glucosaminidase activity also had *N*-acetylgalactosaminidase activity and that in each case the total hydrolysis in mixtures of the two substrates was less than the sum of those of the individual substrates. There was therefore no evidence of a specific site for either substrate.

It cannot, however, be concluded from these results that the same enzyme is responsible for the hydrolysis of both substrates in every case. Fractionation and differential inactivation of the activities in emulsin indicated the presence of more than one active protein. More detailed investigations are required before any further conclusion can be drawn about the relationship of *N*-acetyl- β -glucosaminidase and *N*-acetyl- β -galactosaminidase activity in each case.

The increased *N*-acetylglucosaminidase activity in the *C. perfringens* enzyme preparation in the presence of albumin appears to be another example of protection by surface-active agents against inactivation in purified enzymes. This has been described for *N*-acetylglucosaminidase (Woollen *et al.* 1961), for β -glucuronidase (Bernfeld, Bernfeld, Nisselbaum & Fishman, 1954), for acid phosphatase (Tsuboi & Hudson, 1955) and cellulase (Myers & Northcote, 1959).

C. perfringens antitoxin has been shown to inhibit the hyaluronidase (McClean & Hale, 1941) and the lecithinase (Macfarlane & Knight, 1941) of this organism, the latter non-competitively (Cinader, 1953). The antitoxin has been shown here to act non-competitively with *N*-acetylglucosaminidase.

SUMMARY

1. The *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities of a wide variety of enzyme preparations have been measured.

2. Where *N*-acetylglucosaminidase was detected it was accompanied by *N*-acetylgalactosaminidase but the ratios of the two activities covered a wide range.

3. Experiments with mixed substrates did not indicate a specific site for either activity in any of the preparations.

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The Metabolism of Protocatechuic Acid by a *Vibrio*

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Recent work with micro-organisms has suggested that the mechanisms of biological ring-fission may be considerably more diverse than was originally suspected. Not only has a new mode of fission of the benzene nucleus been reported

(Dagley & Stopher, 1959; Trippett, Stopher & Dagley, 1960), but additional pathways have been formulated whereby the aliphatic products resulting from ring-fission are channelled into terminal oxidation cycles (Ribbons & Evans, 1959). This paper describes the metabolism of protocatechuic acid by *Vibrio* O1 (Happold & Key, 1932)

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and, in particular, the participation in this scheme of a lactone previously found only as an intermediate in the bacterial degradation of catechol.

EXPERIMENTAL

Organisms. *Vibrio* O1 (N.C.I.B. 8250) was maintained by monthly subculture both on nutrient agar and slopes of the defined medium (see below) solidified with agar. *Neurospora crassa* (wild-type) (SY4a) was obtained from Professor W. C. Evans, University College of North Wales, Bangor, Caernarvonshire. Mutants of this mould, Y7655a and Y7655-10575a, with growth requirements for aromatic compounds, were obtained from Professor E. L. Tatum (Tatum & Gross, 1956). Each of the moulds was maintained on standard maltose-peptone medium [maltose (2%, w/v); peptone (1%, w/v); pH 6.0] and also on minimal medium (Ryan, Beadle & Tatum, 1943) with glucose (1%, w/v) as the carbon source, and for the mutants with the aromatic supplement (Gross, Gafford & Tatum, 1956). Vanillic acid (0.02%, w/v) was added as an inducer of protocatechuic acid oxidase in the wild-type strain when required. *Nocardia erythropolis* CA4 was the strain isolated from cultures enriched with *p*-nitrobenzoic acid (Cain, 1958).

Growth conditions. The vibrio was grown for 24–50 hr. with forced aeration in 10 l. flasks, containing 9 l. of defined medium, after inoculation with 50 ml. of a culture grown overnight in the same medium. This medium contained (g./l.): K_2HPO_4 , 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; *p*-hydroxybenzoic acid, 1.0; the pH was adjusted to 7.0–7.2 with NaOH. Growth under these conditions gave cells strongly adapted to oxidize protocatechuic acid. Unadapted cells were grown either in a defined medium where succinic acid (1 g./l.) replaced the aromatic substrate or in a nutrient medium containing (g./l.): Bacto peptone, 2.0; Lab-Lemco, 0.5; the pH was adjusted to 7.0–7.2. Cells were harvested by continuous centrifuging in an Alfa-Laval separator (Alfa-Laval Ltd., Brentford, Middlesex).

