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The Metabolism of Thymol by a *Pseudomonas*

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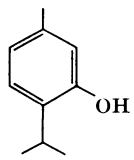
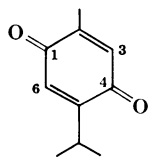
1. *Pseudomonas putida* when grown with thymol contained a *meta*-fission dioxygenase, which required ferrous ions and readily cleaved the benzene nucleus of catechols between adjacent carbon atoms bearing hydroxyl and isopropyl groups. 2. 3-Hydroxythymo-1,4-quinone was excreted towards the end of exponential growth and later was slowly metabolized. This compound was oxidized by partially purified extracts only when NADH was supplied; the substrate for the dioxygenase appeared to be 3-hydroxythymo-1,4-quinol, which was readily and non-enzymically oxidized to the quinone. 3. 2-Oxobutyrate (0·9 mole) was formed from 1 mole of 3-hydroxythymo-1,4-quinone with the consumption of 1 mole of oxygen; acetate, isobutyrate and 2-hydroxybutyrate (which arose from the enzymic reduction of 2-oxobutyrate) were also formed. 4. These products, which were produced only when the catechol substrate contained a third hydroxyl group, appeared to result from the enzymic hydrolysis of the ring-fission product.

Thymol (I) is found in certain essential oils from plants, accounting for about one-third of an oil from *Trachyspermum copticum* (Goryaev & Ignatova, 1959) and for about 7% of the phenolic constituents of oils from *Coleus aromaticus* (Dutt, 1959). Accordingly, the existence of microbes that are capable of degrading thymol is to be expected, despite the powerful bactericidal properties of this compound. We have studied a pathway for the bacterial metabolism of thymol, which involves the formation of a trihydric phenol, 3-hydroxythymo-1,4-quinol (V), before degradation can proceed to completion. This is unusual, since the introduction of only two hydroxyl groups into the benzene nucleus is generally sufficient to initiate the bacterial degradation of an aromatic compound (Dagley, Chapman, Gibson & Wood, 1964). Larway & Evans (1965) reported that the trihydric phenol, hydroxyquinol, is degraded by *ortho*-fission of the nucleus, whereas we find that 3-hydroxythymo-1,4-quinol is metabolized by *meta*-fission.

Derivatives of thymoquinone (II) are central to these studies and it is convenient to number the carbon atoms of the benzene nucleus according to the convention for quinones (Morton, 1965) when we refer to such compounds as (II), (III) or (IV),



(I)



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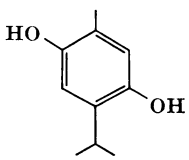

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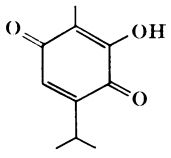

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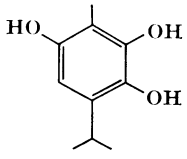

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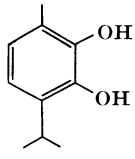


(VI)

which are derived directly from the thymoquinone structure. For all other derivatives of catechol the usual notation is used, as exemplified by 3-isopropyl-6-methylcatechol (VI). A brief account of some aspects of this work has already appeared (Chamberlain, Chapman & Dagley, 1967).

MATERIALS AND METHODS

Isolation and growth of organism. The organism was isolated from garden soil in Leeds by elective growth with thymol. It has been added to the National Collection of Industrial Bacteria (N.C.I.B. 10 014). It is a motile, Gram-negative, short rod with polar flagella, which produced a fluorescent green–yellow pigment on King B medium (King, Ward & Raney, 1954) but no phenazine pigment on King A medium. Pigment production on the former medium was not abundant, and none was produced on nutrient–agar: this led to an erroneous suggestion that the organism should be classified in the non-fluorescent group of pseudomonads (Chamberlain *et al.* 1967). Its position amongst the fluorescent organisms is in agreement with the observation that protocatechuate was metabolized by *meta* ring-fission after growth of the organism with *p*-hydroxybenzoate (Stanier, Palleroni & Doudoroff, 1966). The bacteria did not produce gelatinase and, by this criterion and others proposed by Stanier *et al.* (1966), we consider the organism to be a strain of *Pseudomonas putida*. Stock cultures were maintained on nutrient–agar slopes sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and subcultured monthly.

For experimental use cells were grown, either in shake cultures or with forced aeration, at 30° in a medium adjusted with NaOH to pH 7·2 and containing (per l.): KH_2PO_4, 2g.; $(NH_4)_2SO_4$, 1g.; Lab–Lemco (Oxoid Division of Oxo Ltd., London, E.C. 4), 0·3g.; $MgSO_4,7H_2O$, 0·4g.; thymol, 0·3g. Thymol is volatile and slightly soluble and it was added to the medium after it had been sterilized but before it had cooled below 60°; the $MgSO_4$ in solution in a small volume was sterilized separately and added to the bulk of the growth medium when cool. The cells were harvested after growth for 18hr. and washed once by resuspending them in phosphate buffer, followed by centrifugation at 4° for 15min. at 5000*g*. The phosphate buffer used throughout this work contained KH_2PO_4 (5g./l.) brought to pH 7·2 by the addition of NaOH. The methods used to prepare cell extracts and to measure gas exchanges at 30° were those described by Dagley & Gibson (1965).

