several weeks a smell like that of butyric acid was noticed. The samples prepared for analysis in the above work were therefore kept *in vacuo* or in N_2 .

Infrared spectra

Measurements were made with a modified Hilger D. 209 spectrometer, fitted with a rock-salt prism and working as a single beam instrument. Absorption due to atmospheric water vapour was minimized by enclosing the instrument in an air-tight Perspex box through which dry air was circulated. The samples were ground to fine powders which were moistened with Nujol, the resulting mulls being held as thin films between rock-salt plates for examination.

The intensity of the radiation incident on the samples was determined in the usual way by replacing the samples with two rock-salt plates of the same thickness as those used for supporting the mulls. Absorption curves were then drawn (see Fig. 1), the gaps in which are due to regions of Nujol absorption. For calibration of the spectrometer, hydro-

carbon bands of known frequency were used in conjunction with bands due to CO_2 and a small amount of water vapour present in the instrument. Frequencies below 1750 cm^{-1} are accurate to $\pm 3\text{ cm}^{-1}$, and in the 3300 cm^{-1} region to $\pm 10\text{ cm}^{-1}$.

SUMMARY

The mould metabolites frequentin ($C_{14}H_{20}O_4$) and palitantin ($C_{14}H_{22}O_4$) obtained from *Penicillium palitans* and some atypical strains of *Penicillium frequentans* both give dihydropalitantin ($C_{14}H_{24}O_4$) on reduction with sodium amalgam. Hence the carbon skeletons and positions of the oxygen atoms in the two metabolites are identical.

We are indebted to Mr J. F. Grove for valuable advice, to Dr J. H. Birkinshaw for a sample of the dextrorotatory acid, and to the Directors of I.C.I. Ltd. (Plastics Division) and Mr H. A. Willis for spectrographic facilities.

REFERENCES

- Birkinshaw, J. H. (1952). *Biochem. J.* **51**, 271.
 Birkinshaw, J. H. & Raistrick, H. (1936). *Biochem. J.* **30**, 801.
 Curtis, P. J., Hemming, H. G. & Smith, W. K. (1951). *Nature, Lond.*, **167**, 557.

Oxidation of Pyruvic Acid Oxime by Soil Organisms

By J. H. QUASTEL, P. G. SCHOLEFIELD AND J. W. STEVENSON

Research Institute, Montreal General Hospital, Montreal, and Department of Bacteriology and Immunology, McGill University, Montreal

(Received 13 June 1951)

The possibility that hydroxylamine might be an intermediate in the oxidation of ammonia to nitrite by *Nitrosomonas* has often been discussed. There has been, however, no direct evidence to confirm this view and, indeed, hydroxylamine is well known to be toxic in low concentrations to these and many other organisms. Recently, Lees & Quastel (1946), working on the hypothesis that the hydroxylamine formed might be bound as an oxime, reported that the perfusion of an equimolar mixture of sodium pyruvate and hydroxylamine over a soil enriched with nitrifying organisms through previous perfusions of ammonium chloride, resulted in the formation of nitrate. They suggested that the equilibrium of the reaction between sodium pyruvate and hydroxylamine might be such that only a very low concentration of free hydroxylamine was present at any instant. This

concentration might be sufficiently low to allow oxidation of the free hydroxylamine and yet

eliminate its toxic effects. Subsequently, Quastel & Scholefield (1949) were able to show that neither *Nitrosomonas* nor *Nitrobacter* was responsible for oxidation of pyruvic acid oxime (hereafter called pyruvic-oxime) and predicted the existence of a separate enzymic mechanism. The responsible organisms have been isolated, and a preliminary account of them has been reported (Quastel, Scholefield & Stevenson, 1950). The present communication gives a more detailed account of these organisms and of the metabolism of pyruvic-oxime by one of them. Jensen (1951) has since reported the isolation of other organisms capable of producing nitrite from pyruvic-oxime.

METHODS

Preparation of materials. The preparation of the soil used in these experiments, the general methods and the estimations used in soil perfusion have already been fully described (Lees & Quastel, 1946; Quastel & Scholefield, 1949, 1951). A local soil was used throughout the present experimental work.

D-Arabinose-oxime was kindly supplied to us by Dr S. B. Baker, of this Institute, and formaldoxime, salicyl-

aldoxime, furil dioxime and benzoin oxime were Analar reagents. The oximes of acetone and acetoacetic ester were not isolated, a mixture of 1.1 moles of the ketone with 1.0 mole of hydroxylamine being used for the perfusion experiments.

