The Formation of β -Ketoadipic Acid by Bacterial Fission of Aromatic Rings

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An increasing interest has been shown in recent years in the biological breakdown of aromatic rings, both in the mammalian body and in bacteria. A review of the oxidation of aromatic rings by micro-organisms has been given by Happold (1950) which supplies the historical background to the present investigation. In brief, a study by Evans & Happold (1939) of the metabolism of a vibrio previously isolated from sewage effluent containing spent gas-works liquors (Happold & Key, 1932) demonstrated that phenol was readily metabolized by this organism and could serve as the sole source of carbon and energy, that catechol was formed and disappeared, and that this was followed by the transient appearance of a compound characterized by giving positive Rothera and Gerhardt reactions. It was suspected that this compound might be a keto acid formed after fission of the aromatic ring, but it was not acetoacetic acid as it was much less readily extractable by ether from aqueous solution. The present work was directed towards the isolation and identification of this interesting metabolic intermediate, and some of the results obtained have been briefly reported in a preliminary communication (Kilby, 1948).

MATERIALS

Organism. The vibrio 01 isolated by Happold & Key (1932) from sewage containing gas-works effluent was used. This organism is similar in morphology to *Vibrio cuneata* isolated by Gray & Thornton (1928).

Mineral-salt medium. The mineral-salt medium to which phenol or benzoate was added as a substrate contained $(\text{NH}_4)_2\text{SO}_4$, 2.5 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.0 g.; KH_2PO_4 , 0.5 g.; metal-salt solution 2.0 ml. and distilled water to 1 l. A stock solution of metal salts was prepared containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 22.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.0 ml. of 1% (w/v); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 ml. of 1% (w/v); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5 ml. of 1% (w/v); conc. HCl, 3 ml. and distilled water to 1 l.

β -Ketoadipic acid. Succinic anhydride (Fieser & Martin, 1943) was converted into β -carbethoxypropionyl chloride as described by Reigel & Lilienfeld (1945). Reaction of this with an ethereal suspension of ethyl sodioacetoacetate gave ethyl α -acetyl- β -ketoadipate, b.p. $156^\circ/0.04$ mm. The experimental conditions followed and the methods of purification employed were essentially those described by Bardhan (1936) for the preparation of the corresponding methyl ester. A cooled ethereal solution of ethyl α -acetyl- β -

ketoadipate was treated with dry NH_3 gas, left overnight, shaken with water, cold dil. HCl and finally with water again, dried with Na_2SO_4 , the ether removed, and the product distilled. Ethyl β -ketoadipate was obtained as a colourless oil, b.p. $116\text{--}117^\circ/0.015$ mm. This ester was hydrolysed by mixing with 9 vol. of conc. HCl and evaporating to dryness over solid KOH in a vacuum desiccator. After two recrystallizations from an ethyl acetate-light petroleum (b.p. $40\text{--}60^\circ$) mixture, β -ketoadipic acid was obtained as white crystals, m.p. 121° (decomp.), which were very soluble in water. An intense purple colour was given in the Rothera reaction, which could still be detected when a 0.001% solution of the acid was used. On heating the acid alone or in aqueous solution, decarboxylation occurred to give laevulinic acid. Treatment of β -ketoadipic acid with 2:4-dinitrophenylhydrazine gave the 2:4-dinitrophenylhydrazone of laevulinic acid, m.p. 203° .

RESULTS

Isolation of laevulinic acid from a benzoate culture

Mineral-salt medium to which sodium benzoate (1 g./l.) had been added as a substrate was inoculated with the vibrio 01 (previously subcultured in benzoate medium), gently aerated with sterile air and incubated at room temperature. A profuse growth resulted and, after 3 days, the medium, which had remained almost colourless, gave a good Rothera reaction. This culture (3 l.) was then evaporated down to about 50 ml. under reduced pressure and at a temperature not exceeding 37° . A very strong Rothera reaction was given by this concentrate; boiling a sample of it for a few minutes largely destroyed the compound responsible for this test and the reaction became much weaker. Salts which had separated out during the concentration and the bacterial cells were removed by Seitz filtration, the clear solution acidified and the resultant precipitate of unmetabolized benzoic acid removed.

A part of this filtrate was boiled for a few minutes, cooled and extracted continuously overnight with ether. After removal of the solvent from the extract, the residue appeared to be largely benzoic acid, but had a smell reminiscent of laevulinic acid. It was dissolved by shaking with a mixture of water and light petroleum (b.p. $40\text{--}60^\circ$) and the clear aqueous

layer treated with a solution of 2:4-dinitrophenylhydrazine. A precipitate separated in a few minutes and was recrystallized from methanol forming orange needles, m.p. 203.5°, after drying at 100° *in vacuo*. This product was identified as the 2:4-dinitrophenylhydrazone of laevulinic acid as the melting point was unchanged on mixing with an authentic specimen.