Cell-free extracts and heat-treatment. Extracts of the organism were prepared, either by ultrasonic irradiation for 20–30 min. with the M.S.E. Mullard 60 w apparatus at 20 kcy./sec. with the cell suspension cooled in melting ice, or by crushing in a Hughes press (Hughes, 1951) without abrasive. The crush was taken up in 0.05 M-phosphate buffer, cell debris and particulate material were removed by centrifuging at 16 000 *g* for 1 hr., and the clear supernatant liquid (usually 15–25 mg. of protein/ml.) was used as a crude extract. This supernatant contained all the enzymes required to convert protocatechuic acid into β -oxoadipic acid. For heat-treated extracts, samples of about 10 ml. of the extract in 0.05 M-phosphate buffer were

rapidly heated to 55° in a water bath (with the thermometer agitating the extract), held at $55 \pm 0.5^\circ$ for 15 min., and then cooled in melting ice. The flocculent coagulated protein was usually removed by centrifuging for 30 min. at 16 000 *g*. Appreciable loss of activity occurred if the heat-treatment was performed on extracts made without buffer.

Chemicals. All melting points are uncorrected. β -Oxoadipic acid, m.p. 119°, was synthesized by the method of Bardhan (1936). β -Carboxymuconic acid (tri sodium salt) was prepared biologically from protocatechuic acid by using the purified protocatechuic acid oxidase from *Nocardia erythropolis* (Cain & Cartwright, 1960). Generous gifts of this material were also received from Professor W. C. Evans, Bangor, and Dr D. L. McDonald, Berkeley. The purest available preparations contained 93% of active isomer by spectrophotometric and enzymic assays. *cis-cis*-Muconic acid, m.p. 195°, was prepared by peracetic acid oxidation of pure phenol (Elvidge *et al.* 1950). Gifts of synthetic 4-hydroxyhex-2-enedioic 1 $\rightarrow$ 4-lactone (muconolactone) (I), m.p. 109°, and 2:6-dioxo-3:7-dioxobicyclo-[3:3:0]octane (dilactone) (II), m.p. 127°, were received from Professor Evans.

The unnatural (–)-isomer of muconolactone, m.p. 79° was prepared by incubation of the synthetic (racemic) material with a washed cell suspension of the vibrio, grown on *p*-hydroxybenzoic acid, until half the substrate had disappeared. The use of washed cells, which degrade only the natural isomer completely, is preferable to resolution with extracts or the purified delactonizing enzyme, since it avoids accumulation of β -oxoadipic acid as the end product of the reaction.

3-Carboxy-4-hydroxyhex-2-enedioic 1 $\rightarrow$ 4-lactone (β -carboxymuconolactone; III) was prepared from the corresponding acid by modification of the method of Gross *et al.* (1956). The mycelia of the wild-type *Neurospora* (SY4a), obtained after 3 or 4 days growth with gentle shaking at 30° in horizontal Roux flasks containing 100 ml. of glucose minimal medium + vanillic acid, were harvested by filtration, washed three times, freeze-dried and stored at –14° till required.

The freeze-dried mycelium (1.5 g.) was moistened with 2–3 ml. of water in a precooled mortar and vigorously ground with washed, finely powdered glass. The crushed mycelium was extracted with 5–10 ml. of water for 15–30 min., centrifuged at low speed to remove cell debris and dialysed against several changes of distilled water for 18–24 hr. The dialysed material was centrifuged at high speed (20 000 *g*, 40 min.), and the supernatant solution (about 12 ml.) was made 2 mM with respect to phosphate at pH 6.2 and then treated with 0.1 vol. of calcium phosphate gel (Keilin & Hartree, 1938) for 30 min. with gentle agitation. After removal of the gel by centrifuging, the supernatant was brought to pH 6.9 with 0.1 M-phosphate buffer, pH 7.8, and the gel-treatment repeated; 75–80% of the