The oximes of the sodium salts of pyruvic, oxalacetic, α -ketoglutaric and phenylpyruvic acids were prepared as follows: hydroxylamine hydrochloride (1.0 mole) and the free acid (1.1 moles) were mixed together and a little NaHCO_3 was added. The mixture was kept as a thick paste

and arabinose oxime, do not yield appreciable quantities of nitrite in a period of 6 weeks. Chlorate is added in all cases to inhibit the further oxidation of nitrite (Lees & Quastel, 1945). Representative results are given in Table 1. It will be noted from this table that the oximes of oxaloacetic, phenylpyruvic and α -ketoglutaric acids do not inhibit markedly (if at all) the oxidation of pyruvic-oxime in soil. Similarly, D-arabinose oxime, whilst itself

Table 1. Nitrite accumulation after perfusion of oximes through a soil

(μg . Nitrite N accumulating per ml. perfusate after perfusion of various oximes (0.01 M) through soil in the presence of 0.001 M-sodium chlorate. 0.01 M-Oxime = 140 μg ./ml.)

Time in hr.	Exp. 1 Exp. 2 Exp. 3	42 43	70 66	91 91	114 115	139 138	300
0.01 M-Pyruvic-oxime	Exp. 1 Exp. 2 Exp. 3	48 6	113 16	113 105	110 107	114 110	109
0.01 M-Arabinose oxime	Exp. 1	4	9	9	11	10	
0.01 M-Arabinose oxime + 0.01 M-pyruvic-oxime	Exp. 1	52	112	109	111	111	
0.01 M- α -Ketoglutaric acid oxime	Exp. 2	11	29	89	104	109	
0.01 M- α -Ketoglutaric acid oxime + 0.01 M-pyruvic-oxime	Exp. 2	3	36	163	189	200	
0.01 M-Oxaloacetic acid oxime	Exp. 3						98
0.01 M-Oxaloacetic acid oxime + 0.01 M-pyruvic-oxime	Exp. 3						203
0.01 M-Phenylpyruvic acid oxime	Exp. 1	1	22	40	58	62	
0.01 M-Phenylpyruvic acid oxime + 0.01 M-pyruvic-oxime	Exp. 1	57	132	150	154	153	
0.01 M-Salicylaldoxime	Exp. 3						3
0.01 M-Furil dioxime	Exp. 3						2

by the addition of small quantities of water as needed. Further NaHCO_3 was added until no more CO_2 was evolved and then sufficient water was added to bring the paste into solution at the boiling point in the presence of a slight excess of the alkali. On cooling, the solution deposited crystals of the sodium salts of the oximes of these α -ketoacids and it was in this form that they were used. The oxime of sodium pyruvate was generally recrystallized from boiling water to remove the brown colour normally associated with the pyruvic acid used. The samples of pyruvic-oxime used in this work contained no free pyruvate as estimated by yeast carboxylase and no free hydroxylamine as estimated by MnO_2 (Mann & Quastel, 1946). α -Ketobutyric acid oxime was prepared by Dr L. Martin of this Institute.

RESULTS

Perfusion of oximes through soil

The first oxime which was found to undergo metabolism in soil was pyruvic-oxime (Lees & Quastel, 1946; Quastel & Scholefield, 1949). It was later shown that oxaloacetic acid oxime suffers a similar breakdown (Quastel *et al.* 1950). To these must now be added phenylpyruvic acid oxime and α -ketoglutaric acid oxime, whilst salicylaldoxime, furil dioxime

yielding little nitrite, in no way impedes the oxidation of pyruvic-oxime. Re-perfusion of arabinose oxime yielded no nitrite in 4 days. The stability of other sugar oximes in soil is now under consideration. Whilst only a dozen oximes have been studied to date, it seems likely that only those of the α -ketoacids are able to undergo nitrification in soil. A preliminary experiment has also shown that the hydrazones, unlike the oximes of these acids, are not nitrified.

The oximes of acetone, formaldehyde, acetoacetic ester and benzoin are relatively inactive as nitrite formers in soil and do not inhibit the oxidation of pyruvic-oxime. It has also been found that disodium succinohydroxamate (prepared in this Institute by Dr L. Martin) does not inhibit this oxidation.

Certain antibiotics have been tested as possible inhibitors of pyruvic-oxime oxidation in soil. It has been found that penicillin and streptomycin are relatively inert. Thus, at a concentration of 0.01 % they produce only half a day's lag period with pyruvic-oxime as substrate. Chloromycetin produces a lag period varying from half a day at a concentration of 0.001 % to 6 days at 0.05 %.

The values for the lag periods are obtained by finding the difference between the times required to reach the asymptotic nitrite value in the presence and absence of the inhibitor. It is of interest to note that, at a concentration of 0.025%, chloromycetin entirely prevents oxidation of sodium nitrite in soil for a period of more than 10 days (Quastel & Scholefield, 1951).