Partial purification of cell extracts. The main object of this procedure was to eliminate the ability of extracts to catalyse the oxidation of NADH by air while retaining the enzymes concerned with the metabolism of thymol. In this *Pseudomonas*, as in others (Hayaishi, Taniuchi, Tashiro & Kuno, 1961), fine particles present in extracts appeared to carry the enzymes for NADH oxidation, since activity was decreased to about one-quarter when these particles were removed by centrifugation. The crude extract was centrifuged at 128000$g_{av.}$ for 2hr. at 0° and 0·2vol. of 2% (w/v) protamine sulphate was then added, with stirring, to the supernatant solution at 4°. After centrifugation at 4° to remove the precipitate, the solution was brought to 70% saturation with $(NH_4)_2SO_4$ by addition, with stirring, of a neutralized solution. The precipitate was collected on the centrifuge, dissolved in phosphate buffer (KH_2PO_4, 5g./l.), pH 7·2, and dialysed for 5hr. with stirring at 4° against three changes of the same buffer. If it was not used immediately the partially purified extract was frozen and stored.

Chromatography. Whenever a compound was identified by chromatography its R_F was compared with that of authentic material run on the same chromatogram. 2,4-Dinitrophenylhydrazones of oxo acids were examined by thin-layer chromatography on Kieselgel G as described by Bayly, Dagley & Gibson (1966), with their solvents *A* and *B*. R_F values for the 2,4-dinitrophenylhydrazone of 2-oxobutyrate were: in solvent *A*, 0·33; in solvent *B* (two spots), 0·71, 0·80. One-dimensional chromatography on Whatman no. 1 paper was used to identify 2-aminobutyrate with the following ascending systems: solvent *C*, butan-1-ol–acetic acid–water (12:3:5, by vol.); solvent *D*, butan-1-ol–pyridine–water (1:1:1, by vol.) (Morrison, 1953); solvent *E*, 2-methylpropan-1-ol–formic acid–water (19:2:6, by vol.) (Hassall & Greenberg, 1963); solvent *F*, propionic acid–1M-acetic acid (3:1, v/v). Solvents *C* and *D* were run to the end of the paper; in solvents *E* and *F*, R_F values for 2-aminobutyrate were respectively 0·22 and 0·44. Paper chromatography was also used to confirm the identity of 2,4-dinitrophenylhydrazones (El Hawary & Thompson, 1953). Chromatography on Celite columns was used to separate organic acids (Swim & Krampitz, 1954; Dagley & Gibson, 1965). The identities of these acids were confirmed by gas–liquid chromatography with a Pye series 104 chromatograph model 24. The acids, in ethereal solution, were injected into a column (150cm. × 0·4cm.) packed with 10% diethyleneglycol adipate plus phosphoric acid adsorbed on Celite (80–120 mesh) and maintained isothermally between 90° and 120°; N_2 was used as carrier gas at a flow rate of 45ml./min., and the retention times of acetic acid and isobutyric acid were respectively 12min. and 18min.

Reduction of 2,4-dinitrophenylhydrazones to amino acids. An amount up to 1mg. of the 2,4-dinitrophenylhydrazone of an oxo acid was dissolved in 0·2ml. of acetic acid in a conical centrifuge tube. A knife-point of PtO_2 was added and H_2 was bubbled gently through the solution until the yellow colour had disappeared (about 1hr.). The supernatant solution was used directly for chromatography or high-voltage paper electrophoresis.

Electrophoresis. The identities of various organic acids were confirmed by high-voltage paper electrophoresis as described by Dagley & Gibson (1965). Lactate dehydrogenase and 2-oxobutyrate dehydrogenase were separately demonstrated when cell extracts were examined by disc electrophoresis in polyacrylamide gels, with the apparatus of Davis (1964) and the buffers and gels of Williams & Reisfeld (1964). Electrophoresis was performed at room temperature (about 20°) with a constant current of 4mA/tube at 100–300v. Protein was located by placing the gels for 1hr. in 1% (w/v) Amido Black in 7% (v/v) acetic acid; excess of dye was removed by immersion for 48hr. in 7% (v/v) acetic acid with several changes of solvent. The materials used in disc electrophoresis were from British Drug Houses Ltd., Poole, Dorset. The dehydrogenases were located by using *N*-methylphenazonium sulphate to couple the oxidation of their hydroxy acid substrates (lactate or 2-hydroxybutyrate) to the reduction of nitro-blue tetrazolium. Gels were immersed in 5ml. of a solution of 0·1M hydroxy acid in 0·1M-tris (pH 9·2) containing 2·5mg. of NAD, 0·25mg. of nitro-blue tetrazolium and 0·12mg. of *N*-methylphenazonium sulphate. After 1hr. in the dark, bands

became visible, and excess of reagents was removed with 7% (v/v) acetic acid.

Determination of 2-oxobutyrate. In alkali, the 2,4-dinitrophenylhydrazone of 2-oxobutyrate has $\lambda_{max.}$ 436mμ. Extinctions were therefore measured at this wavelength when the method of Friedemann & Haugen (1943) was used to determine 2-oxobutyrate.

