate	Present	19.2	6.0	47.3	9.5	16.3	8.3	0.63
Theoretical molar relationships:								
Complete combustion of pyruvate	—	—	2.5	1.0	1.0	—	0.0	2.5
Conversion of pyruvate to acetate	—	—	0.5	1.0	1.0	—	1.0	0.5

Table 10. *Oxygen consumption and carbon dioxide production in presence of iodoacetate by Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033M-phosphate buffer. pH 7.4. 38°. Gas: air. O_2 uptake and CO_2 production (free and bound) measured by the method of Warburg & Yabumae (1924).)

Substrate (0.02M)	Dry wt. of bacteria/cup (mg.)	Time (min.)	Iodoacetate (0.002M)	O_2 uptake ($\mu\text{l.}$)	CO_2 production (free and bound) ($\mu\text{l.}$)	CO_2/O_2	'Extra' CO_2 'Extra' O_2	Theoretical R.Q.
Nil	7.0	80	{ Absent	91	72	0.79	—	—
Acetate			{ Absent	220	215	0.98	1.11	1.0
Nil	8.2	60	{ Absent	78	56	0.72	—	—
Pyruvate			{ Absent	275	361	1.31	1.55	1.2
Nil	10.0	120	{ Present	147	115	0.78	—	—
Pyruvate			{ Present	220	266	1.21	2.06	2.0*

* It is assumed that pyruvate is converted into acetate quantitatively in the presence of the inhibitor (iodoacetate).

The data of Table 9 show that pyruvate is oxidized mainly, or solely, to acetate (and CO_2) when the metabolism is deranged by iodoacetate. They also reveal another fact of considerable importance: the utilization of pyruvate is four times as great in the absence of iodoacetate as in its presence. Since the inhibitor practically abolishes the metabolism of acetate (added or formed from pyruvate), this suggests that the normal assimilation of pyruvate may be coupled with the oxidation of acetate, or that there may be an independent oxidative assimilation of pyruvate which is highly sensitive to iodoacetate, and possibly to other inhibitors like arsenite and cyanide.

Additional experiments relevant to the molecular relationships are reported in Table 10. When oxygen consumption and CO_2 production were measured simultaneously in the presence of pyruvate and iodoacetate, the observed CO_2/O_2 ratio (2.06) was consistent with complete blockage of assimilation and quantitative conversion of the substrate into acetate. Control experiments with uninhibited organisms gave CO_2/O_2 ratios which are somewhat higher than the theoretical quotients for complete combustion of pyruvate and acetate. Although such over-all data are difficult to interpret because they represent the resultant of many unknown reactions, the high quotient due to pyruvate oxidation is consistent with the synthesis of cellular lipids.

Possible intermediates of fatty acid breakdown

The essentially aerobic character of the breakdown of fatty acids by *Mycobact. ranae* suggested that a process of β -oxidation might operate. Although ketone bodies could not be detected (Rothera test)

with that of an intermediate in fatty acid catabolism.

The oxidation of pentane-1-carboxylate is not completely abolished by iodoacetate or arsenite (Table 5). On this account accumulation of small quantities of acetoacetate is possible in the presence of inhibitors if β -oxidation occurs. Acetoacetic acid, however, could not be found (Rothera test) after restricted oxidation of pentane-1-carboxylate had proceeded for several hours in the presence of iodoacetate or arsenite.

Cell extracts and broken cells

Acetone powders, clear extracts of crushed cells and suspensions of cell debris fail to oxidize pyruvate and fatty acids, although viable cells of *Mycobact. ranae* do so readily. Attempts to secure active preparations by crushing in the presence of cysteine were nugatory. Failure also attended efforts to reconstitute the systems responsible for the oxidation of pyruvate and acetate by addition of catalytic agents such as cozymase, cocarboxylase, adenosine-triphosphate, fumarate, cytochrome c (heart muscle) and divalent ions (Mg^{++} , Mn^{++} , Co^{++}). Autoxidizable hydrogen carriers such as methylene blue, pyocyanin and riboflavin did not activate the preparations.

In so far as it is labile and cyanide-sensitive, the enzyme system which oxidizes pyruvate and acetate in *Mycobact. ranae* resembles the glucose-oxidizing system of *Mycobact. phlei* (Edson & Hunter, 1947). Both organisms contain a cytochrome system akin to that of baker's yeast (Todd, 1949).

DISCUSSION

Table 11. Oxidation of acetoacetate, β -hydroxybutyrate and crotonate and the inhibitory effect of arsenite by *Mycobacterium ranae*

(Washed bacterial suspension in 3.0 ml. of 0.033 M-phosphate buffer pH 7.4. 38°. Gas: air. Time: 2 hr. Dry wt. of bacteria/cup 7.6 mg. Arsenite 0.0025 M. Inhibition is calculated on the basis of 'extra' oxygen uptake.)

Substrate	Oxygen uptake (μl)		Inhibition (%)
	Arsenite absent	Arsenite present	
Nil	190	135	—
Acetoacetate (0.01 M)	837	226	94
DL- β -Hydroxybutyrate (0.02 M)	550	157	86
Crotonate (0.02 M)	503	169	89

during the metabolism of butyrate and pentane-1-carboxylate, it was found that *Mycobact. ranae* was able to oxidize acetoacetate, crotonate and β -hydroxybutyrate at considerable speed, the oxidations being strongly inhibited by arsenite (Table 11). The rate of oxidation of acetoacetate is consistent

An investigation of the metabolism of *Mycobact. ranae* has made it possible to test certain hypotheses, which have developed from a survey of respiration in acid-fast species (Edson & Hunter, 1943; Franke & Schillinger, 1944), from a more detailed study of *Mycobact. phlei* (Edson & Hunter, 1947) and from additional unpublished work on the metabolism of *Mycobact. phlei* and *Mycobact. smegmatis*.