The remainder of the unboiled acidified concentrate was continuously extracted with ether in order to try and obtain the precursor of the laevulinic acid. Although the material responsible for the Rothera reaction disappeared from the aqueous layer, only a small amount was detected in the ethereal extract, presumably due to decomposition in the ether while this was refluxing. The ethereal extract gave the same 2:4-dinitrophenylhydrazone as obtained above.

Isolation of β -ketoadipic acid from a phenolic culture

In the next attempt to isolate without decomposition the compound responsible for the Rothera reaction, which was believed to be acidic in nature, phenol was used as a substrate instead of benzoate in order to avoid a mixture of unchanged benzoic acid and the unknown acid after extraction. The medium (3 l.) containing phenol (1 g.) was inoculated, aerated and incubated at room temperature for 3 days. Good growth resulted, and the slightly yellow medium gave a Rothera reaction. The cells were removed by Seitz filtration, the filtrate adjusted to pH 7.0 and evaporated at 37° to 150 ml. under reduced pressure. The concentrate, after acidification with dilute sulphuric acid, was extracted by hand repeatedly with ether (40 times, using a total of 1.5 l. ether). The ethereal extract was dried with sodium sulphate and the solvent removed under slightly reduced pressure. The residue was dissolved in ethyl acetate and cooled. White crystals separated (30 mg.) which gave an intense permanganate colour with the Rothera reaction. After a further recrystallization from ethyl acetate, crystals were obtained of m.p. 120° (decomp.), unchanged on mixing with authentic synthetic β -ketoadipic acid of m.p. 121° (decomp.).

Breakdown of β -ketoadipic acid by heat

This experiment was carried out to show that the thermal and non-enzymic decomposition of β -ketoadipic acid to laevulinic acid does not give rise to any succinic acid after oxidation of the product with permanganate. A solution of β -ketoadipic acid (26 mg. in 9 ml. 0.1 M-phosphate buffer, pH 7.4) was heated in a boiling-water bath for 30 min., a treatment previously shown to destroy the keto acid. Potassium permanganate (3 ml. of 6% w/v) and sulphuric acid (1 ml. of 50% v/v) were added and the mixture was allowed to stand at room temperature

for 40 min. It was then extracted for 2.5 hr. with freshly distilled ether in a continuous extractor. Phosphate buffer (2 ml.) was added to the ethereal extract, the ether removed, and the volume made up to 10 ml. with buffer. Succinic acid was estimated manometrically on samples of this solution using the succinic dehydrogenase method (Krebs, 1937). The enzyme preparation used was finely minced and well washed pigeon-breast muscle suspended in five times its volume of phosphate buffer. The succinic acid found in the extract (0.31 mg.) was equivalent to a yield of only 0.03 mol. from 1 mol. of β -ketoadipic acid. (Test runs of the method using 0.5 mg. succinic acid gave 101% recovery.)

Breakdown of β -ketoadipic acid by the vibrio

The cells from a 4 to 5 day old culture were harvested, washed with distilled water and suspended in about three times their volume of phosphate buffer. The mixtures indicated in Table 1

Table 1. *Succinic acid from α -ketoadipic acid in cultures of vibrio 01*

Mixture (see text)	Succinic acid found (mg.)
(A) β -Ketoadipic acid, 20 mg. in 1 ml.; 0.1 M-malonic acid, 1 ml.; cell suspension, 1 ml.; buffer, 1 ml.	15.0
(B) β -Ketoadipic acid, 20 mg. in 1 ml.; cell suspension, 1 ml.; buffer, 2 ml.	15.4
(C) 0.1 M-Malonic acid, 1 ml.; cell suspension, 1 ml.; buffer, 2 ml.	1.17

were incubated at room temperature for 27 hr., in small conical flasks. The cells were removed by centrifugation and washed with 2-3 ml. water, and the combined supernatant and washings heated for 1 hr. in a boiling-water bath. Sodium tungstate (0.25 ml. of 10% (w/v) solution) was added as a protein precipitant, and permanganate oxidations and succinic acid estimations performed as described previously. Deducting the value of the control found in C from the mean of the first two values, the amount of succinic acid found is 14.0 mg. which is equivalent to a yield of 0.95 mol. of succinic acid from 1 mol. of β -ketoadipic acid.

DISCUSSION

It has been shown by Evans (1947) that the metabolism of benzoate by the vibrio and also by *Pseudomonas fluorescens* proceeds through catechol, and this has been confirmed by Stanier (1950) using his simultaneous adaption technique. It has also been shown that phenol is metabolized by the vibrio through catechol, so that either benzoate or phenol cultures can be used to study changes which occur after the catechol stage as the two metabolites are metabolized along the same pathway.