lactonizing activity remained in the supernatant. This dialysis and gel-treatment sequence was repeated until spectrophotometric assay at 225 and 270 $m\mu$ with β -carboxymuconic acid as substrate indicated the absence of delactonizing enzyme. β -Carboxymuconolactone was then prepared on a small scale from β -carboxymuconic acid essentially as described by Gross *et al.* (1956) except that the reaction was performed in 0.1M-phosphate buffer, pH 7.0, to avoid constant pH adjustment. The lactone was extracted from the aqueous solution by continuous ether extraction for 4 days or until the lactone concentration in the aqueous layer was minimal. The product (25 mg., m.p. 163–164°) agreed in properties with those of β -carboxymuconolactone, described by Gross *et al.* (1956). All other organic chemicals were commercial samples generally recrystallized except when used for bulk growth media.

Estimations. Protocatechuic acid and β -oxoadipic acid were estimated manometrically, the former by O_2 uptake with the purified *Nocardia* oxidase (Cain & Cartwright, 1960), the latter by catalytic decarboxylation at 30°, pH 4.0, with 0.1M-aminoantipyrine (Sistrom & Stanier, 1953). Protocatechuic acid, β -carboxymuconic acid and β -carboxymuconolactone were estimated at 290, 270 and 225 $m\mu$ on a Unicam SP. 500 spectrophotometer. At these wavelengths and pH 7.0, the molar extinctions are: protocatechuic acid: $\epsilon_{270\ m\mu}$ 2730, $\epsilon_{290\ m\mu}$ 3890; β -carboxymuconic acid: $\epsilon_{225\ m\mu}$ 9400, $\epsilon_{270\ m\mu}$ 6400, $\epsilon_{290\ m\mu}$ 1590; β -carboxymuconolactone: $\epsilon_{225\ m\mu}$ 8900; $\epsilon_{270\ m\mu}$ 570. Muconolactone and the dilactone were estimated colorimetrically as their hydroxamic acid derivatives. The dilactone forms a hydroxamate under the acid conditions (pH 6.4) described by Lipmann & Tuttle (1945). At this pH, muconolactone fails to react. Both lactones, however, form hydroxamates under the alkaline conditions (pH 9.0) of Hestrin's (1949) method, so that either can be determined in the presence of the other. The final procedure adopted was as follows.

(a) Acid hydroxamate (dilactone). To 1 ml. of the test solution was added 1 ml. of hydroxylamine solution (equal volumes of 4M-hydroxylamine hydrochloride and 3.5M-NaOH) and 1 ml. of acetate buffer (0.05M, pH 5.4), and the solution kept for 10 min. Then 1 ml. of HCl (conc. acid diluted 1 in 3), 1 ml. of trichloroacetic acid (10%, w/v) and 1 ml. of $FeCl_3 \cdot 6H_2O$ (5.0% in 0.1N-HCl) were added successively and the red-purple colour was read at 500 $m\mu$, after 10 min., on a Unicam SP. 600 spectrophotometer.

(b) Alkaline hydroxamate (muconolactone). The procedure was identical, but the hydroxylamine solution contained equal volumes of 2M-hydroxylamine hydrochloride and 3.5M-NaOH, water replaced the acetate buffer, and the HCl was made by diluting the conc. acid 1 in 4. Precipitated protein was removed by centrifuging at 3000 *g* for 5 min. in both methods. Standards were prepared with synthetic specimens of both dilactone and muconolactone; estimations on known amounts under normal experimental conditions have given excellent agreement.

Protein was estimated by the biuret method of Gornall, Bardawill & David (1949) with bovine albumin (Fraction V, Armour Laboratories) as a standard, or by the phenol method of Lowry, Rosebrough, Farr & Randall (1951).

Buffers. Incubations were carried out either in Sørensen Na_2HPO_4 - KH_2PO_4 buffer or in 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer. The molarity and pH used are indicated in the text.

