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The Metabolism of Naphthalene by Soil Bacteria

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SUMMARY: The early stages in the oxidation of naphthalene by five micro-organisms were investigated. In all the organisms, D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, salicylic acid and catechol appeared to be intermediate oxidation products. Washed cells grown on naphthalene oxidized α- and β-naphthol, but whether these compounds are true intermediates remains undecided.

When naphthalene is administered to animals, D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (naphthalene diol), α-naphthol and their conjugates can be isolated from the urine. The diol and its glucuronide are readily dehydrated in dilute acid solution to naphthol and naphthol glucuronide respectively. Corner, Billett & Young (1954) found that most of the glucuronide in the urine of naphthalene-treated rabbits was diol glucuronide and suggested that the naphthol glucuronide reported by previous workers was an artefact. If this suggestion is well-founded, the scheme of naphthalene metabolism presented by Boyland & Wiltshire (1953*b*) can be simplified by omission of the pathway naphthalene $\rightarrow$ α-naphthol, and naphthol can be excluded as an intermediate in naphthalene oxidation by animals. Such a conclusion would be consonant with the conversion of naphthalene to diol, but not to naphthol by rat liver slices (Boyland & Wiltshire, 1953*a*). Walker & Wiltshire (1953) showed that a certain soil bacterium oxidized naphthalene to naphthalene diol but did not produce or oxidize naphthols. Several different pseudomonads which had been grown with naphthalene as sole carbon source gave an immediate oxygen uptake with α- or β-naphthol as substrate (Stanier & Sistrom, personal communication). Treccani (1953) studied another naphthalene-utilizing organism, probably a *Nocardia* species, which produced salicylic acid from naphthalene.

We have therefore compared the pathways of naphthalene catabolism in five different organisms using growth in defined media, the simultaneous adaptation technique (Stanier, 1947) and chemical methods for the identification of intermediate oxidation products. The organisms used were: two Pseudomonas strains, 53/1 and 53/2, kindly supplied by Dr Sistrom; the organism described by Walker & Wiltshire (1953) and here referred to as organism I; a Pseudomonas strain isolated from Rothamsted soil (organism II); a Nocardia strain R, isolated and described by Treccani (1953).

Organism II is a motile Gram-negative rod which produces a greenish fluorescence in nutrient agar. It forms smooth circular greyish colonies on peptone agar and an even turbidity in peptone water. Nitrite is produced from nitrate, and acid but no gas from sucrose, glucose or maltose. It does not

liquefy gelatine and it can attack phenol and naphthalene. This organism grows well at 25° and thus closely resembles *Pseudomonas desmolyticum* described by Gray & Thornton (1928).

METHODS

Media. The following basal inorganic media were used with the relevant organic substrate:

Medium A. Tausson's (1927) medium with or without the addition of agar, and compounded from solution *a* ($Ca(NO_3)_2$, 1 g.; KNO_3, 0·25 g.; $MgSO_4.7H_2O$, 0·25 g.; $FeCl_3$, 2·5 mg.; distilled water, 200 ml.) and solution *b* (KH_2PO_4, 0·08 g.; K_2HPO_4, 0·2 g.; distilled water, 200 ml.) sterilized separately and mixed aseptically in the proportion 4 parts *a* to 1 part *b*.

Medium B. KH_2PO_4, 1 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4$, 1 g.; $CaCO_3$, 0·1 g. in 1000 ml. tap water, pH 6·8–7·0.

Cultural conditions were as described by Walker & Wiltshire (1953) except that sometimes media containing also 1% of crystalline sodium succinate were used.

Manometric method. Organisms were washed in 0·02 M-phosphate buffer (pH 6·98) and resuspended in the same buffer. The concentration of the suspensions was standardized turbidimetrically by means of a photoelectric absorptiometer. The oxygen uptake by 1 ml. cell suspension (containing about 0·3 mg. total-N) was measured in Warburg manometers at 30°. The main cup of the Warburg flask contained the cells and 0·02 M-phosphate buffer (pH 6·98) to give a total volume of 3 ml., the substrates being placed in the side bulb. The centre cup contained 0·2 ml. 20% (w/v) aqueous potassium hydroxide.

Detection of intermediates. Reactions for the detection of naphthalene diol, naphthols and salicylic acid were as described previously (Walker & Wiltshire, 1953). Catechol was detected as described by Evans (1947).