Nitrourea at a concentration of 0.0033M completely inhibits nitrite oxidation for 16 days whilst having no effect on pyruvic-oxime oxidation (Quastel & Scholefield, 1951). Similarly, *p*-aminosalicylic acid produces a 1.5-day lag period in the oxidation of pyruvic-oxime in soil at a concentration of 0.0033M, but will produce a similar effect on the oxidation of ammonium ions at a concentration of 0.001M (Quastel & Scholefield, 1951).

At a concentration of 0.005% the only sulphadiazine drug to have any effect on pyruvic-oxime oxidation is sulphadiazine, six others having relatively little effect.

Manometric experiments on washed suspensions of Achromobacter

As will be seen from the Addendum to this paper, four organisms have been isolated, three from soil and one from faeces, which can bring about the oxidation of pyruvic-oxime to nitrite. Manometric experiments have been carried out using suspensions of these organisms.

Pyruvic-oxime oxidase

The fact that the oxidation of pyruvic-oxime, which takes place in the presence of these organisms, is controlled by a thermolabile enzyme was established by showing that immersion of the organisms in a boiling-water bath for 1 min. completely eliminated their respiratory activity in the presence of pyruvic-oxime. Furthermore, the presence of M/300 sodium cyanide inhibited the oxygen uptake by over 70% and M/300 sodium azide by over 90%. The enzyme controlling the oxidation was termed pyruvic-oxime oxidase (Quastel *et al.* 1950).

Whilst it was quite definite that the three soil organisms and the one isolated from faeces could all metabolize pyruvic-oxime and produce nitrite from this source, their relative activities differed considerably. Suspensions of each organism were therefore prepared and the rates of oxygen uptake in air were measured using potassium hydroxide in the centre wells of the Warburg vessels. In each case the turbidities were made the same, as judged with the Fisher colorimeter using Filter B525, and in each case the endogenous metabolic rate was also estimated. Fig. 1 shows that both *Achromobacter* sp. (I) and the faecal organism actively metabolize pyruvic-oxime. The other species of *Achromobacter* and the species of *Corynebacterium*, on the other hand,

showed only a low rate of oxidation of pyruvic-oxime under these conditions. In all the experimental work discussed in this paper, therefore, the *Achromobacter* sp. (I) has been used. The organisms were always washed twice with distilled water and suspended in distilled water. The suspension contained the growth from one 3-day-old plate per ml. Approximately 5 mg. dry weight washed organisms was used per vessel in all experiments.

Fig. 1. Comparison of the abilities of four organisms to oxidize pyruvic-oxime. Contents of flasks: 1.0 ml. washed suspension of organisms (5 mg. dry wt.), 1.0 ml. 0.1M-phosphate buffer, pH 7.2; 0.2 ml. 0.02M-pyruvic-oxime; water to total vol. 3.2 ml. Air 37°. KOH in centre wells. (I) *Corynebacterium* sp. from soil; (II) *Achromobacter* sp. (2) from soil; (III) *Achromobacter* sp. (1) from soil; (IV) *Achromobacter* sp. (3) from faeces; O, no substrate added; ●, 0.02M-pyruvic-oxime present.

In view of our previous studies concerning the effects of hydrogen-ion concentration on the production of nitrite in soils (Quastel & Scholefield, 1949, 1951) it seemed of interest to investigate the effects of changes of pH on the oxidation of pyruvic-oxime. It will be seen from Fig. 2 that in the range of pH 6–9 there is no appreciable variation in the rate of O₂ consumption, but that a shift of 0.5–1.0 pH unit outside this range causes a very sharp drop in activity. In all our experiments on the production of nitrite from pyruvic-oxime in soil, therefore, pH may be eliminated as a factor in-

fluencing metabolic activity since the buffering power of soil is great enough to maintain conditions well within the region of pH 6-9.

Fig. 2. The variation of activity of pyruvic-oxime oxidation with pH. ○, phosphate buffer (0.066M); ●, borate buffer (final concentration of H_3BO_3 , and KCl 0.05M; pH adjusted with NaOH). For experiment: 2.0 ml. buffer solution; 1.0 ml. bacterial suspension; 0.2 ml. 0.25M-pyruvic-oxime or 0.2 ml. water tipped.

Oxygen consumption during the oxidative metabolism of pyruvic-oxime

It was of interest to discover the number of molecules of O_2 consumed by the organism during its metabolism of one molecule of pyruvic-oxime and whether the ratio of O_2 consumed to pyruvic-oxime used remained constant during the course of an experiment. It has been found convenient to estimate the amount of nitrite formed, as this is a measure of the metabolism of pyruvic-oxime.