Enzymic assays. In the nomenclature of these enzymes, the system employed by Sistrom & Stanier (1954) has been used: 'lactonizing enzyme' describes the system synthesizing a lactone and 'delactonizing enzyme', one which degrades it. Protocatechuic acid oxidase and the lactonizing enzyme of *Vibrio* O1 were assayed spectrophotometrically by disappearance of their respective substrates. The assay system contained: substrate, 0.5 μ mole; tris-HCl buffer, 100 μ moles, pH 7.4; up to 0.2 mg. of enzyme protein; water to a total volume of 3 ml. Cuvettes of light-path 10 mm. were used with a constant-temperature cell carrier at 30°. The delactonizing enzyme was assayed either by disappearance of substrate (muconolactone) at 30°, pH 7.4, or because of the smaller quantities of substrate required for an equally accurate assay, by manometric estimation of the β -oxoadipic acid produced. Both methods gave similar results in trial runs. The reaction mixture in Warburg flasks for manometry contained: (+)-muconolactone, 10 μ moles; tris-HCl buffer, 100 μ moles, pH 7.4; about 3–4 mg. of enzyme protein; water to a total volume of 3.0 ml. At given times (after incubation for 15, 20 or 30 min. at 30°) the reaction was stopped by the addition of 0.1 ml. of acetic acid, and the β -oxoadipic acid formed was estimated by decarboxylation. A linear relationship between time and β -oxoadipic acid produced was established.

Chromatography. All solvent proportions are by volume. The aliphatic acids were run in solvent A [ethanol-aq. NH_3 soln. (sp.gr. 0.88)-water (80:4:16)] and were detected by spraying with ethanolic bromothymol blue (0.05%, w/v) made just alkaline with dil. NH_3 soln. This solvent gave very satisfactory separation of *cis-cis*-muconic acid and β -carboxymuconic acids from each other and from their respective lactones, which, because of their free carboxyl groups, also react as acids. Muconolactone and the dilactone were run as their hydroxamates in solvent B [pyridine-acetic acid-water (50:35:15)] or, as the unchanged lactone, in both solvent C [pyridine-ethyl acetate-water (1:1:1)] and solvent D [diethyl ether-benzene-formic acid (98%, w/v)-water (70:30:14:10)]. When run as the hydroxamates, the lactones were detected with $FeCl_3$ solution (5% in 0.1N-HCl diluted 1 in 4 with ethanol) and showed up as reddish-purple spots. More intensely coloured and clearly defined spots were obtained by forming the hydroxamates on the paper after running as the unchanged lactone. The procedure adopted employed the reagents for alkaline hydroxamate estimation (except the water) applied in the described order (after diluting each reagent 1 in 4 with ethanol) by means of a fine spray to avoid over-wetting the paper with resulting streaking of the spots. It was essential, after forming the hydroxamate on the paper under alkaline conditions, to spray with sufficient ethanolic HCl to render the chromatogram completely acidic. Without this precaution, the lactone spots were disguised by $Fe(OH)_3$ formation when ethanolic $FeCl_3$ was applied. Dinitrophenylhydrazones were run in solvent E [2-methylbutan-2-ol-propan-1-ol-aq. NH_3 solution (sp.gr. 0.88) (65:5:30)], and the chromatograms examined under ultraviolet light or sprayed with 5% (w/v) Na_2CO_3 . All chromatograms were run by the descending method on Whatman no. 542 paper.

Manometric procedures. Uptake of O_2 and evolution of CO_2 were followed by the conventional manometric procedures, (Umbreit, Burris & Stauffer, 1949). Evolution of

CO₂ was often measured under anaerobic conditions, in which case the flask and manometer were gassed with O₂-free N₂ for 15 min. before temperature equilibration.

RESULTS

Conversion of protocatechuic acid into β -oxoadipic acid

p-Hydroxybenzoic acid is a well-known inducer of protocatechuic acid oxidase in many bacteria (Stanier & Ingraham, 1954; Durham, 1956; Cartwright & Cain, 1959), but the nature of the degradation products of protocatechuate has not previously been investigated in this vibrio, although it has been used in many experiments with aromatic compounds. Crude extracts of 24 hr.-old cells grown on *p*-hydroxybenzoic acid rapidly oxidized protocatechuic acid, but not catechol, with the theoretical O₂ uptake and production of a material giving a strong Rothera-positive reaction (Table 1). This material was isolated from a larger-scale experiment and identified by melting point (117–118°) and mixed melting point (119°) with authentic β -oxoadipic acid; the identity was confirmed by co-chromatography as the dinitrophenylhydrazone in solvent *E*.

