2 hr. after injection, *c.* 5 % of the injected S^{35} being in the circulation; during the next hour it fell to about half this figure and then steadily declined over the next 5 hr.

In both this and the previous experiment, it must be remembered that the BAL content has been calculated from the amount of S^{35} found. The figures, therefore, necessarily give an upper limit to the BAL concentration, since there is the possibility of exchange and irreversible oxidation to non-thiol compounds.

DISCUSSION

There is general agreement in the distribution of S^{35} between the amounts obtained by Simpson & Young (1945) 6 hr. after injection of radioactive BAL and those found by us. With the exception of the small intestine where the percentages of S^{35} were approximately the same (3·7 and 3·61) the concentrations in the other tissues after 6 hr. were, as would be expected, consistently lower. In our experiments there is also fair agreement between the concentrations found in the blood of the rat and rabbit 1 hr. after injection; thus the rat which received twice the dose given to the rabbit had nearly twice the concentration of BAL in the blood.

From the therapeutic point of view it is desirable to determine the rate of absorption of BAL, the level of BAL in the blood stream and for how long an adequate concentration is maintained. The figures given in Table 2 show that 80 % of the injected BAL had already entered the circulation within 1 hr. of injection. At this time the maximal concentration had been reached and was maintained for not less than 2 hr. (Table 3); assuming, therefore, that all the S^{35} is due to BAL itself, the data indicate that these doses repeated at intervals of 3 hr. are sufficient to ensure maximum therapeutic effect in rats. This frequency of dosage is consistent with the American recommendations embodying initial dosing of BAL at intervals of 4 hr. for patients suffering from arsenical dermatitis, a treatment which was based upon studies in rabbits (Eagle, 1943) and injection experiments in human volunteers (Sulzberger, Baer, Kanoff & Eagle, 1944).

A further point of therapeutic importance is the confirmation that a single dose of BAL is largely excreted within 6–24 hr. after injection; this means that any possible toxic effects of a single dose should be temporary.

SUMMARY

1. A method is given for the preparation of radioactive 2:3-dimercaptopropanol (BAL).

2. It has been shown that the S^{35} of radioactive BAL is largely excreted in the neutral sulphur fraction of post-injection urine.

3. The distribution and persistence in the circulation of S^{35} following injection of radioactive BAL has been determined.

The radioactive sulphur used in this work was obtained in the form of $BaSO_4$ through the good offices of the Chemical Defence Research Department of the Ministry of Supply from Dr F. C. Henriques of Harvard University, U.S.A., to whom our thanks are due.

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Oxidation of Phenol and Benzoic Acid by Some Soil Bacteria

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The utilization of aromatic compounds by certain bacteria is now well known, and numerous workers have described the isolation of micro-organisms from various sources, capable of metabolizing phenolic substances. Thus, Sen Gupta (1921) has studied quantitatively the disappearance of phenols and cresols from soils of diverse origin, and showed clearly the biological nature of the process. Störmer (1908) had tested the effect on soil of a number of organic compounds, including toluene, xylene, phenol and *p*-cresol, and claimed to have isolated organisms that could destroy these compounds, but

details of this part of the work are not given. Fowler, Ardern & Lockett (1911) suspected the occurrence of phenol-utilizing bacteria on sewage filter beds, because phenol produced in the course of the decomposition of sewage proteins did not accumulate. They isolated from sewage effluent an organism resembling *B. helvolus* (Zimmermann) (*Flavobacterium helvolum*) and found that it grew in a mineral salt medium to which phenol (0·01 g./100 ml. of medium) was added, and destroyed the phenol.

In recent years, the wide use of aromatic compounds as soil insecticides, has stimulated considerable interest in the mechanism of their disappearance. Thus, the Rothamsted workers, Gray & Thornton (1928) and Tattersfield (1928), isolated many types of soil bacteria capable of destroying the following aromatic compounds: phenol, *o*-, *m*- and *p*-cresol, naphthalene, phloroglucinol, resorcinol and toluene, which are commonly used as soil sterilizing agents. Pure cultures of these organisms were capable of utilizing these compounds as sole source of organic carbon, when incorporated into a mineral salt medium, containing an inorganic nitrogen supply. In a study of the distribution of these micro-organisms, the above authors showed that they were most often found in arable soil, but rarely occurred in unmanured or 'wild' soil. From a study of their morphology and growth characteristics, the strains were classified into the following six families: *Coccacae*, *Mycobacteriaciae*, *Bacteriaceae*, *Pseudomonadaceae*, *Spirillaceae* and *Bacillaceae*, respectively.

In a study of the bacterial purification of gasworks' liquor, Happold & Key (1932) isolated from sewage a Gram-negative vibrio (vibrio 01) which was capable of attacking monohydroxyphenols. The present author has isolated phenol-utilizing organisms from the faeces of a large variety of animals, viz. man, horse, cow, sheep, and pig, and they appear to be normal inhabitants of the intestine.

Little is known of the mechanism and of the intermediate products through which these micro-organisms metabolize benzene derivatives. Happold (1930) has studied the capacity of bacterial suspensions to catalyze the oxidation of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine. He found that the oxidation of catechol with the formation of *o*-benzoquinone, ran parallel with the ability of the organism to give the dimethyl-*p*-phenylenediamine oxidase reaction, as applied by Gordon & McLeod (1928). *Vibrio tyrosinatica* (Beijerinck) was the only organism which possessed tyrosinase, and which consequently catalyzed the oxidation of monohydroxyphenols, with subsequent formation of more complex components. None of the phenol-utilizing organisms isolated by Gray & Thornton (1928), Happold & Key (1932), or the present author, contain a demonstrable tyrosinase system, if the criterion employed be the capacity to oxidize tyrosine, giving the well-known colour sequence, leading to melanin formation, characteristic of the tyrosine-tyrosinase reaction. Tyrosine is however utilized by most of these organisms, but the products formed are quite colourless, and eventually cleavage of the benzene ring occurs.

