factor is not replaceable by methylene blue, pyocyanine, glutathione, coenzyme II, yeast extract, or boiled or unboiled whole yeast.

3. The effect of hydrogen-donator concentration on the rate of DHA reduction, as compared with the rate of methylene-blue reduction, indicates that the rate of hydrogen transfer may be greatly reduced in the DHA-reducing system.

4. Growth on lactate or formate in place of glucose increases the hydrogenase activity, but decreases the DHA-reducing activity.

5. When more than one hydrogen donator is present, the rates of DHA reduction are not additive. The rate of reduction when formate, lactate and hydrogen are present together is not as great as when glucose is present alone.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The writer wishes to thank Dr M. Ingram and Dr L. W. Mapson for the interest they have taken in the work.

REFERENCES

- Bovarnick, M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **47**, 191.
Esselen, W. B. & Fuller, J. E. (1939). *J. Bact.* **37**, 501.
Green, D. E., Stickland, L. H. & Tarr, H. L. A. (1934). *Biochem. J.* **28**, 1812.
Gunsalus, I. C. & Hand, D. B. (1941). *J. biol. Chem.* **141**, 853.
Mapson, L. W. & Ingram, M. (1951). *Biochem. J.* **48**, 551.
Stewart, A. P. & Sharp, P. F. (1945). *Industr. Engng Chem.* **17**, 373.
Stickland, L. H. (1929). *Biochem. J.* **23**, 1187.
Tkachenko, E. S. (1936). *Biochimia*, **1**, 579. Cited in *Chem. Abstr.* (1937). **31**, 7461.
Yudkin, J. (1937). *Biochem. J.* **31**, 865.

Oxidation of Phenylacetic Acid by *Penicillium chrysogenum*

By D. J. D. HOCKENHULL, A. D. WALKER, G. D. WILKIN AND F. G. WINDER
Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks

(Received 4 June 1951)

Phenylacetic acid (PA) has been shown by Singh & Johnson (1948) to be almost wholly consumed by *Penicillium chrysogenum* in fermentations producing penicillin. Only a small fraction entered the penicillin produced. The present study has indicated that the rest is wholly or partly oxidized, beginning with the acetic acid residue.

This type of oxidation in moulds has not hitherto been described. Supniewski (1942) has, however, claimed that his 'Bacille pyocyanique' was capable of oxidizing PA by way of benzoic acid.

EXPERIMENTAL

Methods

(1) *Determination of phenylacetic acid.* As acetic acid was a normal contaminant of the fluids examined, it was necessary to steam-distil them before extraction with ether, following the procedure described by Friedemann (1938), but using Hockenhull's (1949) modification of the apparatus. Results had a precision of $\pm 5\%$. Benzoic acid interfered because it behaved in almost the same way as PA.

(2) *Growth conditions.* The material for use in these studies was obtained by growing the mould upon the synthetic medium of Jarvis & Johnson (1947). (Lactose, 22.5; glucose, 7.5; ammonium acetate, 3; PA, 2; KH_2PO_4 , 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.10; $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004; Na_2SO_4 , 0.5; CaCO_3 , 10 g/l.

distilled water.) The medium was adjusted to pH 6.3 with 10N-NaOH before sterilization for 15 min. at 15 lb./sq.in. It was distributed in 60 ml. quantities into 250 ml. conical flasks. The flasks were each inoculated with 3% of a vegetative inoculum and incubated at 25° on a rotary shaker at 180 rev./min.

The inoculum was prepared as follows: A suspension of 10^7 to 10^8 spores/ml. in 0.1% aqueous Calsolene oil HS (Imperial Chemical Industries Ltd.) was made up from a 6- to 8-day culture on glycerol-molasses-peptone agar of the organism which had been derived from *P. chrysogenum* Q176 by ultraviolet light mutation. This suspension (3 ml.) was inoculated into 60 ml. of a development medium (containing corn steep liquor (C.S.L.), brown sugar and 0.1% PA) in a 250 ml. conical flask. This was incubated for 40 hr. at 25° on a rotary shaker at 180 rev./min.

(3) *Respiration of mould suspensions in presence of PA.* The preparations used for respirometric work with Warburg apparatus were made as follows. Mycelium from the 60 ml. culture described above was centrifuged, washed in 60 ml. 0.05M-sodium phosphate buffer, pH 7.2, centrifuged and again suspended in 60 ml. buffer in a 250 ml. conical flask. Four 6 mm. glass beads were added and the flask was shaken at 25° for 16 hr. to reduce O_2 consumption in the absence of substrate. The felt was again centrifuged off and finally resuspended in a further 60 ml. of buffer. In the presence of PA this suspension gave an increase in respiration of 50–200% over the blank.