Materials. Thymol and thymoquinone were from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Thymoquinol was prepared by adding 4% (w/v) $Na_2S_2O_4$ to 1g. of thymol dissolved in 20ml. of ethanol until the solution became almost colourless. Thymoquinol was extracted with three 25ml. lots of ether, the solution was dried with anhydrous Na_2SO_4 and the solvent was removed by evaporation. The residual solid was crystallized twice from benzene; yield, 0·4g. of white crystals, m.p. 142° [Hodgman, Weast & Selby (1957) give 143°]. The following catechols were purified by sublimation before use: catechol, 3-methylcatechol and 4-methylcatechol (from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.); 3-isopropylcatechol, 4-isopropylcatechol and 3-isopropyl-6-methylcatechol (these compounds, and also 3-chloro-5-hydroxy-4-isopropyltoluene, were the kind gifts of Coalite and Chemical Products Ltd., Bolsover, Chesterfield, Derbys.). L-2-Aminobutyric acid, sodium 2-oxobutyrate and 2-hydroxybutyric acid were from Sigma (London) Chemical Co. Ltd., London, S.W. 6. We thank the following for gifts of compounds: Dr F. M. Dean for 3-hydroxythymo-1,4-quinone, prepared as described by Dean, Jones & Sidisunthorn (1962); Dr P. K. Bhattacharya for cumic acid and 2,3-dihydroxycumic acid isolated from culture fluids of a micro-organism.

Spectroscopy. Infrared-absorption spectra and nuclear-magnetic-resonance spectra were obtained as described by Dagley & Trudgill (1965).

RESULTS

Isolation and some physical properties of 3-hydroxythymo-1,4-quinone. Cultures became purple when thymol was utilized for growth: the colour reached maximum intensity towards the end of the exponential phase and then disappeared gradually to leave a colourless culture after about 24hr. The purple compound was extracted from 8l. quantities of culture fluid, centrifuged to remove bacteria, when the colour ($\lambda_{max.}$ 522mμ) was most intense. Each litre of the fluid, which became yellow after acidification with 3ml. of conc. hydrochloric acid, was extracted with 200ml. of ether, the ether layers were pooled and dried over anhydrous sodium sulphate, and the ether was removed by distillation. The solid yellow residue was dissolved in the minimum amount of ethanol, precipitated by adding ice-cold water, filtered, dried in a vacuum desiccator and crystallized from cyclohexane. The yield of yellow crystals, m.p. 163°, was about 0·3g. from 10l. of culture fluid (Found: C, 66·1; H, 6·5; $C_{10}H_{12}O_3$ requires C, 66·7; H, 6·7%). The compound reacted as a phenol (Folin & Ciocalteu, 1927) but not as an *o*-dihydroxyphenol (Evans, 1947). The infrared-absorption spectrum was identical with that of authentic 3-hydroxythymo-1,4-quinone (IV) and showed the presence of a hydroxyl group by absorption at 3300cm.$^{-1}$, and of a quinonoid structure by peaks at 1620, 1640 and 1665cm.$^{-1}$. The isolated and authentic compounds both gave absorption maximum in ethanol at 266mμ (ϵ 12100) and in ethanol plus sodium ethoxide at 272mμ (ϵ 7960). In the visible region of the spectrum, a solution in phosphate buffer (5g. of potassium dihydrogen phosphate/l., pH 7·5) gave a single peak having $\lambda_{max.}$ 522mμ (ϵ 1930), which proved useful in determining concentrations of 3-hydroxythymo-1,4-quinone. This peak was lost on acidification and a smaller one appeared at 405mμ. From spectroscopic determinations of the purple form present in solutions at pH 4·6–7·6 it was found that equal concentrations of the two species were in equilibrium at pH 5·3.

The nuclear-magnetic-resonance spectra of isolated and synthetic compounds were also identical and were readily interpreted in terms of the protons of structure (IV). When D_2O was added, one signal at 2·9τ ascribed to the hydroxyl proton was removed and replaced by another at 5·4τ due to HDO formed in the exchange reaction. A knowledge of the position of this hydroxyl group is important when one is deciding between possible alternative modes of enzymic fission of the benzene nucleus, and the nuclear-magnetic-resonance spectrum gave this information. Thus when the hydroxyl group is at C-3, as in (IV), C-6 bears a proton that can couple with the lone proton of the isopropyl group to give a doublet, which was, in fact, found at 3·5τ. The isomeric quinone with the hydroxyl group at C-6 would carry a proton at C-3 and this, when coupled with those of the adjacent methyl group, would give rise to a quadruplet; such a signal was not observed.

Reduction of 3-hydroxythymo-1,4-quinone. When a few crystals of sodium borohydride were added to an ethanolic solution of 3-hydroxythymo-1,4-quinone the peak at 266mμ was eliminated and another appeared briefly at 348mμ, to be replaced by a third at 283mμ. The transitory appearance of an unstable reaction intermediate when a quinol is formed from a hydroxyquinone was noted by Morton (1965). A solution of 3-hydroxythymo-1,4-hydroquinol (V) was prepared by adding 2ml. of water slowly with shaking to 20μmoles of 3-hydroxythymo-1,4-quinone in 2ml. of ethanol, followed by the slow addition of solid sodium dithionite until the solution was colourless. The u.v. spectrum at pH 3 showed $\lambda_{max.}$ 283mμ (ϵ 2700). The quinol was oxidized very rapidly to the quinone by air above pH 6·5 and attempts to isolate the compound from solution did not succeed.

When freshly prepared, extracts of *Pseudomonas* grown with thymol oxidized both synthetic and

biologically isolated 3-hydroxythymo-1,4-quinone, but when extracts were stored additions of NADH were required: partially purified extracts likewise oxidized the quinone only when NADH was present. It therefore appeared to be necessary for the quinone to be reduced to the quinol before it could be degraded, and that fresh extracts could oxidize without supplementation because the NADH required for the reductive step could be formed endogenously by these extracts.