A scheme for the oxidation of catechol by tyrosinase proposed by Nelson & Dawson (1944) was: catechol $\rightarrow$ *o*-benzoquinone $\rightarrow$ 1:2:5-trihydroxybenzene $\rightarrow$ 5-hydroxy-*o*-benzoquinone $\rightarrow$ coloured products. The first two of these compounds appear in the medium when phenol is metabolized by the vibrio (Evans, 1947). The similarity between the two systems led the author (Kilby, 1948) to suggest that the metabolism of phenol by the vibrio followed the same pathway as far as 5-hydroxy-*o*-benzoquinone, and that in the presence of tyrosinase this compound was converted into coloured products, while with the vibrio, which is devoid of tyrosinase, a hydrolytic ring fission occurred to give β -keto-adipic acid, which was subsequently broken down to succinic acid and probably acetic acid:

This scheme received support by the isolation soon afterwards of the third postulated intermediate, 1:2:5-trihydroxybenzene, from vibrio cultures growing on catechol (Evans, 1948). Subsequently, Evans and his co-workers have expressed the view (see Happold, 1950) that this compound was not a true bacterial intermediary as it was not oxidized at a sufficient rate to be included in the main metabolic pathway, when oxidation rates were compared using the simultaneous adaption technique (Stanier, 1947).

Since β -keto-adipic acid was first reported as a product of bacterial metabolism (Kilby, 1948), its formation has been confirmed by Evans (1948) and by Stanier, Sleeper, Tsuchida & Macdonald (1950). The latter have shown that specific enzymes capable of oxidizing either catechol or protocatechuic acid to β -keto-adipic acid can be isolated from dried cells adapted to aromatic substrates but not from dried

unadapted cells. The rate of oxidation of β -keto-adipic acid by living cells of *Ps. fluorescens* was found by Stanier and his associates always to be much less than that of presumed aromatic precursors, perhaps because the highly polar acid penetrates slowly into the cell.

It appears, therefore, that β -keto-adipic acid has been established as an intermediate in the bacterial metabolism of some aromatic compounds, but the nature of its immediate precursors is still an open question. The almost quantitative conversion of β -keto-adipic acid into succinic acid which was observed suggests that a hydrolytic split of the keto acid into succinic and acetic acids is the next stage in the metabolic pathway. This would be analogous to the fission of acetoacetic acid into acetic

acid observed by Lehninger (1942) working with *Escherichia coli*. Whether the fission of the keto acid is followed by oxidation appears to depend on the experimental conditions; when left at room temperature without shaking, very little of the succinic acid formed was metabolized, and addition of malonate had little effect. Shaking in Warburg flasks at 37° gave oxygen uptakes which varied from about 3 to 11.3 atoms of oxygen/molecule of keto acid depending on the sample of cells used (for complete oxidation, 11 atoms are required).

The metabolism of aromatic compounds in the mammal may follow a somewhat similar pathway. Phenylalanine and tyrosine are metabolized by rat-liver slices through homogentisic acid, and Lerner (1949) believes that ring fission occurs at one point to give an 8-carbon dicarboxylic acid from which acetoacetate and malic acid are subsequently formed.

SUMMARY

1. β -Keto adipic acid has been shown to arise by ring fission in the metabolism of phenol and benzoic acid by a vibrio species.

2. The keto acid is subsequently degraded by the vibrio to succinic acid.

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Comparison of Haem *a*, the Dichroic Haem of Heart Muscle, and of Porphyrin *a* with Compounds of Known Structure*

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In his classical studies on the photochemical absorption spectrum of the respiratory ferment, Warburg (for review see Warburg, 1949) concluded that its prosthetic group is a haem with a formyl side chain, related to chlorocruorohaem ('Spirographis' haem) or to phaeohaem *b₆* (iron complex of compound, formula III). The absorption spectra of the cytochromes *a*, *a₁* and *a₂* (the last named probably identical with cytochrome oxidase and the respiratory ferment) suggest that these are all derived from the same type of haem and possibly the same haem (cf. Lemberg & Legge, 1949). Attempts to isolate this haem from muscle have led to somewhat controversial results. It has been possible to isolate such a haem from heart muscle (Negelein, 1933; Roche & Bénévvent, 1936), but there has been no complete agreement as to its properties. The porphyrin ('cryptoporphyrin') isolated by Negelein (1932*a*) from pigeon-breast muscle was later considered an artifact by this same author (1932*b*); this may well be so, but we have not been able to confirm Negelein's opinion that it arose through the action of light on protoporphyrin in acid solution during the

isolation (cf. also Rawlinson & Hale, 1949). The divergences were probably due to the difficulties of isolation and to the lability of haem *a*, and not to the existence of a variety of haems *a* derived from different cytochromes, although such a possibility has not yet been definitely excluded.

At the time of Warburg's investigations only a small number of porphyrin derivatives with carbonyl groups in the side chains was known, but it has been greatly increased since, particularly through the work of Fischer. It appeared necessary to compare their properties and the properties of their haem compounds systematically with those of porphyrin *a* and haem *a*. For this it was necessary to re-investigate the properties of the latter compounds. This study had just begun when, through the kindness of Dr W. A. Rawlinson, we were informed of the isolation of haem *a* and porphyrin *a* from ox-heart muscle and from *Corynebacterium diphtheriae* by an improved method (Rawlinson & Hale, 1949). We have, therefore, concentrated mainly on the comparison of these products with porphyrins and haems of known structure containing carbonyl groups in conjugation with the nucleus in one or two pyrrole rings. The three schools working on haem *a* (at the Department of Chemical Pathology, University

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