The Warburg vessels usually contained 2 ml. suspension (20–30 mg. dry wt.). The standard substrate concentration

was 0.01 M. The Q_{O_2} (PA) (μ l. O_2 absorbed/mg. dry wt. mycelium/hr.) was found by the usual method; Q_{O_2} (PA) = Q_{O_2} (total) - Q_{O_2} (blank). In presence of 0.01 M-substrate the rate of O_2 uptake was almost constant for at least 1 hr.

(4) *Disappearance of PA in presence of the mould.* A grown culture was harvested, filtered, washed and resuspended in pH 7.2 0.05 M-sodium phosphate buffer.

PA was added at about 2.5 mg./ml. Tests were usually carried out in a volume of 60 ml. in 250 ml. conical flasks at 25° on the shaker (180 rev./min.) for a period of 16 hr. About 30–40% of the added PA was utilized at a substantially constant rate.

RESULTS

Examination of PA metabolism in typical fermentations

Standard fermentations were set up on Jarvis & Johnson (1947) medium (60 ml. in each of twenty-four 250 ml. flasks). At intervals up to 100 hr. pairs of flasks were withdrawn, the contents were

Fig. 1. Relationship between enzymic activity, growth and medium constituents in shaken cultures of *P. chrysogenum* Q176 at 25°. Graphs marked PA and HPA represent total Q_{O_2} in presence of PA and *p*-hydroxyphenylacetic acid respectively. □—□, whole broth; ○—○, HPA; ●—●, PA; ◆—◆, blank.

washed and starved suspensions prepared (§3 of Methods). The filtrates were analysed for PA. The activity of the suspension was tested against PA and also against the broth filtrate at its original strength (whole broth).

Fig. 1 shows that the respiratory activity against PA and broth constituents was greatest after about 18 hr. By this time the PA level had begun to fall

rapidly. The mycelial weight was then about 75% of its maximum.

Accordingly, preparations for further study were harvested at 16–18 hr.

Relation of Q_{O_2} (PA) to substrate concentration

Respirometer experiments were carried out using different levels of PA (Table 1).

Table 1. Relation of Q_{O_2} (PA) to substrate concentration

(Incubation mixture: washed starved suspension prepared as described in section 3 of Methods, 2 ml. (25–30 mg. dry wt.); substrate, 0.2 ml. in side bulb.)

PA (Na salt) (M)	Q_{O_2} (PA) 0–20 min.
Exp. 1 0.00640	3.90
0.00250	3.55
0.00100	3.45
0.00040	3.45
0.00016	3.01
0.000064	3.12
0.000026	2.55
(Endogenous respiration)	(2.45) Q_{O_2} (blank)
Exp. 2 0.100	2.93
0.04	3.00
0.016	4.36
0.0064	4.10
0.0026	4.21
0.0010	3.98
0.0004	3.88
0.00016	3.48
(Endogenous respiration)	(1.62) Q_{O_2} (blank)

At the lowest concentrations employed there was a decrease in Q_{O_2} during the 20 min. reaction time, which, however, could be mainly accounted for by exhaustion of substrate. The differences during the 5 min. between readings were too small, compared with the inherent error of the method, to show whether any change in respiration was associated with change in substrate concentration. At this lowest level the oxygen consumption for total oxidation was calculated and found to be 8.6 mol. oxygen/mol. PA as against a theoretical value of 9.0 mol./mol. for complete conversion of PA to carbon dioxide.

At 0.000064 M and above, the respiratory rate was almost constant except at the very high (0.10 and 0.04 M) concentrations where there was a decline, possibly due to toxicity.

Relation of enzyme activity to pH value

Respirometer experiments were carried out in 0.05 M-sodium phosphate buffers from pH 5.6 to 8.4. Controls without substrate were carried out at each pH value. The Q_{O_2} (PA) was found to increase towards the highest pH value (Fig. 2).

*Accelerated reduction of methylene blue
in presence of PA*

A response to PA was obtained when the mould was incubated anaerobically with methylene blue in Thunberg tubes. For this, 5 ml. of washed, starved suspension (about 25 mg. dry wt./ml.) were employed in the main tube. The side bulbs each contained 1.0 ml. of 0.1% methylene blue with or without 1.0 ml. of 0.1 M-PA. Incubation was carried out at 26°. Decolorization times were: blank, 10.5 min.; with substrate, 7.5 min. (means of four values).