Conversion of 3-hydroxythymo-1,4-quinone into 2-oxobutyrate. In a typical experiment, several Warburg respirometers each contained, at 30°: 8 μmoles of 3-hydroxythymo-1,4-quinone, 10 μmoles of NADH, 14mg. of extract protein and 1·2mM-ferrous sulphate in a total reaction volume of 3ml. of phosphate buffer, pH 7·2. In other experiments with fresh (untreated) extracts it was possible to omit both NADH and ferrous sulphate, but partial purification removed the loosely-bound ferrous ions required by the ring-fission oxygenase. When no more oxygen was consumed in the reaction and the purple colour of the substrate had disappeared, protein was removed and 2,4-dinitrophenylhydrazones were formed, extracted and chromatographed on Kieselgel G in solvents *A* and *B*. The only spots given were those that corresponded to the 2,4-dinitrophenylhydrazone of 2-oxobutyrate. Since schemes of metabolism alternative to the one we propose would predict the formation of pyruvate or 4-methyl-2-oxopentanoate, these oxo acids were also used as markers. The identity of 2-oxobutyrate was confirmed by using paper chromatography and also by reducing the 2,4-dinitrophenylhydrazone to 2-aminobutyrate. The amino acid was identified by paper chromatography with solvents *C*, *D*, *E* and *F* and the following markers: isoleucine, alanine and the reduced 2,4-dinitrophenylhydrazone of pyruvate; leucine and the reduced derivative of 4-methyl-2-oxopentanoate; glutamate and the reduced derivative of 2-oxoglutarate; 2-aminobutyrate and the reduced derivative of 2-oxobutyrate. The identity of 2-aminobutyrate was confirmed by high-voltage paper electrophoresis.

The stoicheiometry of the conversion of 3-hydroxythymo-1,4-quinone into 2-oxobutyrate was investigated as follows. Each of a series of Warburg flasks contained, in 3ml. of phosphate buffer, pH 7·2: 8 μmoles of 3-hydroxythymo-1,4-quinone, partially purified extract (16·3mg. of protein) and 1·2mM-ferrous sulphate. Additions were made of 2, 4, 6 and 8 μmoles of NADH respectively and the coenzyme was omitted from one flask to confirm its requirement in the reaction. Uptake of oxygen was measured; when uptake ceased, 2ml. of phosphate buffer was added to 0·5ml. of the reaction mixture to precipitate ferric phosphate, which was removed by centrifuging. The concentration of unaltered 3-hydroxythymo-1,4-quinone was then obtained by measuring its extinction at 522mμ, and the amount of substrate consumed in the reaction was calculated for each flask. The protein in each remaining reaction mixture was precipitated by conc. hydrochloric acid and removed, and amounts of 2-oxobutyrate formed were determined. For each 1·0 μmole of quinone utilized, 1·0 μmole of oxygen was consumed and, when the amount of substrate metabolized did not exceed 4 μmoles, about 0·9 μmole of 2-oxobutyrate was formed (Fig. 1). The amount of NADH required was somewhat greater than that needed to reduce the quinone (about 1·7 μmoles of NADH/1·0 μmole of substrate). However, some reduction of 2-oxobutyrate by NADH occurred, as later experiments showed.

Enzymic formation of acetic acid, isobutyric acid and 2-hydroxybutyric acid. The oxidation of 5 μmoles of 3-hydroxythymo-1,4-quinone and 7·5 μmoles of NADH was catalysed by partially purified extract (17mg. of protein) in each of 10 Warburg flasks, which contained 3ml. of a mixture of phosphate buffer, pH 7·4, and 1·2mM-ferrous sulphate. After incubation for 20min.,

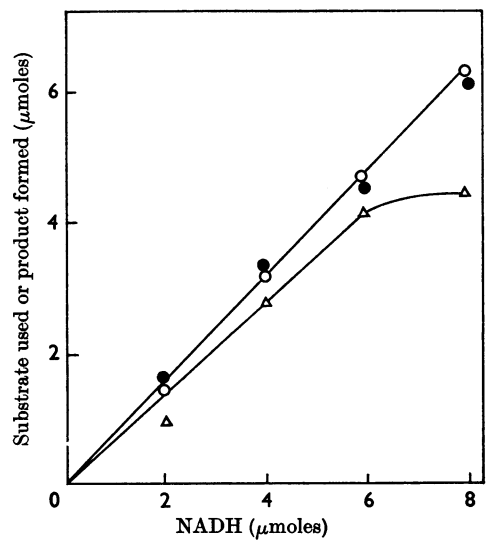


Fig. 1. Oxygen utilized and 2-oxobutyrate formed enzymically from 3-hydroxythymo-1,4-quinone in the presence of various amounts of NADH. Each of a series of Warburg vessels contained, in 3ml. of phosphate buffer (KH_2PO_4, 5g./l.), pH 7·2: 8 μmoles of 3-hydroxythymo-1,4-quinone, 16·3mg. of extract protein and 1·2mM-ferrous sulphate. NADH was added and mixtures were incubated at 30° until uptake of O_2 ceased, when the following were determined as described in the text: ○, 3-hydroxythymo-1,4-quinone utilized; ●, total uptake of O_2; △, 2-oxobutyrate formed.