RESULTS

Growth experiments

To test whether they could serve as carbon sources for growth, the following substances, in sterile solution to give the final concentrations shown, were added to 25 ml. volumes of medium B in 100 ml. Erlenmeyer flasks: sodium succinate (0·5%), sodium salicylate (0·25%), catechol (0·02%), α-naphthol (0·001%), β-naphthol (0·001%). Growth took place on naphthalene, salicylate or succinate but not on catechol or the naphthols. Although there was no growth on catechol, this compound was detected in all cultures growing on salicylate. Viable cells were still present in the naphthol-containing cultures at the end of the experiment (8 days). In medium A neither strain R nor organism II grew on the naphthols at 0·003% or 0·006%.

A further experiment was carried out using strain 53/1, naphthalene-grown cells of which had been found to oxidize both naphthols rapidly. Naphthalene-grown cells of strain 53/1 were inoculated into 50 ml. lots of medium A in 500 ml. Erlenmeyer flasks to which α-naphthol, β-naphthol or salicylic acid was added to give a final concentration of 0·02%, 0·01% or 0·0067%; control

flasks contained no added carbon compound. Only salicylic acid permitted growth though the cells remained viable up to at least 10 days in the naphthol cultures as was verified by making transfers into nutrient broth.

Detection of intermediate products. Various products were detected in liquid cultures containing naphthalene, grown in either Roux bottles without agitation or in flasks with stirring. We have already reported the isolation of naphthalene diol and salicylic acid from cultures of organism I in medium A (Walker & Wiltshire, 1953); catechol was also detected in the present work. Naphthalene diol, salicylic acid and catechol were all found in cultures of organism II and of strains 53/1 and 53/2 in media A and B. Strain R accumulated the same compounds in medium A, but in medium B no diol was found. In no case was a naphthol detected at any stage of growth.

Comparison of oxygen-uptake of organisms grown on succinate and naphthalene

All five strains were grown on plates of medium A solidified with agar and containing naphthalene, succinate or a mixture of naphthalene and succinate, in order to compare naphthalene-adapted and non-adapted cells. Organisms grown on succinate + naphthalene behaved like those grown on naphthalene. Strains 53/1 and 53/2 were indistinguishable in their behaviour. When grown on succinate none of the strains immediately oxidized naphthalene or any of the postulated intermediates, although in the case of organism II there was only a short lag before the cells began to oxidize naphthalene. When grown on naphthalene or a mixture of naphthalene and succinate, all strains oxidized immediately naphthalene, naphthalene diol, salicylic acid and catechol; in addition strains 53/1 and 53/2 oxidized α- and β-naphthol. Results typical for organisms I and II and strain R are shown in Fig. 1 A and for strains 53/1 and 53/2 in Fig. 1 B. The oxygen uptake by washed cells alone has been deducted, and the uptake with β-naphthol is omitted because it was much the same as for α-naphthol.

Oxidation of naphthols

When grown on naphthalene, organism II was very sensitive to small changes in α-naphthol concentration, with a maximum rate of oxidation at $1{\cdot}33 \times 10^{-3}$ M. The optimal concentration for organism I and strain R was lower (Fig. 2 A, B). Strain 53/1 oxidized naphthols more rapidly than any other strain, and it was not inhibited by any concentration of naphthol used. However, cells of this strain, when grown on succinate alone, also oxidized naphthols at a suitably low concentration, the respiration being slightly inhibited by higher concentrations (Fig. 3).

Inhibition of oxygen uptake by naphthols

Naphthols at $1{\cdot}66 \times 10^{-3}$ M inhibited the oxidation of naphthalene by adapted cells of strain 53/1, organism I or organism II (Fig. 4). β-Naphthol was less inhibitory than α-naphthol especially in the case of strain 53/1. Salicylic acid at the same concentration did not inhibit. The oxygen uptake by salicylate-grown cells with salicylate as substrate was also inhibited, especially by α-naphthol, but not at all by naphthalene.