Fig. 2. Relationship between pH and PA oxidation by washed suspensions of *P. chrysogenum* Q176 at 26°.

Substrate specificity of the oxidizing systems

Under standard conditions with the Warburg respirometer the following substrates showed marked enhancement of respiration over the

blanks: hydrocinnamic, benzoylformic, DL-hydratropic and *p*-hydroxyphenylacetic acids. Smaller increases were shown with benzoic, DL-mandelic, D-hydratropic acids. The responses of β -phenylisobutyric and *p*-hydroxybenzoic acids were negligible (Table 2).

It must be emphasized that the above results were obtained from different preparations and should, therefore, be interpreted only in a qualitative sense.

Inhibitor studies

Effects on respiration. The effects of various compounds (brought to pH 7.2 with sodium hydroxide or hydrochloric acid) at 0.0001 M were tested upon the standard respiration systems (0.01 M-PA). The substances tested were chosen either for structural similarity to PA or because they had been shown to have inhibitory activity against special types of enzymes. The following substances were tested: *p*-hydroxyphenylacetic, *p*-bromophenylacetic, *p*-aminophenylacetic, *p*-nitrophenylacetic, DL- α -chlorophenylacetic, DL- α -aminophenylacetic, D or DL- α -methylphenylacetic (hydratropic), $\alpha\alpha$ -dimethylphenylacetic (β -phenylisobutyric), benzoic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, *o*-aminobenzoic, *p*-aminobenzoic, *p*-methoxybenzoic, *o*-chlorobenzoic, *m*-chlorobenzoic, *p*-chlorobenzoic, *p*-bromobenzoic, *p*-iodobenzoic, *p*-nitrobenzoic, benzene-sulphonic, toluene- α -sulphonic, toluene-*p*-sulphonic, naphthalene-2-sulphonic, hexahydrophenylacetic, 2-naphthylacetic, cinnamic, hydrocinnamic, DL-mandelic, monochloroacetic, trichloroacetic, monoiodoacetic and dichlorophenoxyacetic acids, phenylethylamine, phenylacetamide, benzylcyanide, *N*-phenylacetyl- α -alanine, *N*-phenylacetyl- β -alanine, *p*-fluorophenylethylamine, sulphanilamide, α -alanine, β -alanine, phenylalanine, *p*-cresol, a commercial mixture of *o*-, *m*- and *p*-cresols, *p*-nitrophenol, 2:4-dinitrophenol, sodium azide, hydroxylamine, sodium fluoride, mercuric chloride, copper sulphate, sodium arsenite, sodium arsenate, quinine, acriflavine, methylene blue, semicarbazide, 2:3-dimer-

Table 2. *Specificity of the oxidizing systems*

(Incubation mixture: suspension as § 3 of Methods, 2 ml.; substrate, 0.2 ml. of 0.1 M-Na salt. Each figure is the mean of two readings not differing from each other by more than 0.10. The respiration of the system in presence of PA is given each time as this varied from preparation to preparation, as these had to be prepared afresh for each experimental run.)

Substrate (S) 0.01 M	Q_{O_2} (S)	Q_{O_2} (control)	Q_{O_2} (no substrate)
Benzoic acid	0.25	2.34	3.35
DL-Mandelic acid	1.30	4.89	2.40
Hydrocinnamic acid	0.24	1.30	3.21
D-Hydratropic acid	1.16	1.17	1.93
DL-Hydratropic acid	0.39	1.17	1.93
β -Phenylisobutyric acid	1.00	1.30	3.21
<i>p</i> -Hydroxybenzoic acid	0.15	1.3	5.40
<i>p</i> -Hydroxyphenylacetic acid	0.12	2.34	3.35
Benzoylformic acid	9.19	4.89	2.40
	0.62	1.09	2.38

captopropanol (BAL), dithizone, 8-hydroxyquinoline and salicylaldehyde. The only significant inhibitions (per cent residual activity given in parentheses) were produced by *p*-fluorophenylethylamine (60), cinnamate (69), fluoride (65), iodoacetate (23) and mercuric chloride (0).

Effects of disappearance of PA. Investigations were also carried out by the acidimetric technique described in § 1 of Methods. Owing to the inability of the analytical techniques to distinguish between PA and benzoic acid, blockage of benzoic acid oxidation with consequent accumulation would be indistinguishable from failure to bring about the initial oxidation of PA. The compounds listed above were employed at 0.0015M. Inhibitions (per cent residual activity given in parentheses) were obtained with *p*-fluorophenylethylamine (51), sodium cinnamate (47), sodium iodoacetate (51), mercuric chloride (48), *p*-cresol (47), 'mixed cresols' (4), sodium azide (8) and hydroxylamine (8). The last four had no effect on the oxygen uptake due to PA. The effect of azide and hydroxylamine may indicate the participation of a metallo-enzyme in the system, possibly at the benzoic acid level.