So far as the observations go, the metabolic pattern of the genus *Mycobacterium* is strictly aerobic; the various species oxidize the same substrates, and their activities differ in degree rather than in kind. The metabolism leads to formation of major cellular components, including lipids which are peculiar to the genus. One feature of the metabolism which is re-emphasized by this study of *Mycobact. ranae* is partial oxidation of substrates by washed suspensions (Table 3). In this respect the *Mycobacteria* conform to the general conception of Clifton's (1946) assimilation hypothesis, oxidative catabolic reactions being coupled to synthetic reactions. The primary task in elucidating the meta-

bolism of the acid-fast bacteria is to discover means of dissociating the catabolic reactions from the complex without destroying or inhibiting too many enzymic activities.

Iodoacetate has proved to be an agent of the desired type, since small concentrations act selectively on *Mycobact. ranae*, inhibiting the oxidation of acetate almost completely without abolishing the catabolism of pyruvate. Under these circumstances washed suspensions convert pyruvate into acetate (and CO_2) in a manner which is almost quantitative (Table 9). Unpoisoned cells, however, rapidly oxidize pyruvate and acetate at approximately equal rates. For these reasons it may be assumed that

of the excess pyruvate fails (Table 9). The simplest hypothesis which will embrace these facts is indicated by the continuous arrows in Fig. 2, where pyruvate is shown to break down to acetate, the assimilation of which is linked to its own combustion. The experiments, however, do not exclude the possibility of pyruvate being assimilated as a 3-carbon compound in reactions linked to the oxidation of acetate; nor do they exclude an independent oxidative assimilation of pyruvate, linked to its own combustion and paralysed by arsenite and iodoacetate but not by hydroxylamine. Although hydroxylamine blocks the metabolic route via acetate, it does not hinder oxygen consumption in

Fig. 2. Scheme of pyruvate and acetate metabolism in *Mycobacteria*. Reaction blocked by arsenite I, by iodoacetate, arsenite and hydroxylamine II, by arsenite and iodoacetate III.

acetate is an intermediary in the normal breakdown of pyruvate, although there is as yet insufficient evidence to show how much of the pyruvate takes this pathway. An hypothesis consistent with the facts presented in this paper is illustrated in Fig. 2.

Several additional observations are relevant to the scheme: (1) the molecular ratio of oxygen uptake/acetate or pyruvate disappearance is less than that required for total combustion (Table 3); (2) the metabolism of added pyruvate or acetate is abolished by arsenite (Tables 5 and 6) as it is in *Mycobact. phlei* (Edson & Hunter, 1947); (3) extra oxygen uptake due to the presence of pyruvate is not affected significantly by hydroxylamine, whereas that due to acetate is inhibited almost completely (Tables 5 and 6); and (4) the utilization of pyruvate is much greater in the absence of iodoacetate than in its presence (Table 9) when the sole reaction occurring is

It follows that the above reaction is sensitive to arsenite.

The observations can be explained if iodoacetate interferes not only with the enzymes responsible for combustion of acetate but also with coupled reactions of assimilation. When acetate is not oxidized, it is not metabolized in any other way: thus acetate assimilation is blocked under anaerobic conditions and by arsenite, iodoacetate and hydroxylamine. When a portion of added pyruvate is oxidized to acetate in the presence of iodoacetate, assimilation

of the excess pyruvate fails (Table 6). For this reason an alternative assimilation of pyruvate has been shown by dotted arrows in Fig. 2.

Up to the present, efforts to pursue the analysis by means of broken cells or cell extracts have been unsuccessful, due, perhaps, to lability of the enzymes including the cytochrome system. It is evident that elucidation of the coupled reactions, which are probably the key to an understanding of the metabolism of acid-fast bacteria, must await the preparation of pyruvate oxidases as amenable to experiment as those obtained from *Neisseria gonorrhoeae* (Barron & Hastings, 1933), *Lactobacillus delbrückii* (Lipmann, 1939), *Escherichia coli* (Still, 1941) or *Proteus vulgaris* (Stumpf, 1945).

Oxidation of higher homologues of acetic acid is rapid and accompanied by assimilation (Table 3). The enzymes are labile and the mechanism of fatty acid breakdown is obscure since no intermediates have been detected, although acetoacetic acid is oxidized at a rate compatible with the function of an intermediary metabolite (Table 11). The endogenous formation of acetic acid under the influence of iodoacetate is also noteworthy, but speculation regarding the precursor would be premature.

SUMMARY

1. *Mycobacterium ranae* oxidizes pyruvate and acetate at approximately equal rates, which are about four times greater than the rate of endogenous

respiration. Other *n*-fatty acids (up to C₆) are oxidized at even faster rates.

2. The action of inhibitors on the metabolism of *Mycobact. ranae* has been investigated, notably that of iodoacetate which suppresses metabolism of acetate without completely inhibiting the dissimilation of pyruvate.

3. In the presence of iodoacetate, pyruvate is

oxidized to acetate quantitatively, assimilation processes being abolished. These facts have been used to construct a hypothesis of normal pyruvate metabolism.

4. Preparations of broken cells and cell extracts were unable to oxidize pyruvate or acetate.

We are much indebted to Messrs Merck and Co., Rahway, New Jersey, for a very generous gift of cocarboxylase.

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