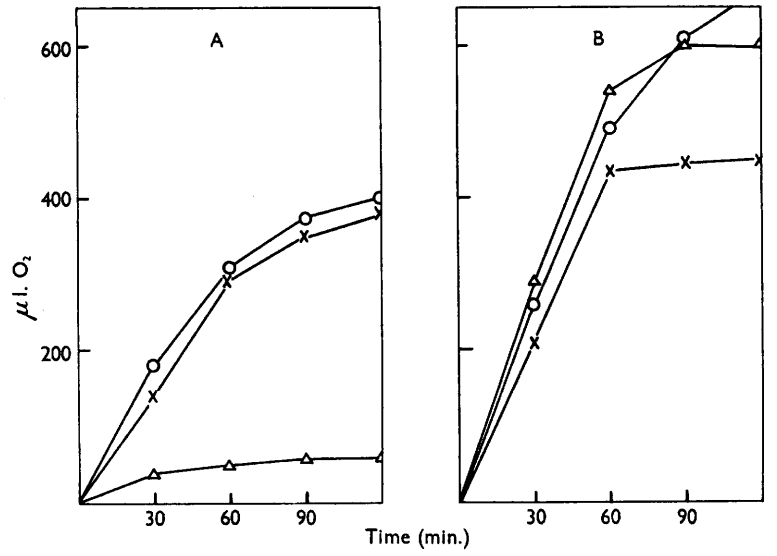

Fig. 1. Comparison of different naphthalene-oxidizing bacteria. Rate of oxygen uptake by washed cells in 0·02 M-phosphate buffer pH 7·0 in presence of: 5 μmole naphthalene (O——O), salicylic acid (×——×), α-naphthol (△——△); A, organisms I, II and R; B, strains 53/1 and 53/2.

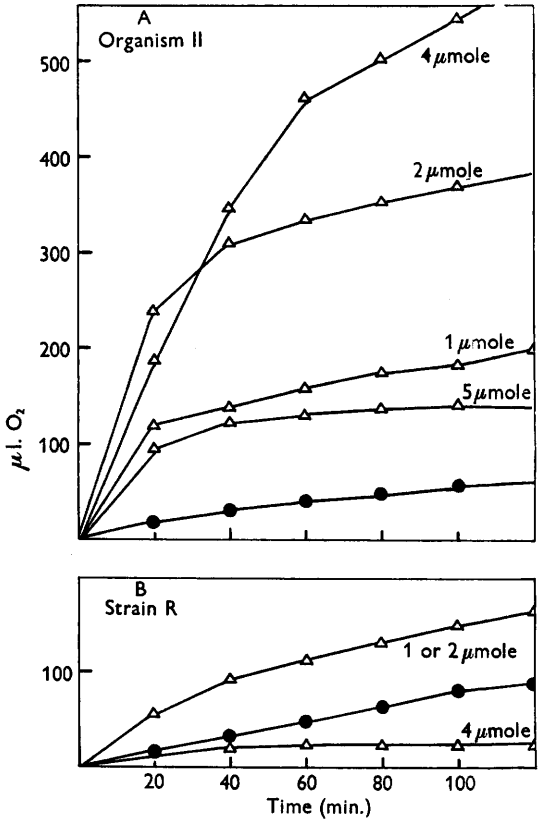

Fig. 2. Effect of naphthol concentration on the rate of oxygen uptake by naphthalene-adapted cells. Oxygen uptake by washed cells (●——●) and in presence of several concentrations of α-naphthol (△——△). A, organism II; B, strain R.

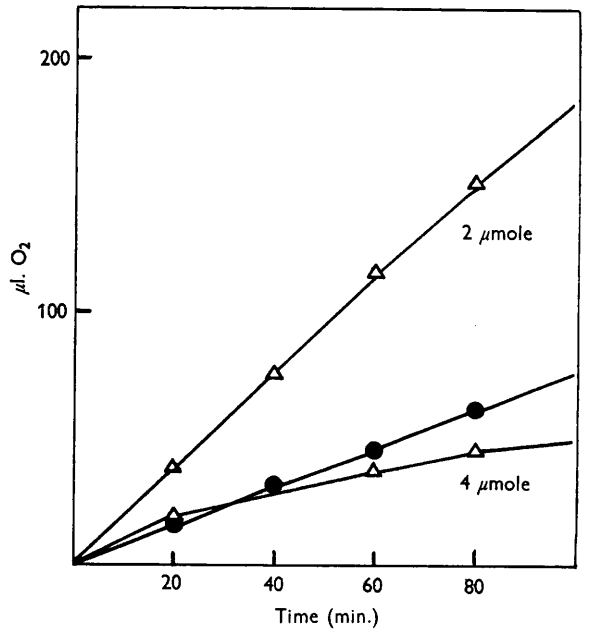

Fig. 3. Effect of naphthol concentration on the rate of oxygen uptake by succinate-grown cells of strain 53/1. Oxygen uptake by washed cells (●——●) and in presence of two concentrations of α-naphthol (△——△).