when uptake of oxygen had ceased, the flask contents were pooled, protein was precipitated with 5N-sulphuric acid, and organic acids present in the reaction mixture were extracted continuously with ether for 24hr. and then removed by shaking with a solution of sodium hydroxide, as described by Dagley & Gibson (1965). The solution was concentrated, acidified and applied to a column of Celite; fractions, eluted with butan-1-ol–chloroform mixtures of increasing polarity, were then titrated. In addition to 2-oxobutyrate, determined separately in the relevant fractions (Friedemann & Haugen, 1943), three acids were present (Fig. 2*b*), which gave the same pattern on elution as authentic acetic acid, isobutyric acid and 2-hydroxybutyric acid (Fig. 2*a*). The experiment was repeated for a reaction in which 60μmoles of 3-hydroxythymo-1,4-quinone were incubated for longer (60min.) with an excess of NADH (120μmoles). In this experiment the yield of 2-oxobutyrate was less, but the amount of acid eluted as 2-hydroxybutyrate was increased (Fig. 2*c*). Later experiments showed that these extracts contained enzymes that catalysed an NADH-dependent reduction of 2-oxobutyrate to 2-hydroxybutyrate.

The identities of acetic acid and isobutyric acid were confirmed by oxidizing 24μmoles of 3-hydroxythymo-1,4-quinone plus 30μmoles of NADH in a series of Warburg vessels as before. After acidification, the reaction products were extracted as previously described, first into ether, then into aqueous sodium hydroxide and finally into a small volume of ether for examination by gas–liquid chromatography. The retention times for the products were those of acetic acid and isobutyric acid; 2-oxobutyrate and 2-hydroxybutyrate were retained by the column. The acids present in the relevant fractions from Celite chromatography also gave spots having the mobilities of acetic acid, isobutyric acid, 2-oxobutyric acid and 2-hydroxybutyric acid when examined by high-voltage paper electrophoresis.

The extracts used in this work had been fractionated to remove 'NADH oxidase' activity without impairing at the same time the multienzyme system that degraded the ring-fission substrate into the various products identified. Undoubtedly the extracts contained many additional enzymes, including dehydrogenases that reduced 2-oxobutyrate to 2-hydroxybutyrate: an enzyme-catalysed oxidation of NADH, dependent on additions of pyruvate or 2-oxobutyrate, was readily observed in the spectrophotometer. The extracts showed one major band when they were subjected to polyacrylamide-gel electrophoresis and then stained for lactate dehydrogenase with pyruvate, but a second band appeared when the same gel was stained in the presence of 2-oxobutyrate. Since one of the two bands revealed after exposure to 2-oxobutyrate coincided with lactate dehydrogenase it appears that this enzyme was partly responsible for the observed NADH-dependent reduction of 2-oxobutyrate to 2-hydroxybutyrate; however, extracts evidently contained, in addition, an enzyme that functioned more specifically to reduce 2-oxo-

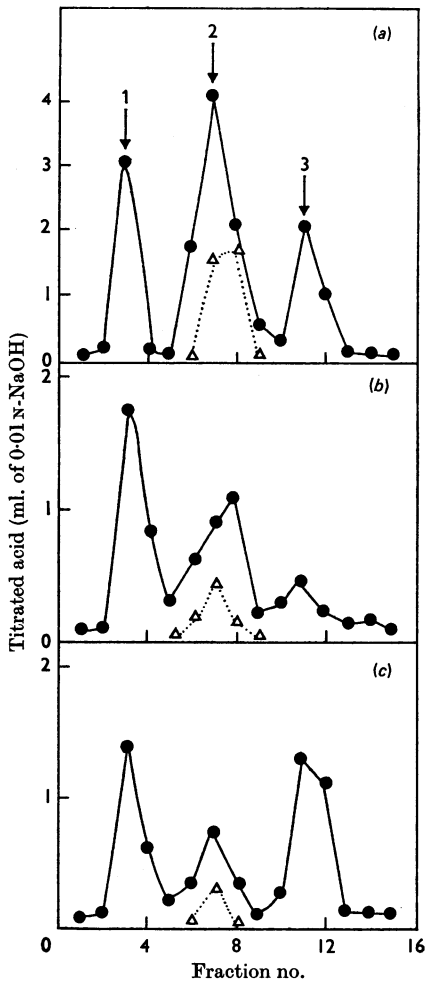


Fig. 2 Chromatography on Celite. Carboxylic acids were extracted from the reaction mixtures after incubation at 30° for 60 min. and were titrated in fractions (10 ml.) after elution. ●, Titration results. (*a*) The following authentic acids were applied to the column: 1, isobutyric acid; 2, acetic acid; 3, 2-hydroxybutyric acid. Reaction products were from: (*b*) 50μmoles of 3-hydroxythymo-1,4-quinone and 75μmoles of NADH; (*c*) 60μmoles of 3-hydroxythymo-1,4-quinone and 120μmoles of NADH. 2-Oxobutyrate was determined separately as described in the text in certain fractions (△).