Arsenite, quinine, acriflavine and thiocyanate were ineffective. Potassium cyanide was itself destroyed by the system. Copper sulphate was ineffective even up to 0.03M. All results were corrected for the effect of inhibitors upon the blanks.

Isolation of intermediate metabolites

A mixture containing 5 mg./ml. PA in 500 ml. of washed suspension was allowed to react according to the technique described above. It was thought that phenolic bodies might arise by oxidation of the ring in a manner analogous with the conversion of phenylalanine to tyrosine in mammals (Jervis, 1947). Filtered samples were therefore tested for phenols, either by coupling with diazotized sulphathiazole (method of Fox, 1922) or by coupling with 2-chloro-4-nitrophenyldiazonium naphthalene-2-sulphonate (by the methods described by Heinrich & Schuler, 1947, 1948). Red-brown or red-purple colours were obtained, respectively. The chromogenic product seemed, however, to pass into ether from neutral or even mildly alkaline aqueous solution, and no 'acidic' product could be isolated. No fraction corresponding to *p*-hydroxyphenylacetic acid could be isolated, although this was not surprising in view of its ready oxidizability (usually thrice that of PA).

Attempts to trap quinones by the addition of aniline to the reaction mixture were also unsuccessful, probably because of a general toxic effect on the mould. The results of inhibitor and other studies indicated that side-chain oxidation was probable. If this were so, the formation of benzaldehyde might be expected and further efforts were directed to its

isolation. The addition of phenylhydrazine oxalate (1 mg./ml.) to the reaction mixture completely inhibited PA oxidation. Sulphite, however, proved non-inhibitory and gave the following results:

Exp. 1. Production of benzaldehyde from PA. Felt from 500 ml. of medium was filtered and resuspended in 500 ml. of solution containing 10 g. KH_2PO_4 , 25 g. Na_2SO_3 and 2.5 g. PA (Na salt). The mixture was shaken at 25° for 47 hr. After 22 hr. a smell of benzaldehyde was detected.

The filtered broth (about 400 ml.) was made blue with thymolphthalein by adding 10% (w/v) Na_2CO_3 and extracted with three 120 ml. portions of ether. After washing with water, the ether extract was concentrated under reduced pressure. The aqueous residue (2–3 ml.) was diluted to 10 ml. with ethanol and divided into two portions. From these, benzaldehyde 2:4-dinitrophenylhydrazone (yield 65 mg., m.p.: isolated material 240–241°) and benzaldehyde semicarbazone (yield 70 mg., m.p.: isolated material 216–218°) were prepared; melting points of the authentic derivatives and mixed melting points were identical with these values. (Semicarbazone: Found: C, 58.3; H, 5.4; N, 25.8, 26.0. Calc. for $\text{C}_9\text{H}_8\text{ON}_3$: C, 58.9; H, 5.6; N, 25.8%.)

Exp. 2. Production of benzaldehyde from DL-mandelic acid. Exp. 2 differed from Exp. 1 in that 2.5 g. of DL-mandelic acid replaced the 2.5 g. of PA. The whole ethanolic extract prepared as in Exp. 1 yielded 108 mg. of semicarbazone (m.p. 210–216°).

Exp. 3. Production of benzaldehyde from benzyl alcohol. An experiment similar to Exp. 2 was carried out with 2.5 g. of benzyl alcohol instead of PA. The whole ethanolic extract yielded 242 mg. of semicarbazone (m.p.: 210–216°).

Exp. 4. Production of acetophenone from DL-hydratropic acid. Exp. 1 was repeated with 2.5 g. DL-hydratropic acid instead of the PA.

After 1 hr. a smell of acetophenone was apparent. After 23 hr. the mixture was extracted as in Exp. 1. All the alcoholic extract was used to make a semicarbazone. Yield 110 mg.; m.p. isolated material, 190–196°; authentic acetophenone semicarbazone, 198–199°; mixed, m.p. 193–197°; benzaldehyde semicarbazone, 216–218°. (Found: C, 60.6; H, 6.2; N, 23.4; Calc. for $\text{C}_9\text{H}_{11}\text{ON}_3$: C, 61.0; H, 6.3; N, 23.7%.)