Table 2. Comparison of oxygen uptake and nitrite production during metabolism of pyruvic-oxime

(Details given in text.)

Time (min.)	Extra oxygen uptake (i) (μl.)	Nitrite produced (ii) (1 g.mol. = 22.4 l.) (μl. equivalent)	Ratio: (i)/(ii)
15	4.1	2.24	1.8
30	7.4	4.62	1.6
45	13.6	7.54	1.8
60	21.8	—	—
75	30.0	15.3	1.95
90	35.7	20.2	1.8
105	55.4	31.2	1.9
120	71.0	37.6	1.9
135	79.5	41.5	1.9
		Mean	1.82

The results of a typical experiment are given in Table 2. Several manometer vessels were set up containing 1.0 ml. bacterial suspension of the standard organisms, 1.0 ml. 0.1M-phosphate buffer, pH 7.2, and 1.0 ml. water; and into each of them was tipped 0.2 ml. 0.2M-pyruvic-oxime. Every 15 min. one vessel was removed and the nitrite content of

the medium was estimated colorimetrically. From the results given in the above table it will be seen that 1.82 molecules of O_2 are consumed per molecule of sodium nitrite produced. The value of this figure indicates that the molecule of pyruvic-oxime is not completely oxidized by these organisms under our experimental conditions. The theoretical value of the ratio in question for complete oxidation to CO_2 , water and nitrite would be 3.5. Furthermore, it is obvious that the ratio of nitrite produced to O_2 consumed is constant within experimental error during the entire experiment.

A likely mode of breakdown of pyruvic-oxime is given by the following scheme:

Such a scheme involves the necessary intermediate formation of pyruvic acid. Experiments were therefore carried out to discover whether the presence of semicarbazide, by its binding of intermediary pyruvic acid, would influence the rate of O_2 uptake of *Achromobacter* in the presence of pyruvic-oxime. The results showed that semicarbazide (0.05M) neither altered the rate of O_2 uptake nor, within experimental error, the total O_2 uptake.

The oxygen uptake due to the addition of other substances

(a) *Nitrogen-containing molecules.* The metabolism of a number of substrates by *Achromobacter* sp. (1) has been studied with the intention of discovering which compounds are possible intermediates in the metabolism of pyruvic-oxime. Thus one pathway of metabolism of pyruvic-oxime might be via alanine by reduction of the oxime to this amino-acid. That this is not the case, however, is shown by the facts: (i) that the metabolism of the oxime in soil does not proceed via ammonia, whereas that of alanine does (Quastel & Scholefield, 1949); (ii) that alanine on incubation with *Achromobacter* sp. (1) produces no nitrite although it is quite readily oxidized. Another possible intermediate is hydroxylamine, but previous evidence has already rendered this possibility unlikely. Again 0.0001M-hydroxylamine does not produce nitrite on perfusion in soils that readily oxidize pyruvic-oxime.

Since it is clear that neither ammonia nor hydroxylamine is likely to be an intermediate in the breakdown of pyruvic-oxime and since the ratio of O_2 uptake to the formation of nitrite is constant from the start of the experiment, it is reasonable to deduce that the nitrite is formed by an initial direct oxidation of the oxime.

(b) *Oxidation of possible intermediates in the metabolism of pyruvic-oxime.* Investigations were made of the rates of O_2 uptake by a washed suspension of

Achromobacter sp. (1), in the presence of a variety of substances which might be possible intermediates in pyruvic-oxime metabolism. Sodium lactate and sodium pyruvate were found to be oxidized by the organism without any initial time lag; the rates of O_2 uptake were not constant but tended to increase

would yield another 1.79×0.75 , or 1.34, atoms of O per molecule of pyruvic-oxime (Table 3). Thus the oxidation of the oxime by these organisms would require $1.50 + 1.34$, or 2.84, atoms of O per molecule and this is in agreement with the observed value of

Fig. 3. The effect of pyruvic-oxime concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but with varying amounts of pyruvic-oxime. $\circ$, no pyruvic-oxime present; $\bullet$, 0.1 ml. 0.02M-pyruvic-oxime; Δ , 0.2 ml. 0.02M-pyruvic-oxime; $\times$, 0.4 ml. 0.02M-pyruvic-oxime.

with time, a phenomenon also shown by pyruvic-oxime. Sodium propionate was also oxidized, but the onset of oxidation is preceded by an initial time lag. Constant rates of O_2 uptake have been obtained with acetate, but usually even this substrate tends to give an increasing rate of O_2 uptake with time. Typical results for pyruvic-oxime and sodium pyruvate oxidation are given in Figs. 3 and 4. The results shown in Table 3 were obtained with a freshly harvested suspension of organisms that had been subcultured from a stock stored for 6 months at room temperature after lyophilization. Enzymic activity with pyruvic-oxime as substrate remained high but the extent of oxidation was somewhat lowered.

