Oxidation of Homogentisic Acid by Cell-free Extracts of a *Vibrio*

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SUMMARY

Acetoacetic acid is a product of degradation of homogentisic acid by cell-free extracts of a vibrio grown with phenylacetic acid as sole carbon source. 4-Maleylacetoacetate was identified as a reaction intermediate; reduced glutathione was a cofactor for this enzymic degradation. These reactions are similar to those catalysed by enzymes of rat liver.

INTRODUCTION

Homogentisic acid is an intermediate in the bacterial oxidation of tyrosine (Suda & Takeda, 1950; Jones, Smith & Evans, 1952), phenylalanine (Dagley, Fewster & Happold, 1953) and phenylacetic acid (Dagley *et al.* 1953; Kunita, 1955*a*). In animal liver 4-maleylacetoacetate is first formed from homogentisate by the single-step addition of O₂, catalysed by homogentisate oxidase; it then isomerizes to 4-fumarylacetoacetate. This reaction, which requires reduced glutathione (GSH), is followed by hydrolysis of 4-fumarylacetoacetate to fumarate and acetoacetate (Knox & Edwards, 1955; Knox, 1960). Many alternative routes exist for the oxidation of aromatic compounds by bacteria (Dagley, Evans & Ribbons, 1960) and further evidence was therefore required before it could be assumed that the same reactions occur when bacteria oxidized homogentisic acid or its precursors. This evidence is now presented.

METHODS

The organism used was the vibrio O1 (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen; NCIB 8250) originally isolated by Happold & Key (1932). Batches of organisms were grown at 30° with forced aeration in a medium adjusted with NaOH to pH 7.2 and containing (g./l.): KH₂PO₄, 2; (NH₄)₂SO₄, 1; MgSO₄.7H₂O, 0.4; phenylacetic acid, 0.7. Organisms were transferred from a slope to 25 ml. of this medium aerated at 30° in a boiling tube (6 in. × 1 in.) and after 24 hr. the whole of the fully-grown culture was used to inoculate 1 l. of medium aerated in a 2 l. flask. The resulting culture was used as inoculum for 8 l. of medium in a 10 l. flask into which passed a glass tube fitted with an aquarium aerator stone. From 9 l. of this culture about 6 g. (wet weight) of organism was obtained after incubation overnight at 30°. All buffer solutions contained 2 g. of KH₂PO₄/l., brought to pH 7.2 with NaOH. Cell-free extracts were prepared from organisms disrupted at -14° without abrasive in the bacterial press

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of Hughes (1951), extracted with buffer and centrifuged at 10,500 g for 45 min. to remove insoluble material. Uptake of O_2 and evolution of CO_2 were followed by the conventional manometric procedures (Umbreit, Burris & Stauffer, 1949). Acetoacetic acid was determined by coupling with diazotized *p*-nitroaniline to give a coloured substituted formazan. This reaction is quantitative when carried out according to Walker (1954) and is specific for compounds containing a reactive methylene group; thus, pyruvic acid and many other metabolites do not react. Acetoacetic acid was prepared from freshly-distilled ethyl acetoacetate by the method of Krueger (1952). *m*-Hydroxyphenylacetic acid was prepared according to King & McMillan (1946). Other materials were commercially available. The protein concentrations of cell-free extracts were determined by the method of Sols (1947) with reference to a calibration curve made by using crystalline bovine serum albumin. Concentrations of maleylacetoacetate and fumarylacetoacetate were calculated from measurements of optical densities at 330 m μ , one part of the given solution being brought to pH 1 and the other to pH 13 by addition of HCl and NaOH, respectively. Knox & Edwards (1955) used the known molar extinction coefficients at these two pH values to derive formulae which give the amounts of maleylacetoacetate and fumarylacetoacetate in mixtures of the two. Absorption spectra were read in a Unicam SP 500 spectrophotometer using silica cells of 1 cm. light path.

RESULTS

Oxidation of homogentisate by crude cell extracts

Cell-free extracts of the vibrio grown with phenylacetic acid catalysed the rapid oxidation of homogentisic acid (Fig. 1*a*) with an uptake of nearly 1.5 mole O_2 /mole homogentisate. Phenylacetic, homoprotocatechuic (3:4-dihydroxyphenylacetic) and mono-hydroxyphenylacetic acids were not oxidized. When uptake of oxygen had ceased the flask contents were cooled in ice, protein precipitated by addition of 0.2 volume of 20 % (w/v) metaphosphoric acid and the precipitate removed by centrifugation and discarded. The contents of the flask containing cell extract but no substrate were treated similarly and served as a control. No reaction was given for homogentisic acid (Neuberger, 1947) but its conversion to a β -oxoacid was shown by a strong positive Rothera reaction (Rothera, 1908) and by the immediate evolution of CO_2 on addition of aniline citrate to solutions in respirometer flasks (Umbreit *et al.* 1949). Similar enzyme preparations from rat liver convert homogentisate to acetoacetate (Knox & Edwards, 1955). When samples of deproteinized reaction mixtures were treated according to the procedure of Walker (1954) the spectrum of the compound formed was very similar to that given by acetoacetic acid similarly treated (Fig. 2). The quantity of acetoacetate agreed with that given by aniline citrate decarboxylation and was equivalent to the homogentisate which disappeared (Table 1).