It would appear, therefore, that the destruction of monohydroxyphenols by these organisms is quite different from the well recognized type of biological oxidation produced by aerobic oxidases, elucidated by Raper and his collaborators (see Raper, 1928).

The present communication is concerned with the further study of the metabolic activities of these micro-organisms, using phenol and benzoic acid as substrates, in an attempt to reveal the mechanism of the oxidative destruction of the benzene ring.

EXPERIMENTAL

Organism. During the initial stages of this work the organism used was the vibrio 01 isolated by Happold & Key (1932); this organism is very similar in morphology and growth characteristics to *Vibrio cuneata* isolated by Gray & Thornton (1928). When the initial stages in the oxidation of the benzene ring became clear, it was deemed desirable to determine how general the metabolic pathway was, by employing the other phenol decomposing bacteria. The following organisms described by Gray & Thornton (1928), and obtained from the National Collection of Type Cultures were used for this purpose: *Mycobacterium crystallophagum* (2566), *Micrococcus sphaeroides* (2559), *Vibrio cuneata* (2583), *Pseudomonas rathonis* (2577) *and Bacillus closteroides* (1584).

Media. The following mineral salt medium has been used throughout: $(NH_4)_2SO_4$ 1·0 g.; $MgCO_3$ 0·5 g.; K_2HPO_4 0·5 g.; $CaCl_2$ 0·1 g.; NaCl 0·1 g.; $FeCl_3$ 0·01 g. dissolved in distilled water (1 l.). The pH was adjusted to 7·2, and the substrate concentration was 50–100 mg./100 ml. medium.

When phenol was used as the metabolite, the concentration used was 50 mg./100 ml. medium; higher concentrations prolonged the initial lag phase in growth, over 100 mg./100 ml. medium being definitely toxic to the organism. Much higher concentrations of benzoic acid, however, were tolerated by the organism, but the usual concentration used was 100 mg. sodium benzoate/100 ml. medium.

Isolation of phenol-utilizing organisms. Soil or faeces (0·5 g.) was inoculated into 100 ml. of sterile medium, contained in tubes that could be aerated with a current of sterile air. Almost invariably growth occurred within a few days, and transplants were made into fresh aeration tubes containing the same medium. After obtaining cultures by this elective method, sub-cultures were made on to nutrient agar plates containing 0·05 g. phenol/100 ml. of nutrient agar. Representative colonies were inoculated into phenol liquid media, and only those organisms that could grow under these conditions, with phenol as the sole organic carbon source, were used for further experiments. The cultures were usually incubated at 35°, but for large scale work at room temperature.

Manometric methods. The oxygen consumption of cell suspensions was measured in the usual way in Warburg manometers at 37° in the presence of 0·1 M-phosphate buffer at pH 6·8. Conical cups (about 20 ml. capacity) with a central well were used; substrates were added from the side arm; O_2 uptake, and CO_2 output were measured. The approximate volume of well-centrifuged bacterial cells used in the respiration experiments was 0·2 ml./manometer cup.

Estimations. Phenol was estimated by the bromination method of Redman, Weith & Brock (1913), and also by the colorimetric method of Folin & Ciocalteau (1927). Catechol substances were estimated by the molybdate colorimetric method of Rae (1930).

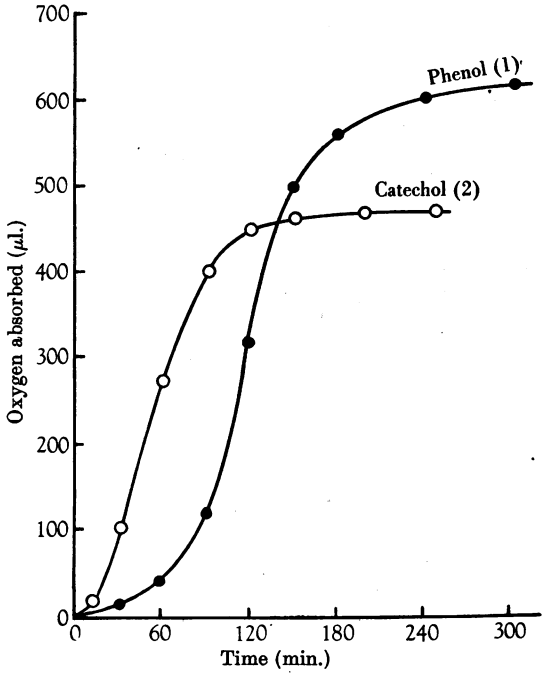

Fig. 1. Oxygen uptake curves with phenol (1) and catechol (2) as substrates using a suspension of the vibrio 01.

Contents of manometer cups, 1 ml. washed suspension (0·2 ml. packed cells) in 0·1 M-phosphate buffer, pH 6·8, and 1 ml. (0·5 mg.) of substrate solution in 0·1 M-phosphate buffer, pH 6·8. Gas phase, air; temperature of bath, 37°.

Separation of mono- and dihydroxyphenolic compounds. A method found extremely useful for the separation of mono- and dihydroxyphenol derivatives, and which has been widely employed during the present investigation, is based on the following observations. Monohydroxy derivatives of benzene, e.g. phenol, *p*-cresol, *p*-hydroxybenzoic acid, and tyrosine, do not give an insoluble lead compound with lead acetate in neutral solution. Dihydroxy derivatives, e.g. catechol, homocatechol, 3:4-dihydroxybenzoic acid, dopa, and adrenaline, in neutral or slightly acid solution with neutral lead acetate give an insoluble lead compound immediately. This behaviour can be made the basis of the quantitative separation of these two types of substances.