In this experiment the nitrite yield was 75% of the theoretical. It would therefore be expected that 1.50 atoms of O would be taken up per molecule of pyruvic-oxime added, on the assumption that oxidation proceeds initially to pyruvate and nitrite. The further oxidation of the pyruvate produced

Fig. 4. The effect of sodium pyruvate concentration on O_2 uptake by *Achromobacter* sp. (1). Contents of flasks as in Fig. 1 but no pyruvic-oxime present. $\circ$, no sodium pyruvate present; $\bullet$, 0.1 ml. 0.02M-sodium pyruvate; Δ , 0.2 ml. 0.02M-sodium pyruvate; $\times$, 0.4 ml. 0.02M-sodium pyruvate.

2.81. This observation conforms with the view that pyruvate is an essential intermediate in the oxidation of pyruvic-oxime.

Table 3. Atoms of oxygen taken up per molecule of substrate, after lyophilization and storage of *Achromobacter* sp. (1)

Pyruvic-oxime	2.81
Sodium pyruvate	1.79
Sodium acetate	2.24

It will be noted from the results given in Table 3 that the O_2 uptake per molecule of substrate is of the same order with sodium acetate as with sodium pyruvate. This result we consider explicable on the basis that part of the pyruvate (about 40%) is assimilated in synthetic processes in the organism, the remainder undergoing further oxidation.

(c) *The metabolism of other substrates.* When the sodium salts of succinic, fumaric and malonic acids were tested as substrates, the curves shown in Fig. 5

Table 4. *The effect of various oximes on the metabolism of pyruvic-oxime*

(Present in all flasks: 1.0 ml. suspension; 1.0 ml. 0.1M-phosphate buffer, pH 7.2; 0.2 ml. 0.02M-pyruvic-oxime; 0.2 ml. 0.02M-inhibitor and water to total vol. of 3.2 ml. Air. 37°. KOH in centre wells.)

Inhibitor	Oxygen uptake in 45 min. (μ l.)	Correction for O_2 uptake in the absence of pyruvic-oxime and in the presence of the inhibitor (μ l. O_2)	Percentage inhibition of rate of O_2 uptake
None	120	19	0
Phenylpyruvic acid oxime	110	43	33
D-Arabinose oxime	80	25	46
α -Ketoglutaric acid oxime	98	30	32

were obtained. They indicate that malonate is not metabolized by this organism, succinate after a short lag period, and fumarate but feebly.

Fig. 5. The oxidation of various carboxylic acids by *Achromobacter* sp. (1) compared with that of pyruvic-oxime. Contents of flasks as in Fig. 1 with the following substrates replacing pyruvic-oxime. ●, water or sodium malonate (0.2 ml. 0.02M); ○, sodium fumarate (0.2 ml. 0.02M); ×, sodium succinate (0.2 ml. 0.02M); △, sodium pyruvate (0.2 ml. 0.02M); ○, pyruvic-oxime (0.2 ml. 0.02M).

(d) *The effects of other oximes.* The effects of pure samples of the oximes of α -ketobutyric acid, phenylpyruvic acid, α -ketoglutaric acid and arabinose on pyruvic-oxime oxidation were investigated (Quastel *et al.* 1950). The results are shown in Table 4 and indicate inhibitory effects of three of these oximes.

Phenylpyruvic acid oxime solution usually shows

no free nitrite, and yet after 2 hr. incubation with the organism this substance consistently gave 5–10% of its total nitrogen content as free nitrite. Some increase in O_2 uptake is obtained (Table 4), but this is also seen to some extent with the other two oximes which yield no nitrite. This activity is so feeble, however, that it has been classified as approximately zero in considering enzyme substrate specificities. The other two oximes have never yielded any nitrite with this organism.

Table 5. *Metabolism of oximes by Achromobacter sp. (1)*

(Conditions as in Table 4)

Substrate added (μ moles)		Oxygen uptake in 60 min. (μ l.)	Nitrite formed finally (μ moles)
Pyruvic-oxime	α -Ketobutyric acid oxime		
0	0	30	0
10	0	225	7.39
0	10	117	9.36
10	10	184	16.76

It is evident, therefore, that whilst the oxidation of various oximes to nitrite in soil is confined to those of the α -ketoacids, the ability of isolated organisms to oxidize the oximes is more restricted. Thus *Achromobacter* sp. (1) can metabolize pyruvic-oxime but not (or only very feebly) the oximes of the α -ketoacids mentioned above, although some of these are quite readily metabolized on perfusion through soil. One other oxime is, however, metabolized by this organism, i.e. the oxime of α -ketobutyric acid. Evidence for this is presented in Table 5. Both oximes are readily oxidized, but admixture causes the rate of O_2 uptake to fall between the values given by each oxime alone. This would indicate that the two substrates compete for a single enzyme system. Nitrite formation at the completion of the experiment accounts for 74% of the added pyruvic-oxime nitrogen and 94% of the added α -ketobutyric acid oxime nitrogen. In a mixture of the two oximes the nitrite yield is ultimately completely additive.