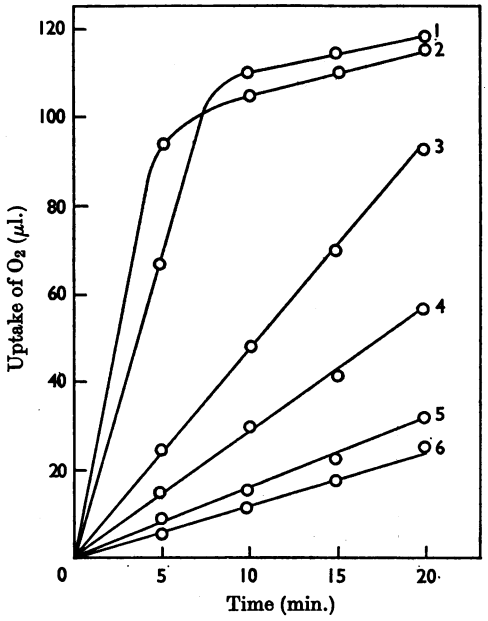


Fig. 3. Oxidation of catechols by crude cell extracts. Uptake of O_2 at 30° was measured in Warburg vessels containing 16.5 mg. of protein of crude cell extract and 5µmoles of each of the following: 1, 3-isopropylcatechol; 2, 3-isopropyl-6-methylcatechol; 3, 3-methylcatechol; 4, 4-methylcatechol; 5, catechol; 6, 4-isopropylcatechol. Uptake of O_2 in the absence of substrates (10µl. of O_2 in 20min.) has been subtracted.

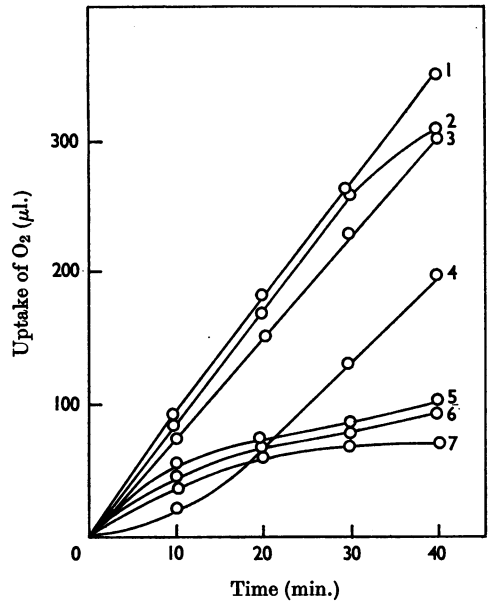


Fig. 4. Oxidation of catechols by intact cells grown with thymol. Warburg vessels at 30° contained, in 3ml. of phosphate buffer, 30mg. dry wt. of cells and 5µmoles of each of the following (except thymol, 2·5µmoles): 1, thymol; 2, 3-isopropyl-6-methylcatechol; 3, 3-isopropylcatechol; 4, catechol; 5, 3-methylcatechol; 6, 4-methylcatechol; 7, 4-isopropylcatechol. Uptake of O_2 in the absence of substrates (130µl. of O_2 in 40min.) has been subtracted.

butyrate. Recoveries of isobutyric acid and acetic acid from Celite chromatography were only about one-half and one-third respectively of the quantities calculated on the assumption that 1 mole of of 3-hydroxythymo-1,4-quinone gives rise to 1 mole of each of the acids. Some loss may have occurred during extraction, and it is also probable that the extracts contained enzymes that catalysed the further metabolism of these compounds. Warburg respirometers were set up for measurement of carbon dioxide evolved during the metabolism of 3-hydroxythymo-1,4-quinone, but no significant evolution of gas was detected. No volatile aldehydes were found by the methods that Dagley & Gibson (1965) and Bayly *et al.* (1966) employed for the isolation of acetaldehyde and propionaldehyde formed from catechols.

Oxidation of substituted catechols. Crude extracts of *Pseudomonas* grown with thymol rapidly oxidized 3-isopropylcatechol and 3-isopropyl-6-methylcatechol (VI) with a final uptake of 1 mole of oxygen/mole of substrate; 3-methylcatechol and 4-methylcatechol were oxidized respectively at about one-third and one-fifth of this rate, and catechol and 4-isopropylcatechol were oxidized very slowly (Fig. 3). The compounds formed by oxidation of 3-isopropylcatechol and 3-isopropyl-6-methylcatechol were respectively red–yellow and pale yellow at pH 7·2, and they were not metabolized further. Their spectra were those expected for *meta*-ring-fission products, with a peak in alkali near 400mµ abolished on acidification to give another peak at a lower wavelength (Dagley *et al.* 1964). Light-absorption maxima for the metabolite of 3-isopropylcatechol were: at pH 10, 394mµ; at pH 7, 325mµ; at pH 3, 317mµ; for the metabolite of 3-isopropyl-6-methylcatechol they were: at pH 10 and at pH 7, 398mµ; at pH 3, 235mµ. Thymol and thymoquinol were not attacked by these extracts.

Although their ring-fission compounds were not metabolized by extracts, 3-isopropylcatechol and 3-isopropyl-6-methylcatechol were oxidized by intact cells almost as rapidly as thymol, probably to completion and certainly well beyond the point of ring-opening. 3-Methylcatechol and 4-methylcatechol were incompletely oxidized, and the shape of the oxygen-uptake curve for catechol suggested

that the cells were induced to metabolize the substrate during the period of incubation (Fig. 4).

Metabolism of some phenolic compounds related to thymol. Cells grown with thymol oxidized thymo-1,4-quinol almost as rapidly as the growth substrate.