Respirometric experiments. The vibrio 01 was grown in the phenol mineral salts medium. In 4 days, under continuous aeration of the culture, at room temperature, heavy growth was obtained. The bacterial cells were centrifuged aseptically, and washed twice by resuspension in distilled water. They were finally suspended in 0·1 M-phosphate buffer, pH 6·8, in a dilution of 2·0 ml. packed cells/10 ml. One ml. of this suspension was used/manometer cup, whilst the side arm contained 1 ml. substrate solution (0·5 mg.) in the same phosphate buffer, making a final volume of 2·0 ml./respirometer cup. Oxygen uptake and CO_2 output were measured at 37°.

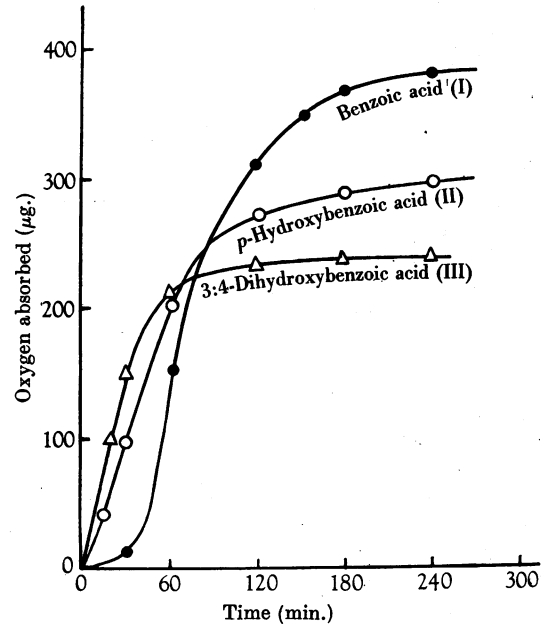

Fig. 2. Oxygen uptake with benzoic acid (I), *p*-hydroxybenzoic acid (II), and 3:4-dihydroxybenzoic acid (III) as substrates, using a suspension of the vibrio 01.

Contents of manometer cups, 1 ml. cell suspension in 0·1 M-phosphate buffer, pH 6·8, and 1 ml. (0·5 mg.) of substrate solution in 0·1 M-phosphate buffer, pH 6·8. Air; 37°.

RESULTS

The curves of O_2 uptake during the course of the oxidation of phenol and catechol can be seen in Fig. 1; for benzoic *p*-hydroxybenzoic and 3:4-dihydroxybenzoic acids in Fig. 2. The resting respiration of the bacterial cells alone have been subtracted, but in no case was this appreciable.

A definite latent period is evident with benzoate and the monohydroxy compounds, but this is appreciably reduced in the case of the dihydroxy compounds. After about 2–3 hr. the initial fairly rapid oxidation rate diminishes considerably, and then subsequently a small O_2 uptake continues for a much longer period, at a rate of approximately

10 μl./hr. This probably indicates that the intermediates accumulating at the end of the rapid oxidation phase, are slowly oxidized further.

The gaseous exchange has been computed with reference to the end of the rapid oxidation stage, and is summarized in Table 1.

Table 1. *Substrates oxidized by suspension of cells of vibrio* 01, O_2 *consumption and* CO_2 *output*

Substrate (0·5 mg.)	O_2 uptake (μl.)	Atoms O/ molecule substrate	CO_2 output (μl.)	Molecule CO_2/ molecule substrate
Phenol	610	10	470	4
Catechol	460	9	400	4
Benzoic acid	370	8	365	4
p-Hydroxybenzoic acid	290	7	325	4
3:4-Dihydroxybenzoic acid	230	6	290	4

The fact that catechol takes up one oxygen atom/molecule of substrate less than phenol is strong evidence that it is the first oxidation product of the latter. Similarly it may be tentatively deduced that the oxidation of benzoic acid proceeds via the *m*- or *p*-hydroxy compound to protocatechuic acid, since salicylic acid is not oxidized by the bacterial suspension. These views were confirmed later by the actual isolation of these intermediate compounds in large-scale culture experiments.

The utilization of phenol

On seeding the medium containing phenol (50 mg./100 ml.) with the vibrio 01 and aerating with sterile air at room temperature, growth is usually visible after 2 days, becoming profuse on the third day. At neutral pH hardly any pigmentation is visible through the dense, opaque bacterial culture. Previous experience of the course of the biological oxidation of phenol (e.g. by the monophenolase, tyrosinase) coupled with results obtained by feeding experiments with animals, made it appear probable that the introduction of more hydroxy groups might be a preliminary step to the disruption of the aromatic ring. A young culture of the phenol organism, as above, when spun to remove bacterial cells, gave a distinct qualitative test for 'catechol' substances, when tested by the delicate tungstate or molybdate colour reaction.* The rate of disappearance of phenol in a culture, and the formation of 'catechol' substance, is given quantitatively in Fig. 3. As will be seen, after the beginning of growth, phenol disappears from the medium fairly rapidly, the intermediate giving the 'catechol' reaction having a transient existence. Phenol has completely disappeared from the culture at room temperature, within 7 days.