No period of adaptation seems to be necessary before the oximes are metabolized, and in no manometric experiment has any evidence been obtained which would indicate that *Achromobacter* sp. (1) may become adapted to oxidize any oxime other than those of the α -keto fatty acids.

SUMMARY

1. Oximes of the α -ketoacids (pyruvic, phenylpyruvic, oxaloacetic and α -ketoglutaric) are readily nitrified in soil.

2. The oximes of salicylaldehyde, furil, arabinose, acetone, formaldehyde, acetoacetic ester, and benzoic acid are inert in soil as nitrite-formers and do not inhibit nitrification of pyruvic-oxime in soil.

3. Among six sulphanilamide derivatives tested, sulphadiazine (0.005%) has the most marked inhibitory action on nitrite formation from pyruvic-oxime in soil. Chloromycetin (1 in 40 000) is highly inhibitory, but penicillin and streptomycin are relatively inert under soil conditions. *p*-Aminosalicylic acid has a small inhibitory action, but nitrourea (0.0033M), which completely inhibits nitrite oxidation in soil, has no inhibitory effect on nitrite formation from pyruvic-oxime.

4. Details are given of the isolation and characterization of organisms in soil and in faeces capable of oxidizing pyruvic-oxime. Three species of organisms, attacking pyruvic-oxime, have been isolated from soil. Two of the species fall into the genus *Achromobacter*; the third is identified as a *Corynebacterium*.

5. Among twenty-two stock cultures of bacteria tested for their abilities to proliferate on a pyruvic-oxime medium, a few gave sparse growth and none formed nitrite.

6. Using washed suspensions of *Achromobacter* sp. (1) it is found that during the oxidation of pyruvic-oxime, the ratio of nitrite production to oxygen uptake remains constant.

7. The results of perfusion and manometric experiments indicate that alanine is not an intermediate in pyruvic-oxime oxidation. Hydroxylamine and ammonia are also unlikely to be intermediates.

8. Suspensions of resting *Achromobacter* sp. (1) oxidize acetate and pyruvate readily, succinate after a short lag period and fumarate very slowly. Malonate is not attacked.

9. The evidence would indicate that pyruvic-oxime is oxidized to pyruvate and nitrite, the pyruvate then undergoing further breakdown. The enzyme system responsible for the oxidation of pyruvic-oxime is termed pyruvic-oxime oxidase. Sodium azide and sodium cyanide markedly inhibit the activity of pyruvic-oxime oxidase.

10. Pyruvic-oxime oxidation by suspensions of resting *Achromobacter* sp. (1) is inhibited by the oximes of α -ketoglutaric acid, phenylpyruvic acid and arabinose. These oximes are apparently either feebly or not attacked by pyruvic-oxime oxidase. The oxime of α -ketobutyric acid is also oxidized by washed suspensions of *Achromobacter* sp. (1), probably by the pyruvic-oxime oxidase.

REFERENCES

- Jensen, H. L. (1951). *J. gen. Microbiol.* **5**, 360.
 Lees, H. & Quastel, J. H. (1945). *Nature, Lond.*, **155**, 276.
 Lees, H. & Quastel, J. H. (1946). *Biochem. J.* **40**, 803.
 Mann, P. J. G. & Quastel, J. H. (1946). *Nature, Lond.*, **158**, 154.
 Quastel, J. H. & Scholefield, P. G. (1949). *Nature, Lond.*, **164**, 1068.
 Quastel, J. H. & Scholefield, P. G. (1951). *Bact. Rev.* **15**, 1.
 Quastel, J. H., Scholefield, P. G. & Stevenson, J. W. (1950). *Nature, Lond.*, **166**, 940.