Later experiments showed that sulphite was necessary for the production of acetophenone.

Exp. 5. Exp. 1 was repeated with 2.5 g. benzoylformic acid instead of PA. Although a slight smell of benzaldehyde appeared on making the solution alkaline, no derivative could be isolated by the above technique.

Nature of penicillins produced on synthetic medium

A complete fermentation was carried out on a 60 ml. scale by the method described above. The broth filtrate was assayed at 6 days for the various penicillins (paper chromatography). The relative percentage activities of the various components was as follows: (*Staphylococcus aureus* units): *p*-hydroxybenzyl (X) 0.1; X_1 , 0.9, benzyl (G) 63.4; amyl, 5.6; *n*-heptyl (K), 23.7; K_1 , 0.2; pentenyl (F), 6.1%. This extremely low proportion of penicillin X indicated the formation of no substantial quantity of *p*-hydroxyphenylacetic acid from PA.

DISCUSSION

The isolation of benzaldehyde has shown that the initial oxidation of PA chiefly takes place in the side chain, although ring oxidation cannot be completely excluded. The methylene-blue technique indicated that the first oxidative attack on the side chain was probably a dehydrogenation.

Possible routes to benzaldehyde might be as follows:

Since benzyl alcohol and mandelic acid have both given rise to benzaldehyde, neither can be excluded as an intermediate. Benzoylformic acid, however, although oxidized by the system, has not been found to give rise to significant quantities of benzaldehyde and is thus unlikely to be an intermediate. The finding that hydratropic acid may be converted to acetophenone indicates the possibility of a homologous reaction for PA (without prior oxidation to the keto acid). The above is in contrast to the finding of Gunsalus, as reported by Stanier (1950), that benzoylformic acid is an oxidation product of mandelate and may be further transformed to benzaldehyde by *Pseudomonas fluorescens*.

The possibility of direct oxidation of PA to

benzaldehyde must not be ignored, although such reactions ('perhydroxylations') generally require molecular oxygen or peroxide.

The transformation of benzaldehyde to benzoic acid, whether spontaneous or enzymically catalysed, is presumed to be the next stage in PA degradation. Possible mechanisms for further oxidation might resemble those discussed in the reviews by Happold (1950) and Stanier (1950).

SUMMARY

1. Phenylacetic acid is oxidized completely to carbon dioxide by mycelial suspensions of a mutant of *Penicillium chrysogenum* Q 176.

2. The oxidative activity of such suspensions was greatest when the parent culture had achieved three-quarters of its maximum growth, the total respiratory activity being highest at this time.

3. Respiration in presence of phenylacetic acid increased over the pH range 5.6–8.4.

4. Benzaldehyde has been characterized as a product of PA oxidation.

5. Similar suspensions oxidized benzoic, hydrocinnamic, DL-mandelic, DL- and D-hydratropic, *p*-hydroxyphenylacetic, *p*-hydroxybenzoic and benzoylformic acids and benzyl alcohol, but not β -phenylisobutyric acid. Benzaldehyde has been isolated after biological oxidation of DL-mandelic acid or benzyl alcohol: acetophenone has been obtained by oxidation of hydratropic acid. Benzoylformic acid does not yield benzaldehyde in the presence of the mould.

6. The oxidation of phenylacetic acid is inhibited by mercuric or iodoacetate ions.

We wish to thank Dr B. A. Hems and Dr J. Elks for providing many of the compounds tested as inhibitors. We acknowledge also the technical assistance of Mr D. Goodinson and Miss E. Theakston.

REFERENCES

- Fox, J. J. (1922). *J. Soc. chem. Ind., Lond.*, 173 T.
 Friedemann, T. C. (1938). *J. biol. chem.* 123, 161.
 Happold, F. C. (1950). *Biochem. Soc. Symp.* 5, 85.
 Heinrich, P. & Schuler, W. (1947). *Helv. chim. Acta*, 30, 886.
 Heinrich, P. & Schuler, W. (1948). *Helv. chim. Acta*, 31, 320.
 Hockenhull, D. J. D. (1949). Ph.D. Thesis, University of Manchester.
 Jarvis, F. G. & Johnson, M. J. (1947). *J. Amer. chem. Soc.* 69, 3010.
 Jervis, G. A. (1947). *J. biol. chem.* 169, 651.
 Singh, K. & Johnson, M. J. (1948). *J. Bact.* 56, 339.
 Stanier, R. Y. (1950). *Bact. Rev.* 14, 179.
 Supniewski, J. (1942). *C.R. Soc. Biol., Paris*, 90, 1111.