Oxidation of protocatechuic acid to β -carboxymuconic acid

McDonald, Stanier & Ingraham (1954) prepared from a *Pseudomonas* sp. a protocatechuic acid oxidase which catalysed the conversion of protocatechuic acid into β -carboxymuconic acid. This latter compound has since been found as the primary ring-opening product in protocatechuic acid oxidation by *Neurospora crassa* (Gross *et al.* 1956), *Nocardia erythropolis* (Cain & Cartwright, 1960) and a pseudomonad isolated from phthalic acid enrichments (Ribbons & Evans, 1960). It was likely therefore that the vibrio also possessed

the enzyme, although no evidence of β -carboxymuconic acid accumulation was detectable by spectrophotometric analysis of the course of protocatechuic acid oxidation with crude extracts. With the following scheme, however, a protocatechuic acid oxidase, free from enzymes degrading β -carboxymuconic acid, was obtained and was used to follow accumulation of this product.

A Hughes-press extract of cells grown with *p*-hydroxybenzoic acid was taken up in 0.05 M-phosphate buffer and heat-treated for 16 min. at 55°. This removed considerable protein without appreciably affecting the activity of the oxidase. The coagulated protein was centrifuged off, and the supernatant solution dialysed for 24 hr. against 1 M-ethylenediaminetetra-acetate (EDTA) at pH 7.0 and then against two changes of distilled water for 48 hr., all at 4° in a cold room. The dialysed material was treated with neutral, saturated (NH₄)₂SO₄ solution; all the activity was precipitated in the 0–0.45 saturation range. This fraction was taken up in water, dialysed to remove all salts, brought to pH 5.5 with 0.05 M-acetate buffer and treated twice with 0.3 vol. of calcium phosphate gel for 15 min.

Table 1. *Oxidation of protocatechuic acid to β -oxoadipic acid by crude extracts*

Each Warburg flask contained: phosphate buffer, pH 7.0, 150 μ moles; substrate as shown; enzyme protein, 3.8 mg.; KOH, 0.2 ml. in centre well; water to a final volume of 3 ml. Temp., 30°; gas phase, air. At completion of O₂ uptake, the reaction was terminated by addition of 0.1 ml. of acetic acid, the KOH swabbed from centre well, and 0.4 ml. of 0.1 M-4-aminoantipyrine added to side arm. After temperature re-equilibration, this catalyst was tipped and β -oxoadipic acid estimated by the resulting evolution of CO₂. Results are corrected for gas changes in the absence of substrate.

Protocatechuic acid added (μ moles)	O ₂ uptake (μ moles)	β -Oxoadipic acid produced (μ moles)
2.0	1.8	1.7
3.0	2.8	2.7
5.0	4.9	4.7

Table 2. *Purification of protocatechuic acid oxidase from Vibrio O1*

The unit of activity is defined as that amount of enzyme causing an extinction decrement of 0.01/min. at 290 m μ under the following assay conditions: phosphate buffer, 200 μ moles, pH 7.0; protocatechuic acid, 0.5 μ mole; enzyme, 0.3 ml. Fe²⁺ ions (0.1 ml.), to give a final concentration of 0.33 mM, were incubated with the enzyme for 20 min. before addition of substrate. Supplementation with Fe²⁺ ions was later found unnecessary.

Material	Activity (units/ml.)	Protein (mg./ml.)	Specific activity (units/mg. of protein)
Crude extract	27.0	10.80	2.5
Heat-treated and dialysed extract	26.0	7.50	3.5
(NH ₄) ₂ SO ₄ fraction, 0–45 % saturation	18.5	2.20	8.4
1st gel eluate, pH 7.8, 0.1 M-buffer	5.5	0.28	19.5
2nd gel eluate:			
(i) pH 6.4, 0.1 M-buffer	0.06	0.05	1.2
(ii) pH 7.2, 0.1 M-buffer	4.1	0.17	24.0*

* This fraction is devoid of lactonizing activity.