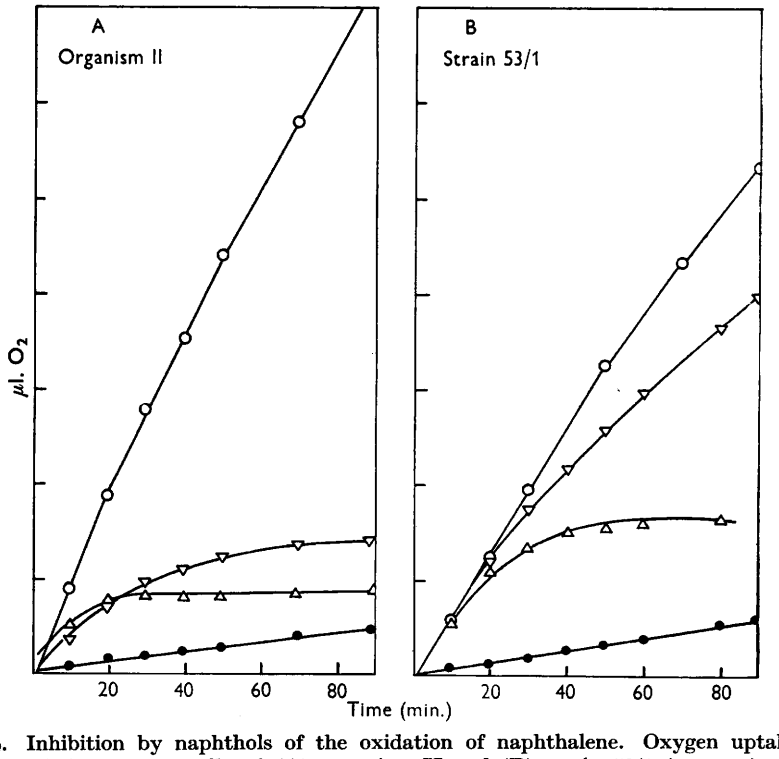

Fig. 4. Inhibition by naphthols of the oxidation of naphthalene. Oxygen uptake by naphthalene-grown cells of (A) organism II and (B) strain 53/1 (●——●) and in presence of 8 μmole naphthalene (○——○), 8 μmole naphthalene + 5 μmole α-naphthol (△——△), and 8 μmole naphthalene + 5 μmole β-naphthol (▽——▽).

Inhibition of oxygen uptake by β-naphthoquinone

The ability of organism II to oxidize low but not high concentrations of naphthols suggested that both naphthols might be converted to some product having a toxic effect on the cells. Non-enzymic oxidation might produce 1:2-naphthoquinone or the related 1:2-dihydroxynaphthalene. Neither of these compounds at 10^{-4} to 10^{-3} M was oxidized at all rapidly by organism I (Walker & Wiltshire, 1953). There was no oxygen uptake by organism II with naphthoquinone in this range of concentration (Fig. 5) but instead a marked

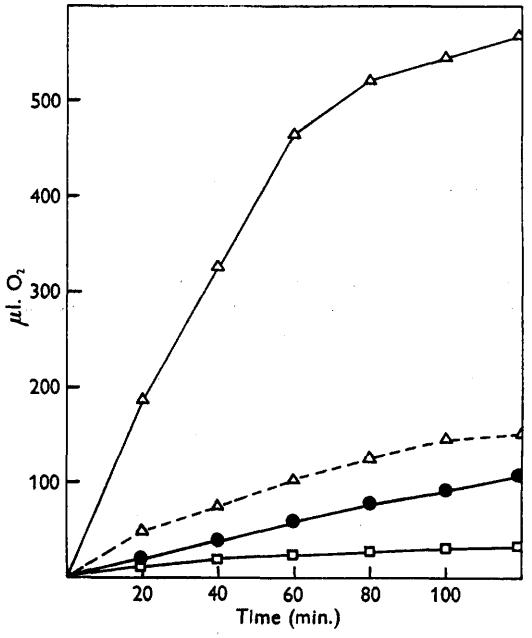

Fig. 5. Inhibition by β-naphthoquinone of the oxidation of α-naphthol by naphthalene-adapted cells of organism II. Oxygen uptake by washed cells (●——●), and in presence of 4 µmole α-naphthol (△——△), 1 µmole β-naphthoquinone (□——□) and a mixture of 4 µmole α-naphthol and 1 µmole β-naphthoquinone (△- - -△).

inhibition of the endogenous respiration. Moreover, 1:2-naphthoquinone decreased the oxygen uptake with $1{\cdot}33 \times 10^{-3}$ M-α-naphthol. Since it is known that primary aromatic amines can react with quinones to form insoluble indirect quinonoid addition compounds, an attempt was made to abolish the naphthoquinone inhibition by aniline; this attempt was unsuccessful however. Also, aniline failed to abolish the inhibition by α-naphthol of the oxidation of naphthalene.