Oxidation of homogentisate by dialysed cell-free extracts

When crude cell extracts were dialysed for 6 hr. at 5° against phosphate buffer the oxidation of homogentisate was catalysed only when ferrous ions were added and the total oxygen uptake was also decreased but was still greater than 1 mole O_2 /mole substrate (Fig. 1*b*). After reaction, solutions were deproteinized with

metaphosphoric acid, neutralized with 2 N-NaOH and equal volumes diluted with distilled water, 3 N-H₂SO₄ and 5 N-NaOH for determination of absorption spectra at pH values of 7, 1, and 13 respectively (Fig. 3). The spectra are similar to those of maleylacetoacetate (Knox & Edwards, 1955) with a maximum at 330 m μ at pH 13, abolished at pH 1. From the published extinction coefficients, about 1.0 μ mole

Fig. 1

Fig. 1. Oxidation of homogentisic acid by extracts of vibrio O1 grown with phenylacetic acid as carbon source. The extract used was either (a) untreated or (b) dialysed for 6 hr. Warburg flasks at 30° contained extract (equiv. 20 mg. protein) and buffer (pH 7.2) to a total volume of 3 ml. and KOH in centre well. The substrates (5 μ mole) added from side bulbs are shown by numbers; 1, homogentisate; 2, homoprotocatechuate; 3, phenylacetate; 4, *o*-, *m*- and *p*-phenylacetate; extract, but no substrate was added in 5. In Fig. 1b, mM-FeSO₄ was added to mixtures shown by blacked-in circles.

Fig. 2

Fig. 2. Absorption spectra of solutions in ethyl acetate after reaction with diazotized *p*-nitroaniline. Acetoacetate was coupled under standard conditions (Walker, 1954) and the reaction product extracted completely into 4 ml. ethyl acetate. Product of enzymic oxidation of homogentisate by untreated vibrio O1 extract (O—O); 8.5 μ g. authentic acetoacetic acid (●—●).

Table 1. *Formation of reaction intermediates in the enzymic oxidation of homogentisate*

Reactions took place in Warburg flasks at 30° containing 5 μ mole homogentisate, cell-free extract of vibrio O1 (20 mg. protein) and buffer, pH 7.2, to a total volume of 3 ml. When O₂-uptake ceased, mixtures were deproteinized by addition of 0.3 ml. 20% (w/v) metaphosphoric acid. The amounts of acetoacetate (by coupling with diazotized *p*-nitroaniline) and of 4-maleylacetoacetate and 4-fumarylacetoacetate (from extinctions at 330 m μ) were then determined.

Expt.	Enzyme	4-Maleylaceto- acetate (μ mole)	4-Fumarylaceto- acetate (μ mole)	Acetoacetate (μ mole)
1	Untreated	0	0	4.80
2	Dialysed for 6 hr.; mM-FeSO ₄ added	1.02	0.18	—
3	Dialysed for 12 hr.; mM-FeSO ₄ added	2.63	0.32	—
4	Dialysed for 12 hr.; mM-FeSO ₄ + 0.3 mM-GSH added	0	0	4.85

maleylacetoacetate and 0.2 μ mole fumarylacetoacetate accumulated; the latter absorbs ultraviolet radiation strongly in acid solution and contributes to the spectrum at pH 1 in Fig. 3. In a similar experiment an extract was dialysed for 12 hr. and the yield of maleylacetoacetate was more than doubled. When reduced glutathione was added, no ultraviolet absorbing compound accumulated and homogentisate was converted almost quantitatively to acetoacetate (Table 1).

The identity of the compound formed from homogentisate by the bacterial extract was confirmed by comparing its reaction with *o*-phenylenediamine to that of maleylacetoacetate obtained by means of the liver preparation. To each 2 ml. of a solution containing about 5 μ mole maleylacetoacetate were added 6 ml. of a freshly made solution of 200 mg. *o*-phenylenediamine dissolved in 25 ml. of the following

Fig. 3

Fig. 4

Fig. 3. Absorption spectra of the product of oxidation of homogentisate by a dialysed vibrio O1 cell extract. Spectra were read at: pH 7 (●—●), pH 1 (△—△), pH 13 (○—○).