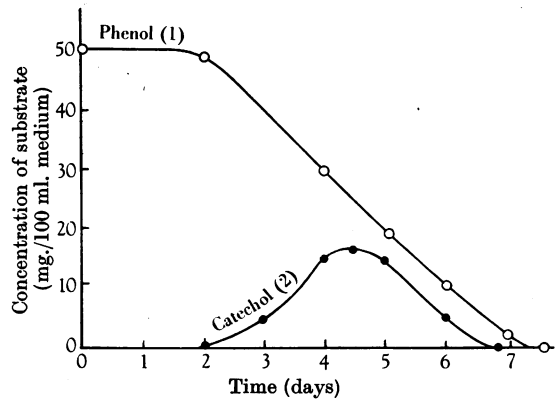

Fig. 3. The rate of disappearance of phenol (1) and the formation of catechol (2) in a phenol culture medium of the vibrio 01.

Initial concentration of phenol 0·5 mg./ml. of medium; medium inoculated by loop, aerated with sterile air, at room temperature (approximately 18°).

Isolation of the 'catechol' intermediate

A phenol culture (10 l., 5 g. phenol), was carefully observed until the 'catechol' reaction was intense. The whole culture fluid was then acidified to pH 3 with H_2SO_4, and filtered through a thin layer of kieselguhr, giving a clear filtrate. This was extracted with ether (3 times) until the mother liquid failed to give the colour reaction. The ether extract was dried with Na_2SO_4 and evaporated to dryness giving an oily residue, which gave an intense 'catechol' reaction. It was taken up in water (50 ml.), filtered, and excess lead acetate solution (20 %) added. A white precipitate immediately separated, which was centrifuged, and washed with distilled water. The precipitate was resuspended in distilled water (20 ml.), and decomposed by H_2S, the PbS filtered off, and the aqueous filtrate re-extracted with ether (3 times). Drying the combined ether extracts with Na_2SO_4 and evaporation of the ether, left a small residue (300 mg.) which crystallized on standing in the cold. It was recrystallized from light petroleum, and then showed m.p. 104°. It afforded a dibenzoyl derivative, m.p. 84° alone and on admixture with authentic dibenzoylcatechol.

The production of o-benzoquinone

A phenol culture (5 l., 2·5 g. phenol) was aerated until growth was visible, and phenol had started disappearing. Freshly distilled aniline (1·0 g.) was

* *Colour test for catechol substances.* The test solution (1 ml.) is made neutral, 1 ml. of a 10 % sodium tungstate or sodium molybdate solution is then added, followed respectively with 0·5 ml. HCl (0·5 N), and 1·0 ml. of a solution of sodium nitrite (0·5 %). A yellow colour immediately develops if 'catechol' substances are present. On addition of 1·0 ml. of NaOH (0·5 N), the yellow colour becomes cherry red.

then added to the culture, and slow aeration continued. Within a few hours, the culture developed a reddish haze, and after a further 2 days, a fine red precipitate was deposited on the sides of the culture vessel. The culture was made distinctly acid to Congo red with HCl, the reddish precipitate filtered off and washed with distilled water. The precipitate was purified by solution in ether, which was filtered and evaporated to dryness. The residue was recrystallized from light petroleum affording bright red needles, m.p. 193°, of dianilino-*o*-benzoquinone and on admixture with an authentic specimen the m.p. was not depressed. (Found: N, 9·63; calc. for $C_{18}H_{14}N_2O_2$: N, 9·66%.)

Utilization of benzoic acid: the isolation of 3:4-dihydroxybenzoic acid

The vibrio 01, in common with most of the other types of organisms mentioned previously, also grew well in the inorganic salt medium to which benzoic acid had been added as the sole source of organic carbon. This substrate was considerably less toxic than phenol to the bacteria, and was normally used at a concentration of 0·1 g. sodium benzoate/100 ml. medium.

Benzoate culture medium (5 l., 5 g. sodium benzoate) was inoculated, and aerated with sterile air at room temperature in the usual manner. Growth was noticeable after 1 day and was very heavy on the second day. After this period, the sensitive colour test for 'catechol' substances was definitely positive. When this test was at its maximum, the whole culture was made definitely acid to Congo red with H_2SO_4, and filtered through kieselguhr. The clear filtrate was exhaustively extracted with ether, and the combined ether extracts washed with distilled water, followed by excess of a 20% neutral lead acetate solution. There appeared immediately in the aqueous layer a white precipitate which was filtered off, well washed with water, and decomposed with H_2S. After filtration of the PbS, the aqueous solution was re-extracted with ether, the ether solution dried with Na_2SO_4, and evaporated to dryness. A white solid remained (0·5 g.) which was recrystallized from ethanol-water mixture, giving white needles of m.p. 195° (alone or mixed with authentic 3:4-dihydroxybenzoic acid). It afforded a diacetyl derivative, m.p. 153°.

Both *m*- and *p*-hydroxybenzoic acid, when employed as substrates, gave rise to protocatechuic acid from the bacterial culture, but the organism was without action on salicylic acid.

Attempts to isolate a derivative of the 3:4-quinone of protocatechuic acid (e.g. a dianilino, or sulphone derivative) were unsuccessful.

Other intermediate compounds formed by the bacterial oxidation of phenol and benzoic acid

The substance responsible for the 'Rothera reaction'. Very early on in the investigation of the metabolic products resulting from the bacterial oxidation of phenol or benzoic acid, it was noticed that at a certain stage the culture fluid gave a very definite Rothera reaction. This colour reaction usually began to appear after the 'catechol' test had become negative. At this stage, the culture filtrate also gave definite positive results in Gerhardt's test (Bordeaux red with $FeCl_3$ solution) and Hurtley's reaction (to 10 ml. culture fluid, 2 ml. conc. HCl were added, followed by 1 ml. of $NaNO_2$ solution (1%); after 2 min., 15 ml. conc. ammonia (sp.gr. 0·880) were added, followed by 5 ml. of a ferrous sulphate solution (10%). A violet coloration was produced.)