ADDENDUM

The Isolation of Bacteria from Soil Perfused with Pyruvic Acid Oxime

By J. H. QUASTEL, P. G. SCHOLEFIELD AND J. W. STEVENSON

Research Institute, Montreal General Hospital, Montreal, and Department of Bacteriology and Immunology, McGill University, Montreal

(Received 13 June 1951)

Approximately 2 g. of perfused soil 'crumbs' were brought into a finely particulate suspension in 6 ml. of sterile distilled water by gently grinding in a mortar. Larger soil particles were allowed to settle out and the supernatant fluid which contained the smaller particles in suspension was cultured. The culture medium, which contained pyruvic-oxime as the only source of assimilable carbon and nitrogen, was pre-

pared as follows: K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.2 g.; $MnSO_4 \cdot 4H_2O$, trace; $FeCl_2 \cdot 6H_2O$, trace; Granular Agar (Difco), 15 g.; pH adjusted to 7.0 with NaOH. Dispensed in 100 ml. lots and autoclaved at 120° for 20 min. 0.25M-Pyruvic-oxime (10 ml.) rendered sterile by Seitz filtration was added to each 100 ml. lot of molten agar-salt solution base and plates were poured.

An inoculum of 0.1 ml. of the soil suspension per plate, spread evenly over the surface of the agar, provided cultures in which colonies were, for the most part, discrete. Plates were incubated at 25 and 37°. Despite the fact that perfusion had been carried out at 21° optimal growth was obtained at 37°. There was no selective effect of incubation temperature, plates incubated at 25 and 37° showing the same flora. Incubation for 72 hr. was required to bring out characters by which various colony types might be distinguished one from the other. No new forms appeared on continued incubation beyond this line.

A number of picks of each colony type were grown in pure culture on pyruvic-oxime agar in order to test their ability to attack pyruvic-oxime with the liberation of free nitrite. The test consisted in removing a small square of the agar bearing the growth (0.5 sq.cm.) which was dropped into Ilosvay reagents in a tube. All picks gave strong tests for nitrite. Control squares of sterile pyruvic-oxime agar incubated for the same period of time showed no trace of nitrite. The various picks upon taxonomic study were resolved into three distinct species. It is of interest to note that all of the organisms isolated from the soil on the pyruvic-oxime medium were capable of oxidizing pyruvic-oxime with the production of nitrite. This would tend to suggest a process of selection or adaptation or perhaps a combination of both phenomena during perfusion.

Two of the species isolated from the perfused soil fall into the genus *Achromobacter*; the third has been identified as a *Corynebacterium*. A third species of the genus *Achromobacter* capable of oxidizing pyruvic-oxime with the formation of nitrite has been isolated from a sample of faeces. None of the species which were isolated conforms with species described under the genera *Achromobacterium* and *Corynebacterium* in Bergey's Manual (1948). Due to the unsettled state of classification of species within these genera the organisms isolated have not been given specific designations.

The following are the characteristics of the various species.

Achromobacter sp. (1)

From perfused soil.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0.5 by 1.3 μ . No endospores. Not encapsulated. Motile, possessing peritrichous flagella. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2–3 mm. in diameter, low convex, circular with entire edges, grey, smooth and glistening, translucent, mucoid. Growth in peptone broth turbid with pellicle and sediment, aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. Cannot use salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (2)

From perfused soil.

Morphology. Rods with rounded ends occurring singly and in pairs. Coccoid and oval forms 0.5–1 by 1–3 μ . No

endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, grey, smooth and glistening, translucent. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates rapidly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Achromobacter sp. (3)

A third species of *Achromobacter* capable of oxidizing pyruvic-oxime with nitrite production was isolated from faeces and characterized as follows.

Morphology. Straight rods with rounded ends occurring singly and in pairs. 0.5 by 1.3 μ . No endospores. Not encapsulated. Non-motile. Gram-negative.

Cultural characteristics. Colonies on peptone agar 2–3 mm. in diameter, low convex, circular with entire edges, grey to faint cream colour (no pigment on colourless media), smooth and glistening, translucent. Non-mucoid. Growth in peptone broth: general turbidity with sediment. Aerobic, facultative. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates very slowly reduced to nitrites. H_2S not produced. Indole not formed. Acetylmethylcarbinol not produced. May utilize salts of citric acid as a sole source of carbon. Cellulose not digested. Starch not hydrolysed. Acid from dextrose and galactose. No acid from arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, levulose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

The essential points of difference upon which the separation of the foregoing organisms as distinct species is based are outlined in Table 1.

Table 1. *Distinguishing features of three species of Achromobacter which oxidize pyruvic-oxime*

Test	<i>Achromobacterium</i> sp. (1)	<i>Achromobacterium</i> sp. (2)	<i>Achromobacterium</i> sp. (3)
Motility	+	—	—
Nitrate reduction	Rapid	Rapid	Very slow
Acid from dextrose	—	—	+
Acid from galactose	—	—	+
Use of salts of citric acid as sole source of carbon	—	+	+

Corynebacterium sp.

From perfused soil.