with gentle agitation. All the oxidase was absorbed on the gel, which was washed with water. The oxidase could be eluted with 0.1 M-phosphate buffer at pH 7.8, but spectrophotometric analysis showed that lactonizing enzyme was still present. The eluate was therefore dialysed, brought to pH 5.5 and reabsorbed on more gel. The gel was washed with water and eluted with 0.05 M-phosphate buffer, pH 6.4; the eluate, containing inactive protein, was discarded and the gel further eluted with 0.1 M-buffer at pH 6.4, again removing enzyme of low activity. The oxidase

was finally eluted with 0.1 M-phosphate buffer at pH 7.2 or higher. No attempt was made to obtain a high specific activity or maintain a large proportion of the total enzyme units. Table 2 shows that the overall purification is small but that the final product is free of the enzymes which degrade β -carboxymuconic acid and could be used to accumulate this compound from protocatechuic acid.

Fig. 1. Oxidation of protocatechuic acid by the partially purified oxidase. Cuvettes contained: phosphate buffer, 200 μ moles, pH 7.0; protocatechuic acid, 0.3 μ mole; enzyme protein, 0.085 mg. (0.5 ml.); total volume, 3 ml. Temp., 30°. ●, Protocatechuic acid; ○, β -carboxymuconic acid.

Fig. 2. Spectra of the oxidation product of protocatechuic acid formed by the partially purified oxidase, and that of β -carboxymuconic acid before, and after, heating for 30 min. at 100°. —, Oxidation product before (A) and after (B) heating; ---, authentic β -carboxymuconic acid before (C) and after (D) heating.

Fig. 1 shows the course of the oxidation followed spectrophotometrically and Fig. 2 the spectrum of the reaction product. This spectrum is identical with that of authentic β -carboxymuconic acid in all respects including the shift in position and increase in extinction of the absorption peak on boiling for 30 min. Manometric experiments showed that for every mole of protocatechuate oxidized, 1 mole of O_2 was consumed, no CO_2 was evolved and 1 mole of β -carboxymuconic acid, estimated spectrophotometrically, was produced by the purified oxidase; the reaction was essentially irreversible. Chromatography of the reaction mixtures at the completion of such oxidations showed a compound which moved an identical distance to authentic β -carboxymuconic acid and failed to separate from mixtures with it in solvent A.

Lactone production by heat-treated extracts of the vibrio

The enzymic conversion of protocatechuic acid into a compound reacting as a lactone was first observed during a class experiment designed by Dr S. Dagley to demonstrate accumulation of β -oxoadipic acid. A crude bacterial extract, which had been stored with intermittent freezing and thawing, catalysed the oxidation of protocatechuic acid, but β -oxoadipate was not produced; a lactone accumulated instead. The reason for this was not clear, and, accordingly, extracts showing no such lactonizing activity, freshly prepared from adapted cells, were treated in various ways by the author to reproduce similar behaviour. The most significant results were those obtained by heat-treatment of extracts for various periods. When extracts were maintained at 55° for 15 min., the delactonizing enzymes were completely destroyed, whereas those catalysing formation of the lactone retained their activity (Fig. 3). The lactone which accumulated in such incubations fulfils the requirement of a true intermediate since addition of crude extract led to its rapid disappearance (Fig. 4). The lactone formed in these incubations gave a spectrum similar to that of authentic muconolactone (Fig. 5, ϵ_{max} at 207 $m\mu$) and ran as muconolactone in chromatograms with solvents C and D. No evidence for the anticipated carboxymuconolactone (ϵ_{max} at 216 $m\mu$) was found, nor did the vibrio produce any lactone forming a hydroxamate under acid conditions (e.g. the dilactone).

Fig. 3. Lactone accumulation as a result of heat-treating crude extracts of the vibrio. The incubations were carried out in boiling tubes vigorously shaken at 270 strokes/min., 5 cm. throw, aerobically. Tubes contained: phosphate buffer, 1 m-mole, pH 7.0; sodium protococatechuate, 100 μ moles; protein extract, 12.8 mg.; total volume, 12.0 ml. Temp., 30°. Control tubes contained (i) enzyme boiled for 10 min. and (ii) no substrate. Lactone estimations were made at 30-min. intervals for 2 hr. The following treatments were given to fresh crude extracts: ●, 50°, 25 min.; ×, 55°, 5 min.; ○, 55°, 15 min.; △, untreated and 50°, 15 min.; ▲, boiled enzyme or with no substrate.