It was originally thought that these colour reactions were due to certain intermediates formed after fission of the aromatic ring, most probably either acetoacetic acid or acetone. However, as these tests are not likely to be specific, and only indicative of certain particular chemical groupings, the matter was further investigated.

A phenol culture which had reached the stage at which a good Rothera reaction was given, was filtered through kieselguhr, and the clear filtrate divided into three portions as follows:

(i) 100 ml. filtrate, made acid to Congo red with H_2SO_4.

(ii) 100 ml. filtrate made alkaline to litmus with NaOH.

(iii) 100 ml. filtrate made alkaline to litmus with $NaHCO_3$.

All of these solutions were separately extracted with ether (acetone free) repeatedly (10 times), and the residual aqueous phase tested with Rothera's reagents. The first solution (i) now gave a much weaker reaction than a control kept at this pH, whilst solutions (ii) and (iii) gave the reaction practically unchanged in intensity. It is evident, therefore, that the unknown intermediate must be of an acidic nature, but even so it is extracted with ether only with difficulty from the aqueous phase.

A comparison with acetoacetic acid and acetone was made as follows. Acetoacetic acid was prepared by the method of Shaffer (1921), and an acidic (to Congo red) aqueous solution adjusted so that the intensity of the Rothera reaction given by it was approximately equal to that of an acidified phenol culture filtrate. Similarly an aqueous acetone solution was adjusted in an identical manner. Upon repeated extraction of the three solutions in parallel, with ether, the following results were obtained (Table 2).

Table 2. *Relative ease of extractability of the 'Rothera substance', acetoacetic acid, and acetone*

Solution	Number of ether extractions	Rothera reaction on mother liquor
Acetoacetic acid	3	Negative
Acetone	3	Negative
Phenol culture filtrate	3	Positive—Rothera test slightly less
Phenol culture filtrate	10	Very pronounced diminution in the Rothera reaction

Close comparison of the Rothera colour reaction given by the bacterial filtrate, with substances containing the S—S linkage like glutathione, or sulphides, show very marked differences between the nature of the colour, its development rate, and persistence, leading to the view that it is not a substance of this nature. The above evidence also seems to exclude identity with acetoacetic acid, but suggests rather that we are dealing with an unstable ketoacid with some similar properties. Further work is in progress with the object of identifying this interesting intermediate of the fission of the benzene ring.

Isolation of formic acid. A phenol culture (2 l. containing 1 g. phenol) was allowed to proceed until the 'catechol' reaction was negative, and all the phenol had disappeared, according to the Folin method of estimation. The cells were removed by filtration through a large Berkefeld candle, and the clear filtrate made distinctly acid to Congo red. To determine the amount of volatile fatty acids, the filtrate was boiled for 15 min., the distillate being collected in 25 ml. of 0·1N-NaOH. Two l. of culture filtrate gave a volatile fatty acid fraction equivalent to 10 ml. of 0·1N-NaOH. The distillate gave the following reactions characteristic of a very weak solution of sodium formate: (i) a red colour with $FeCl_3$; (ii) it reduced ammoniacal silver nitrate; (iii) it decolorized acid $KMnO_4$; (iv) partition of the copper salts between *iso*amyl alcohol and water gave a distinct coloration in the water layer but none in the alcohol, indicating the absence of appreciable amounts of propionic and butyric acids.

As the quantities in the above experiments were small it was repeated, with 20 l. of phenol culture, the distillate being collected in 50 ml. N-NaOH solution. This solution was extracted with ether, then made definitely acid to Congo red, and re-extracted several times with ether. These latter ether extracts were combined, dried with Na_2SO_4, and evaporated to dryness. A small oily residue remained, with a pungent odour (0·5 g.). *p*-Toluidine (2 g.) was added to this residue, and the mixture heated in a bath at 220° for 1 hr., cooled and dilute HCl added. The precipitate which separated was filtered, washed thoroughly with water, and re-crystallized from glacial acetic acid; its m.p. was then 53° alone or mixed with the *p*-toluidide of formic acid.

The enzymatic mechanism in the bacterial oxidation of aromatic compounds

The biological oxidation of phenolic compounds has been the subject of extensive investigation, and fairly well defined enzyme systems are known which participate in one or other of the intermediate stages. Thus, the oxidation of monohydroxy to dihydroxyphenol compounds is catalyzed by only one well defined enzyme, the copper-containing monophenolase, tyrosinase. This enzyme, however, has no action on benzoic acid. Oxidation of a dihydroxyphenol derivative to the corresponding quinone is catalyzed by a variety of seemingly different enzymes, viz. (*a*) The copper-protein enzymes, e.g. tyrosinase, laccase, polyphenol oxidase and dopa (3:4-dihydroxyphenylalanine) oxidase. (*b*) The iron-protein enzymes, e.g. peroxidase, or the cytochrome system.

Molecular or 'active' oxygen is required for all these enzymatic oxidations. In no case has it been clearly demonstrated that a hydrogen acceptor other than molecular oxygen will suffice.

According to the evidence presented in this work, the course of the bacterial oxidation of phenol and benzoic acid can be represented as shown in the diagram on p. 379.

Action of bacterial cell suspensions on tyrosine and dopa. It is known that dopa is oxidized to melanin much more readily than tyrosine, by tyrosinase (Evans & Raper, 1937). In view of the pathway followed by phenol and benzoic acid when metabolized by these micro-organisms, it was of considerable interest to study their effect on tyrosine and dopa. A cell suspension (approximately 2 ml. packed cells/10 ml.) of the phenol-splitting bacteria was prepared by centrifugation of a benzoic acid medium culture. The following five aeration tubes were prepared: (1) tyrosine 25 mg. in 50 ml. 0·1M-phosphate buffer, pH 6·6; bacterial suspension (10 ml.). (2) Similar to (1) but with a boiled bacterial suspension. (3) Dopa 25 mg. in 50 ml. 0·1M-phosphate buffer, pH 6·5; bacterial suspension (10 ml.). (4) Similar to (3) but with a boiled bacterial suspension. (5) Control tube: cell suspension (10 ml.) alone.