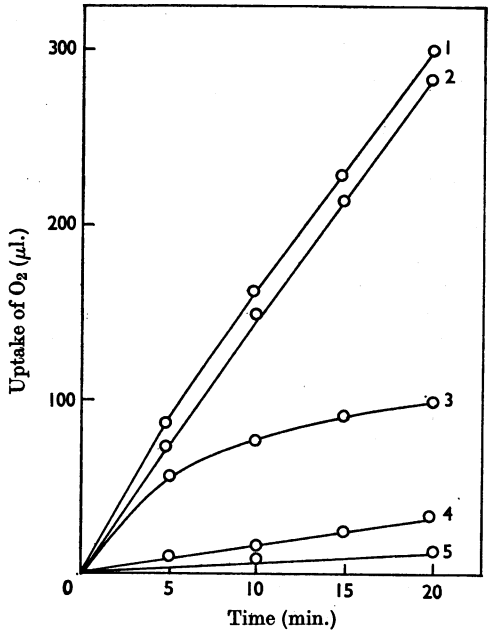
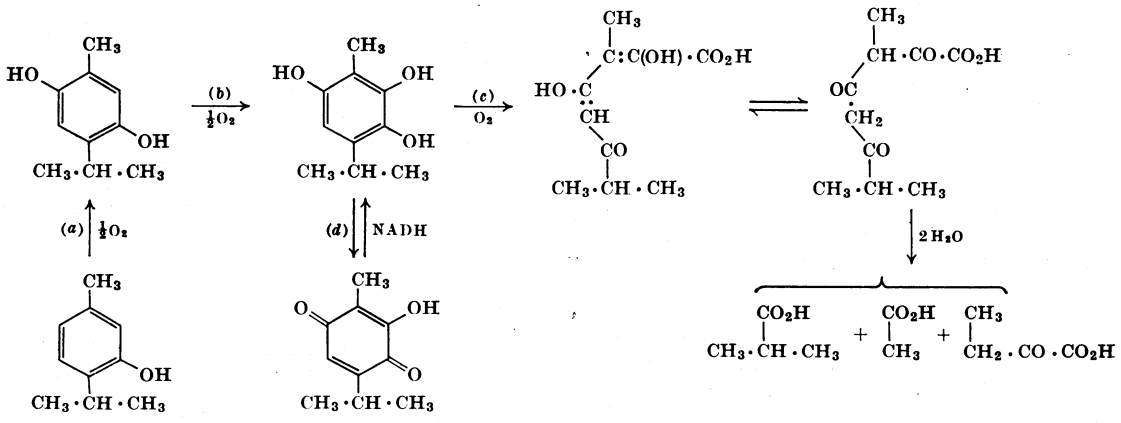


Fig. 5. Oxidation of some phenolic compounds by intact cells grown with thymol. Warburg vessels at 30° contained, in 3ml. of phosphate buffer, 30mg. dry wt. of cells and 2·5μmoles of each of the following: 1, thymol; 2, thymo-1,4-quinol; 3, carvacrol; 4, 3-chloro-5-hydroxy-4-isopropyl-toluene; 5, cumic acid. Uptake of O_2 in the absence of substrates (80μl. of O_2 in 20min.) has been subtracted.

Carvacrol, an isomer of thymol having the hydroxyl substituent adjacent to methyl, was oxidized to a limited extent: no colours were observed that would indicate the formation of quinones. Cumic acid (4-isopropylbenzoic acid) was not significantly attacked and neither was 2,3-dihydroxycumic acid. 3-Chloro-5-hydroxy-4-isopropyltoluene, in which thymol bears a chlorine substituent *meta* to the hydroxyl group, was attacked very slowly (Fig. 5).

DISCUSSION

In Scheme 1 it is proposed that thymol is hydroxylated twice (reactions *a* and *b*) to give 3-hydroxythymo-1,4-quinol, the benzene nucleus of which is then cleaved by a dioxygenase that catalyses reaction (*c*). The dienolic compound so formed would be expected to exist in equilibrium with a tautomer possessing three carbonyl groups each situated β to at least one of the other two. Such a molecule would be readily hydrolysed to yield acetic acid, isobutyric acid and 2-oxobutyric acid. We have shown that these three acids are in fact formed from 3-hydroxythymo-1,4-quinone, the yield of 2-oxobutyrate being stoicheiometric provided that high concentrations of NADH are not required in the reaction (Fig. 1). The present investigations were rendered possible by the fact that, towards the end of exponential growth, the *Pseudomonas* excreted 3-hydroxythymo-1,4-quinone in amounts sufficient to be isolated and characterized. However, this compound was not the substrate for the ring-fission oxygenase: it was attacked by partially purified extracts only in the presence of NADH required for its enzymic conversion into 3-hydroxythymo-1,4-quinol. We showed that at pH values greater than 6·5 the last-named compound was oxidized very rapidly and



Scheme 1. Proposed pathway of metabolism of thymol.

non-enzymically to the quinone; and this would therefore be excreted by the cells unless the quinol were removed by the dioxygenase (*c*) more rapidly than it was oxidized by the non-enzymic reaction (*d*). Several other observations support Scheme 1, where the benzene nucleus is shown as being cleaved by *meta*-fission. Thus although the actual substrate for the oxygenase, 3-hydroxythymo-1,4-quinol, was too unstable to be studied directly, related catechols were oxidized to compounds having the spectroscopic properties of *meta*-ring-fission products. In principle the nucleus could be opened between adjacent carbon atoms bearing the methyl group and the hydroxyl group respectively, rather than at a point adjacent to the carbon atom bearing the isopropyl group as Scheme 1 proposes; although it may be noted that the most feasible oxo acid to be predicted for this alternative metabolic route would be 4-methyl-2-oxopentanoate, rather than 2-oxobutyrate which was found. However, 3-isopropylcatechol and 3-isopropyl-6-methylcatechol were oxidized by cell extracts much more rapidly than were 3-methylcatechol, 4-methylcatechol or unsubstituted catechol. Although the specificity of the oxygenase cannot be established without further purification, it does appear that the enzyme attacks the nucleus most readily at a point adjacent to the carbon atom bearing the isopropyl group. However, substituents *para* to this carbon atom affect enzyme activity, for hydrogen in this position may be replaced by a methyl group but not by a carboxyl group: 2,3-dihydroxycumic acid was not a substrate for the oxygenase.