DISCUSSION

To decide whether a given compound is an intermediate in the metabolism of a substrate by bacteria, it is desirable to use as many criteria as possible, for example: detection or isolation of the intermediate from growing cultures (the most convincing evidence); growth of the given organism on the supposed

intermediate; the intermediate compound should be oxidized as rapidly as the initial substrate by adapted cells.

In the case of naphthalene metabolism, naphthalene diol, salicylic acid and catechol were isolated or detected in the cultures of all the five organisms we used, and all these substances were oxidized immediately by naphthalene-grown cells, so that there seems little doubt that they are all true dissimilatory products of naphthalene. All five organisms grow on salicylic acid; the failure to grow on catechol may be attributed to the toxic effect of *o*-benzoquinone.

In the case of α- and β-naphthol, we have only the positive evidence of simultaneous adaptation to support the view that these compounds are intermediates. We have not been able to detect free naphthols in any cultures although the reaction used is sufficiently sensitive to detect 1 p.p.m. of α-naphthol. It is of course conceivable that naphthols do not accumulate even to this extent. Neither naphthol supported the growth of any of the organisms. When the concentration of naphthols was low, there was a moderate rate of oxidation of both naphthols by succinate-grown cells of strain 53/1, but the rate was increased by adaptation to grow on naphthalene. It is unlikely that both α-naphthol and β-naphthol are intermediates since enzymes generally exhibit a high degree of steric specificity; it would be strange to find no discrimination in this case. However, an alternative mechanism may be advanced. Boyland & Sims (1953) found that synthetic naphthalene diol yielded both α-naphthol and β-naphthol on treatment with dilute mineral acid. Since the diol is a rather reactive compound it is possible that some of it, once formed in the culture, might be non-enzymically converted to both naphthols, and the cells would then be incidentally exposed to the two naphthols. Against this hypothesis, it should be repeated that we have never detected naphthols in the cultures. Moreover, Boyland & Sims (1953) stated that the diol produced by animals yielded exclusively α-naphthol when boiled with 2N acid. It is strange that there should be this difference in behaviour between a natural product and the synthetic racemic form. More probably diol always yields both naphthols but in different proportions according to the conditions of reaction.

The main difference between the organisms examined can be attributed to varying susceptibilities to toxic effects of the naphthols; when allowance for this is made, the general behaviour of the organisms is similar. The results of manometric experiments with adapted cells suggest that the enzyme system produced in response to growth in the presence of naphthalene and responsible for oxidation of naphthols is an adaptive one. This remains the only evidence supporting the view that α- or β-naphthol is an intermediate in the bacterial metabolism of naphthalene.

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REFERENCES

BOYLAND, E. & SIMS, P. (1953). The action of some free radicals on naphthalene. *J. chem. Soc.* p. 2966.

BOYLAND, E. & WILTSHIRE, G. H. (1953*a*). Metabolism of naphthalene by liver slices. *Biochem. J.* **53**, 424.

BOYLAND, E. & WILTSHIRE, G. H. (1953*b*). Metabolism of polycyclic compounds 7. The metabolism of naphthalene, 1-naphthol and 1:2-dihydroxy-1:2-dihydronaphthalene by animals. *Biochem. J.* **53**, 636.

CORNER, E. D. S., BILLETT, F. S. & YOUNG, L. (1954). Biochemical studies of toxic agents 6. The conversion of naphthalene into 1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid in the rabbit. *Biochem. J.* **56**, 270.

EVANS, W. C. (1947). Oxidation of phenol and benzoic acid by some soil bacteria. *Biochem. J.* **41**, 373.

GRAY, P. H. H. & THORNTON, H. G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zbl. Bakt.* (2 Abt.), **73**, 74.

STANIER, R. Y. (1947). Simultaneous adaptation. A new technique for the study of metabolic pathways. *J. Bact.* **54**, 339.

TAUSSON, W. O. (1927). Naphthalin als Kohlenstoffquelle für Bakterien. *Planta*, **4**, 214.

TRECCANI, V. (1953). Produzione di acido salicilico da naftalina per azione di uno schizomicete isolata da terreni petroliferi. *Ann. Microbiol.* **5**, 232.

WALKER, N. & WILTSHIRE, G. H. (1953). The breakdown of naphthalene by a soil bacterium. *J. gen. Microbiol.* **8**, 273.

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