After the addition of a drop of chloroform to each tube they were aerated with NH_3-free sterile air at 37° for 24 hr. At the end of this period samples were taken for the estimation of the amount of substrate still remaining, the residual reaction mixtures being made alkaline with NaOH; any

NH_3 formed was aspirated into 25 ml. of 0·1N-H_2SO_4 solution. The results are summarized in Table 3.

Table 3. *Action of cell-suspension of vibrio* 01 *on tyrosine and dopa*

Aeration tube contents	Development of pigment	NH_3 liberated (mg.)	Substrate remaining after 24 hr. (%)
(1) Tyrosine (25 mg.) plus cells	Nil	2·0	10
(2) Tyrosine (25 mg.) plus boiled cells	Nil	0·25	98
(3) Dopa (25 mg.) plus cells	Heavy black melanin	0·8	Nil
(4) Dopa (25 mg.) plus boiled cells	Slight melanin production	0·2	90
(5) Control tube cell suspension	Nil	0·15	Nil

As will be seen from Table 3, tyrosine is almost completely deaminated by the cell suspension and disappears from the reaction mixture, with no pigment formation. Dopa, on the other hand is oxidized to black melanin to a considerable extent, undergoing less than 50% deamination. With tyrosine and the bacterial cells, the essential side-chain structure, which, in the oxidation of this substrate with tyrosinase leads to indole substances and eventually to melanin, is preferentially attacked. In consequence, deamination and oxidation of the alanine side chain occurs prior to the oxidation of the aromatic nucleus. In the case of dopa it seems probable that both mechanisms are operative. The non-production of the characteristic pigments of the tyrosine-tyrosinase reaction when these bacteria oxidize tyrosine, should not therefore be taken as evidence of the absence of the monophenolase from the bacterial cells.

The anaerobic oxidation of phenolic compounds by the bacterial suspensions. The phenol-decomposing bacteria do not grow anaerobically, neither is there any appreciable utilization of phenol by a bacterial suspension in absence of oxygen. In view, however, of the clear evidence of the production of *o*-benzoquinone from catechol by the organisms, it was of interest to investigate this dehydrogenation reaction further. It will be recalled that oxygen cannot be replaced by any other hydrogen acceptor when the aerobic oxidases are employed to catalyze this oxidation.

The Thunberg-Keilin technique was used, and a fresh bacterial suspension which had been actively oxidizing phenol prepared by centrifugation followed by thorough washing with distilled water. Reagents were used at the following final concentrations: methylene blue, 0·02%; substrate, 0·1% in 0·1M-phosphate buffer, pH 6·8 (both these solutions were placed in the side arm of the Thunberg-Keilin tubes); and bacterial suspension, 1 ml. packed cells diluted to 10 ml. The tubes were kept in a bath at 37°, evacuated by Hyvac pump, filled with nitrogen (oxygen free) and re-evacuated twice. The times required for the complete decolorization of the methylene blue were observed, and are recorded in Table 4.

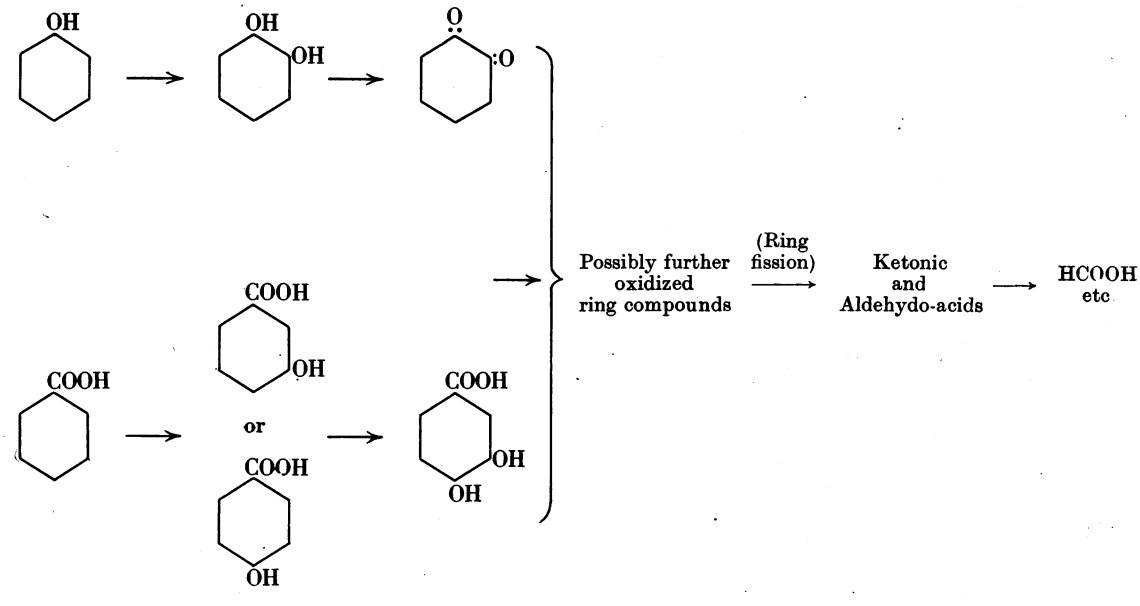

Table 4. *The action of cell-suspensions of vibrio* 01 *on methylene blue and various substrates under anaerobic conditions*

(All tubes contained methylene blue (0·2%), 1 ml. Temp.: 37°. Substrate concentrations: 10 mg./ml.)