All the metabolic pathways initiated by *meta* ring-fission of a catechol that have been reported hitherto are those that give rise to pyruvate as one product. Most of these sequences also furnish an aldehyde, formed by the fission of a hydroxyoxo acid by an aldolase (for reviews see Dagley, 1967; Ribbons, 1965). Neither pyruvate nor aldehydes were detected in the present work and there is no evidence that a hydroxyoxo acid was formed by addition of water to an ethylenic bond: instead, the two molecules of water are shown as participating in hydrolytic fissions after the two hydroxylations that prepare the thymol molecule for metabolism. We have not studied these hydroxylations in isolation from the other reactions of the sequence, but certain conclusions may be drawn. It appears likely, in intact cells, that the carbon atom *para* to the hydroxyl group of thymol is hydroxylated very rapidly. The evidence for this is as follows. Cells oxidized 3-isopropyl-6-methylcatechol at about the same rate as thymol (Fig. 4), whereas when the former compound was exposed to cell-free extracts it was oxidized to a ring-fission product that was not further metabolized. Accordingly we conclude that intact cells, as well as their extracts, would also convert 3-isopropyl-6-methylcatechol into this same non-metabolizable compound, were it not for the fact that they are able to hydroxylate the catechol much faster than they can open its nucleus. However, there is no evidence that 3-isopropyl-6-methylcatechol is in fact formed when thymol is oxidized. An alternative route (Scheme 1) shows metabolism as being initiated by a hydroxylation in the position *para* to the hydroxyl group of thymol. Thymoquinol, the product of such a reaction, is oxidized at about the same rate as thymol by intact cells (Fig. 5). The prior conversion of thymol into thymoquinol would ensure that the additional hydroxyl group, needed for complete metabolism of the ring-fission product, would be introduced before a catechol was formed and hence before the nucleus was opened. Hydroxylation does not appear to occur at the vacant position *meta* to the hydroxyl group of thymol since this would lead to formation of the isomer of 3-hydroxythymo-1,4-quinone. Carvacrol, which bears a hydroxyl group in this position, was incompletely metabolized by cells grown with thymol. None of our cell-free preparations attacked thymol or thymoquinol. even when NADH was added, and it is probable that the hydroxylation enzymes are not readily extracted from disrupted cells.

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REFERENCES

Bayly, R. C., Dagley, S. & Gibson, D. T. (1966). *Biochem. J.* **101**, 293.

Chamberlain, E. M., Chapman, P. J. & Dagley, S. (1967). *Biochem. J.* **103**, 18P.

Dagley, S. (1967). In *Soil Biochemistry*, p. 287. Ed. by McLaren, A. D. & Peterson, G. H. New York: Marcel Dekker Inc.

Dagley, S., Chapman, P. J., Gibson, D. T. & Wood, J. M. (1964). *Nature, Lond.*, **202**, 755.

Dagley, S. & Gibson, D. T. (1965). *Biochem. J.* **95**, 466.

Dagley, S. & Trudgill, P. W. (1965). *Biochem. J.* **95**, 48.

Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.

Dean, F. M., Jones, P. G. & Sidisunthorn, P. (1962). *J. chem. Soc.* p. 5186.

Dutt, S. (1959). *Indian Oil & Soap J.* **25**, 120.

El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.

Evans, W. C. (1947). *Biochem. J.* **41**, 373.

Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.

Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.

Goryaev, M. I. & Ignatova, L. A. (1959). *Izvest. Akad. Nauk Kazakh. S. S. R., Ser. Khim.* **1**, 90.

Hassall, H. & Greenberg, D. M. (1963). *J. biol. Chem.* **238**, 1423.
Hayaishi, O., Taniuchi, H., Tashiro, M. & Kuno, S. (1961). *J. biol. Chem.* **236**, 2492.
Hodgman, C. D., Weast, R. C. & Selby, S. M. (1957). *Handbook of Chemistry and Physics*, 39th ed., p. 1188. Cleveland: Chemical Rubber Publishing Co.
King, E. O., Ward, M. K. & Raney, D. E. (1954). *J. Lab. clin. Med.* **44**, 301.
Larway, P. & Evans, W. C. (1965). *Biochem. J.* **95**, 52P.
Morrison, R. I. (1953). *Biochem. J.* **53**, 474.
Morton, R. A. (1965). In *Biochemistry of Quinones*, p. 43. Ed. by Morton, R. A. London: Academic Press (Inc.) Ltd.
Ribbons, D. W. (1965). *Rep. Progr. Chem.* **62**, 445.
Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966). *J. gen. Microbiol.* **43**, 159.
Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.
Williams, D. E. & Reisfeld, R. A. (1964). *Ann. N.Y. Acad. Sci.* **121**, 373.