Fig. 4. Formation (a), and breakdown (b), of maleylacetoacetate catalysed by a partially purified extract (10 mg. protein/ml.) of vibrio O1. In (a), cuvettes contained, in a final volume of 3 ml., 1.0 μ mole homogentisate, distilled water and either 0.2 ml. extract, (○—○), 0.1 ml. extract (●—●), or 0.05 ml. extract (△—△). In (b), the complete system contained in 3 ml., 0.06 μ mole 'liver' maleylacetoacetate, 1.0 μ mole GSH, distilled water and either 0.05 ml. extract (△—△) or 0.1 ml. extract (□—□). Similar systems contained 'bacterial' maleylacetoacetate with 0.05 ml. extract (●—●) or 0.1 ml. extract (▲—▲). A control cuvette contained maleylacetoacetate and 0.1 ml. of extract but no GSH (○—○).

phosphate + sulphuric acid reagent: 43.5 g. KH_2PO_4 and 20 ml. 5N-orthophosphoric acid were dissolved in 500 ml. of 2.4N- H_2SO_4 and diluted to 1 l. with distilled water. Both solutions of maleylacetoacetate developed a lavender colour which changed slowly to orange (Ravdin & Crandall, 1951).

Formation and disappearance of maleylacetoacetate catalysed by partially purified cell extracts

Saturated ammonium sulphate solution, brought to pH 7.3 with ammonia, was added dropwise with stirring to a crude extract of cells until 60% saturated. The precipitate was centrifuged at 10,000 *g* for 15 min. at 2°, the supernatant fluid discarded and the pellet dissolved in phosphate buffer. The solution was dialysed against changes of buffer at 5° until the dialysis residue no longer gave a reaction for sulphate ions; then 0.4 ml. of 0.033M- FeSO_4 was added to each 10 ml.

of treated cell extract. After incubation for 1 hr. at 5° a slight precipitate was centrifuged down and discarded. In phosphate buffer (pH 7.2) this extract catalysed a linear production with time of maleylacetoacetate; this was followed by measuring the increase in extinction at 330 m μ , from homogentisate at room temperature (Fig. 4*a*). The extract also decomposed both 'bacterial' and 'liver' maleylacetoacetate, but only on addition of reduced glutathione (Fig. 4*b*).

DISCUSSION

The work of Ravdin & Crandall (1951) and Knox & Edwards (1955) established that the following reactions occur in animal liver:

The requirement of homogentisate oxidase for Fe^{++} ions was readily demonstrated by dialysis of extracts of our vibrio O1; Suda & Takeda (1950) made a similar observation for a tyrosine-grown pseudomonad. By contrast, it was necessary to use inhibitors to show participation of Fe^{++} ions in homogentisate oxidation by the enzyme from liver (Knox & Edwards, 1955). These ions may be essential for all known aromatic ring-fission enzymes in bacteria (Dagley *et al.* 1960). Cell-free extracts did not oxidize phenylacetic acid or its monohydroxylated derivatives (Fig. 1); bacterial enzymes which catalyse the introduction of a hydroxyl group into the benzene nucleus are either labile or not readily soluble (Dagley & Patel, 1957). Homoprotocatechuic acid, an isomer of homogentisic acid, is an intermediate in an alternative pathway of oxidation of phenylacetate (Kunita 1955*b*) but it was not oxidized in the presence of extracts of our vibrio O1. Homogentisate was oxidized with the uptake of 1.0–1.5 $\mu\text{moles O}_2/\mu\text{mole}$ substrate; oxygen may be required both for ring-fission and for oxidation of fumarate which is shown as a product of the above reaction sequence. Although it has not been identified as a product, fumarate is readily oxidized by cell-free extracts of the vibrio, with an uptake of 0.5 $\mu\text{mole O}_2/\mu\text{mole}$ substrate.

Further evidence for the above reaction sequence in phenylacetate-grown vibrio O1 is provided by: (i) the adsorption spectra at various pH values of the product of homogentisate oxidase (Fig. 2); (ii) the fact that this product gives the same colour reaction with *o*-phenylenediamine as authentic maleylacetoacetate; (iii) the ability of partially-purified extracts to decompose the latter only when incubated with reduced glutathione, the known cofactor of maleylacetoacetate isomerase (Fig. 4*b*). Homogentisate was converted almost quantitatively to acetoacetate as predicted by the proposed sequence (Table 1). These reactions were also similar to those studied by Lack (1959), who showed that maleylpyruvate was an intermediate in the oxidation of gentisic acid by bacterial cell extracts.

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