Tube no.	Substrate	Cell suspension (10 ml.)	Time for complete decolorization of methylene blue (0·2%)
1	Phenol (1 ml.)	10 ml.	No decolorization in 3 hr.
2	Phenol (1 ml.)	10 ml. (boiled)	No decolorization
3	Catechol (1 ml.)	10 ml.	30 min.
4	Catechol (1 ml.)	10 ml. (boiled)	No decolorization
5	Benzoic acid (1 ml.)	10 ml.	No decolorization
6	Benzoic acid (1 ml.)	10 ml. (boiled)	No decolorization
7	3:4-Dihydroxybenzoic acid	10 ml.	25 min.
8	3:4-Dihydroxybenzoic acid	10 ml. (boiled)	No decolorization

From Table 4 we observe the highly interesting result of an apparent 'catechol dehydrogenase' activity. As far as the author is aware, this represents the first indication of the possibility of a dehydrogenase system being involved in the biological oxidation of *o*-dihydroxy phenols to their respective quinones. It is not known, as yet, which group of dehydrogenating enzymes is responsible for the activation of the catechol derivatives, nor is it clear what is the nature of the intermediate hydrogen acceptors through which it reacts with oxygen.

A heavy suspension of the organism was prepared and investigated for absorption bands by the Hartridge reversion spectroscope. Definite bands were visible in the following regions: (*a*) 530–535 mμ; (*b*) 550–565 mμ; (*c*) 580–585 mμ. It is difficult, at this stage, to ascertain the role of cytochrome systems in the bacterial oxidation of benzene ring compounds which lead to nuclear fission.

Substrates capable of acting as sole metabolites in a simple mineral salt medium by the vibrio 01

Of the organisms described by Gray & Thornton (1928) which were capable of utilizing aromatic compounds in a simple salt medium, a certain amount of specificity towards metabolites was encountered; thus, whilst almost all oxidized phenol, only some were able to grow in the medium containing benzene, toluene or naphthalene. With the vibrio 01 which was the main organism used in this investigation, it is interesting to observe its general oxidative capacity. Table 5 gives a list of the substrates which when present in the salt medium are able to support growth (indicated by a positive sign), compared with related compounds which were found inadequate for growth (indicated by a negative sign).

Table 5. *The effect of various substances as sole metabolites for the vibrio* 01, *in a salt medium at* 30°

(All substrates were used at a final concentration of 0·01 g./100 ml. of the mineral salt medium.)

	Substrates	Growth
Phenols	Phenol	+
	Catechol	+
	p-Cresol	−
	Resorcinol	−
Substituted benzoic acids	Benzoic acid	+
	m-Hydroxybenzoic acid	+
	p-Hydroxybenzoic acid	+
	o-Hydroxybenzoic acid	−
	3:4-Dihydroxybenzoic acid	+
	Phthalic acid	−
	Terephthalic acid	−
	p-Aminobenzoic acid	−
Phenolic amino-acids	Tyrosine	+
	3:4-Dihydroxyphenylalanine	+
Fatty acids (sodium salts)	Acetate	+
	Lactate	+
	Oxalate	−
	Citrate	+
	Pyruvate	±
	Succinate	+
	Formate	±
	Fumarate	+
	trans-trans-Muconate	−
	Adipate	+
	Malonate	−
	Malate	+
	dl-Tartrate	−

Bernheim (1942) compared the oxidative capacities of the tubercle bacillus (bovine) with a non-pathogenic mycobacterium, with regard to benzoic acid and related substances. Table 6 reproduces his results as regards specificity of the substrates oxidized.

Table 6. *The action of cell suspensions of a tubercle bacillus (bovine) and a non-pathogenic mycobacterium on various substrates (Bernheim,* 1942)

	Organism	
Substrate	T.B. (bovine)	Non-pathogen (*Mycobacterium*)
Benzoate	+	+
o-Hydroxybenzoate	+	−
m-Hydroxybenzoate	−	+
p-Hydroxybenzoate	−	+
Tyrosine	−	+
Phenylalanine and other amino-acids	−	−
Phenol	−	+

It is evident that Bernheim's non-pathogenic *Mycobacterium* behaves in much the same way as the vibrio 01.

DISCUSSION

The fate of aromatic compounds in living organisms has long been a topic in intermediate metabolic studies. Thus, Tauber (1878) brought forward some quantitative data of the amount of phenol disposed of by oxidation, as distinct from detoxification mechanisms *in vivo*. He estimated that in the rabbit, 25–50 % of a sublethal dose was broken down by the animal and completely oxidized; traces of pyrocatechol and hydroquinone were isolated from the urine. Deichmann (1944) reinvestigated this problem, also in the rabbit; he found that when the phenol is administered in sublethal doses, detoxication by conjugation is the major route (77 %) by which the compound is eliminated from the body, about 20 % being oxidized completely. When, however, the dose is approaching the lethal level, and the excretion of the conjugated product is diminished, as a result of injury to the kidneys, the destruction of the compound by oxidation is the predominant mechanism. Little light has been thrown on the oxidation mechanism or the intermediate metabolites by these studies.