Morphology. Slightly curved rods, 0.5–1 by 2–4 μ . in young culture. 1 by 6–8 μ . in older culture. Palisading and angular arrangement. Pleomorphic on ageing, with club forms. No endospores. Not encapsulated. Non-motile.

Uniformly Gram-positive in young culture tending to variability in older culture with Gram-negative cytoplasm beaded with Gram-positive granules. Not acid-fast.

Cultural characteristics. Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, white, smooth non-glistening surface. Growth in peptone broth: granular sediment with little general turbidity. Aerobic. Good growth at 25 and 37°.

Physiological characteristics. Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates slowly reduced to nitrites. H₂S not produced. Indole not formed. Cellulose not digested. Starch not hydrolysed. Acid from levulose. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Unless otherwise indicated, *Achromobacter* sp. (1) has been used for all the manometric studies.

In order to determine whether or not species of bacteria other than those isolated from the perfused soil might attack pyruvic-oxime with the production of nitrite, a series of laboratory stock cultures which had been maintained on peptone agar were transferred to pyruvic-oxime agar. The results are outlined in Table 2. It is seen that none of a variety of species held in stock possessed, initially at least, the ability to produce nitrite from pyruvic-oxime. Indeed many of the species tested failed to grow on this medium. Growth where it occurred was in all instances rather sparse requiring from 3 days to 1 week of incubation, depending upon the species. The finding that certain heterotrophic organisms may oxidize pyruvic-oxime to nitrite has been confirmed by the recent work of Jensen (1951).

It was felt that adaptation might be a factor in determining the ability of an organism to attack pyruvic-oxime. In order to test this, four selected species, *Achromobacter hartlebii*, *Achromobacter perolens*, *Agrobacterium radiobacter*

and *Bacterium globiforme*, were carried through six successive transfers on pyruvic-oxime agar. On the sixth transfer the two species of *Achromobacter* produced very small amounts

Table 2. *The ability of various stock cultures of bacteria to grow on pyruvic-oxime agar and to produce nitrite from pyruvic-oxime*

Organism	Growth	Nitrite
<i>Achromobacter hartlebii</i>	+	—
<i>Achromobacter perolens</i>	+	—
<i>Achromobacter</i> sp. (isolated from fish)	+	—
<i>Alcaligenes faecalis</i>	+	—
<i>Agrobacterium radiobacter</i>	+	—
<i>Chromobacterium violaceum</i>	—	—
<i>Pseudomonas aeruginosa</i> (2 strains)	+	—
<i>Escherichia coli</i>	—	—
<i>Aerobacter aerogenes</i>	+	—
<i>Klebsiella pneumoniae</i>	+	—
<i>Serratia marcescens</i>	+	—
<i>Proteus vulgaris</i>	—	—
<i>Micrococcus ureae</i>	—	—
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	—
<i>Micrococcus epidermidis</i>	—	—
<i>Micrococcus agilis</i>	—	—
<i>Sarcina lutea</i>	—	—
<i>Corynebacterium segmentosum</i>	—	—
<i>Bacterium globiforme</i>	+	—
<i>Bacillus mesentericus</i>	—	—
<i>Bacillus cereus</i>	—	—
<i>Bacillus megatherium</i>	—	—

of nitrite. *Agrobacterium radiobacter* and *Bacterium globiforme* failed to produce nitrite. It is of interest to note that the two species which gained, presumably by adaptation, the ability to attack the substrate with the liberation of free nitrite were both members of the same genus as the three active species isolated from perfused soil and faeces.

REFERENCES

- Bergey's *Manual of Determinative Bacteriology* (1948). 6th ed. Edited by Breed, R. S., Murray, E. G. D. & Hitchins, A. P. Baltimore: Williams and Wilkins.
- Jensen, H. L. (1951). *J. gen. Microbiol.* 5, 360.

The Isolation of Androst-16-en-3 α -ol from Women's Urine

By B. W. L. BROOKSBANK AND G. A. D. HASLEWOOD

St Thomas's Hospital Medical School and Guy's Hospital Medical School, London, S.E. 1

(Received 20 December 1951)

The musk-smelling androst-16-en-3 α -ol, first isolated from swine testes by Prelog & Ruzicka (1944), was obtained from a glucuronide fraction from men's urine by Brooksbank & Haslewood (1949, 1950) and from the urine of women with adrenal cortical tumours by Mason & Schneider (1950). When Brooksbank & Haslewood (1950) tried to obtain this steroid from the urine of women

not showing any endocrine abnormality, they were able to isolate from the musk-smelling fraction only a small amount of a substance melting at about 160°, and therefore different from the androstenol. It has seemed of interest to us to repeat this work in order to settle, if possible, the question of the nature of the musk-smelling constituent of (hydrolysed) urine from normal women.