Embden & Balder (1913), in a study of the fate of phenylalanine and tyrosine in the mammalian liver, brought forward evidence of acetoacetic acid formation, but nothing definite was established as to the mechanism of ring cleavage. The studies of Moss & Schoenheimer (1940) using the tracer technique, have made it practically certain that in the rat tyrosine is formed from phenylalanine. Bernheim & Bernheim (1944) have located where this oxidation might take place by their demonstration that rat and guinea-pig liver slices oxidize phenylalanine to give a product which is almost certainly tyrosine. Evidence for the nuclear oxidation of tyrosine is forthcoming from a variety of studies, mainly in connexion with disorders of metabolism; thus an inborn error of metabolism has been described by Medes (1932) called 'tyrosinosis', whence the administration of tyrosine by mouth led to the excretion of 3:4-dihydroxyphenylalanine (dopa) in the urine. The reported formation of small amounts of adrenaline when a tyramine solution is incubated with thin slices of the suprarenal gland by Schuler & Wiedemann (1935), also indicates the presence in mammalian tissues of enzymes capable of the oxidation of the aromatic nucleus.

Turning now to the evidence forthcoming from metabolic work on benzene itself, Drummond & Finar (1938) confirm previous workers, that this substance gives rise to *trans-trans* muconic acid when fed to rabbits. The *cis-cis* acid would be the one directly derived from the opening of the benzene ring, but they suggest that this stereoisomeride is converted into the *trans-trans* acid in the presence of other products formed when benzene is metabolized. Boeseken & Englebeits (1932), however, incline to the view that the *trans-trans* muconic acid is an indirect product of metabolism. Drummond & Finar failed to isolate muconic acid when phenol, catechol or tyrosine was given to rabbits instead of benzene. It will be noted that the phenol utilizing vibrio 01 is unable to metabolize *trans-trans* muconic acid in an inorganic medium.

The study of the oxidative disruption of benzene ring compounds by micro-organisms is, in many ways, a more attractive experimental approach for investigating the mechanism of the fission of the aromatic nucleus in biological oxidation.

Furthermore, these studies are of industrial significance in so far as the purification of chemical waste effluents containing phenolic substances, for example, probably depends for its success on the activities of these types of micro-organism. Also, in the agricultural field, these organisms may well be concerned in the putrefaction of organic matter and in preventing the harmful accumulation of toxic aromatic substances in the soil.

SUMMARY

1. The utilization of phenol and benzoic acid by certain micro-organisms isolated from the soil, sewage and faeces, has been investigated.

2. It has been shown that, using a simple mineral salt medium, with phenol or benzoic acid as the sole source of organic carbon these organisms will grow and completely metabolize these substrates.

3. The following gaseous exchange occurs during the oxidation of these substrates by a washed bacterial suspension of the vibrio 01: (*a*) phenol (1 molecule) utilizes 10 atoms of O, (*b*) catechol (1 molecule), 9 atoms, (*c*) benzoic acid, 8 atoms, (*d*) *p*-hydroxybenzoic acid, 7 atoms and (*e*) 3:4-dihydroxybenzoic acid, 6 atoms/molecule. All liberate 4 molecules CO_2.

4. The fact that catechol takes up one atom of oxygen less than phenol indicates that it is the first oxidation product of the latter. This was confirmed by direct isolation from a bacterial culture starting with phenol as substrate.

5. Similarly, the observation that *m*- or *p*-hydroxybenzoic acids take up one atom of oxygen less than benzoic acid, indicates that one or the other, is the first oxidation product of the latter.

6. Since 3:4-dihydroxybenzoic acid absorbs one oxygen atom less than either *p*- or *m*-hydroxybenzoic acid, it is likely to be the direct oxidation product. This was shown to be the case by actual isolation of protocatechuic acid from the bacterial culture starting with benzoic acid as substrate.

7. During the bacterial oxidation of phenol or catechol, it has been shown that *o*-benzoquinone is produced, by the isolation of a di-anilino derivative.

8. Another intermediate compound, having a transient existence, gives the Rothera reaction. This substance has not been characterized, but evidence is presented which indicates that it may be a keto-acid, although not identical with acetoacetic acid.

9. Formic acid has been isolated from a bacterial culture in which the phenol or benzoic acid had disappeared.

10. It is shown that in the action of a bacterial suspension of vibrio 01 on the two amino-acids, tyrosine and 3:4-dihydroxyphenylalanine, two oxidative mechanisms may operate: deamination leading to oxidation of the side chain, or nuclear oxidation leading to fission of the ring.

11. Evidence is presented for the occurrence of a 'catechol dehydrogenase system' in cell suspensions of these organisms.

12. Our knowledge of the oxidation of the benzene ring within living organisms and the possible role of these micro-organisms in the prevention of aromatic compounds from accumulating in toxic amounts in the soil is discussed.

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Amino-Acids and Proteins in Haemoglobin Formation

I. TRYPTOPHAN*

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Knowledge of protein and amino-acid metabolism is confined almost entirely to those aspects concerned with degradation and excretion. Of the synthetic processes in which amino-acids are utilized little is known. One of the most important of such syntheses is that of haemoglobin, the new formation of which in the adult human being is estimated at about 25 g./day. Red blood cell destruction followed by the irreversible decomposition of the haemoglobin and excretion of part of the resulting products in the form of urinary and faecal pigments takes place continuously in the normal animal and the nitrogenous material thus lost has to be replaced by dietary protein. We have at present, however, very little knowledge of the amino-acids essential for the performance of this function.

Tryptophan, which has an obvious chemical relationship to blood porphyrin, is the only amino-acid which has been extensively investigated from this point of view. The efficacy of tryptophan in the treatment of experimental anaemia was first noted by Hirasawa (1922) and similar results were reported by Okagawa & Tatsui (1931). Fontés & Thivolle (1930) claim to have produced anaemia in rats kept on a tryptophan deficient diet for a period of 10 weeks, and further, to have rendered normal

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