Fig. 4. Intermediate nature of the lactone synthesized by heat-treated extracts. Three identical tubes were set up with incubation mixtures as in Fig. 3. Control tube contained boiled enzyme. Lactone synthesis was followed for 2 hr. then all three tubes were plunged in a boiling-water bath to stop the reaction. After re-equilibration to 30°, to one expt. tube was added 0.5 ml. of crude extract; to the other, 0.5 ml. of boiled crude extract, and the disappearance of lactone followed at 30-min. intervals. ●, Synthesis of lactone by heat-treated extracts (mean of 2 tubes); ○, by boiled extract. △, Disappearance of accumulated lactone by the action of crude extract; ▲, by action of boiled crude extract.

Fig. 5. Extinction curves for (±)-muconolactone (A), the synthetic dilactone (B) and β -carboxymuconolactone (C). All the lactones were measured as freshly prepared aqueous solutions.

Isolation and identification of the lactone

The above results with the heat-treated extracts depended upon the fortunate circumstance that both protococatechuic acid oxidase and the lactonizing enzyme were heat-stable. To conserve limited supplies of the natural substrate, β -carboxymuconic acid, protococatechuic acid was used as starting material in the preliminary experiments and in the isolation procedures. Because the oxidase requires molecular O_2 as reactant, incubations were vigorously aerated in order that the first stage in the sequence: protococatechuate $\rightarrow$ β -carboxymuconate $\rightarrow$ lactone, should not be rate-limiting.

A 1-l., three-necked flask was fitted with an electric stirrer and a narrow-bore tube, through which a rapid air-stream was blown by a pump. The flask, containing 10 m-moles of sodium protococatechuate in 500 ml. of 0.1M-phosphate buffer, pH 7.2, was incubated in a constant-temperature water bath at 30°. A portion (100 ml.) of heat-treated extract (about 1 g. of protein) was added in two separate lots, the first at zero time, the second about 3 or 4 hr. later. The solution was kept saturated with air by agitation with the stirrer and the air stream. Frothing was minimized by the addition of 0.1 ml. of a 0.05% (w/v) solution of Silicone M.S. antifoam emulsion 'B' (Hopkin and Williams Ltd.), at about hourly intervals. A total of 0.7 ml. was added during the course of the experiment. Lactone synthesis was followed on 1 ml. samples throughout the course of the reaction, and, at completion, about 8 hr., the flask contents were brought to pH 2.5 with dil. HCl. The precipitated protein was removed by centrifuging and the supernatant solution, in two portions, extracted twice with 100 ml. of pure ether to remove any residual protococatechuic acid. In the first attempts, the aqueous solution was freeze-dried to about 60 ml., but in later

preparations the volume was decreased by evaporation under reduced pressure with the water-bath temperature not exceeding 40°. The lactone solution was then continuously extracted with pure ether for 48–60 hr., or until the hydroxamate test on the aqueous solution was minimal. Evaporation of the ether extract *in vacuo* left a pale-yellow syrup, which was difficult to crystallize. Crystallization was finally achieved by leaving the syrup in a refrigerator for some hours and scratching the side of the vessel with a glass rod. Subsequent crystallizations were more readily effected by seeding with a crystal of the isolated material. The product (250 mg.) was recrystallized twice from ethyl acetate-benzene as a white crystalline powder, m.p. 74–75°. [Sistrom & Stanier (1954) gave 76–77.5° for the (+)-isomer of muconolactone.] Further less-pure material was obtained from the residual mother liquors. The sample of synthetic (racemic) muconolactone from Professor Evans melted at 109°, and a sample of the (–)-isomer, prepared from it, at 79°. An equimolar mixture of the (–)-isomer and the biologically prepared lactone melted at 108° and did not depress the m.p. of synthetic muconolactone on admixture. The neutralization equivalent of the isolated lactone determined with an automatic titrator (Radiometer, Copenhagen, Denmark) was 142; $C_8H_6O_4$ (monobasic) requires 142. [Found: C, 50.5; H, 4.1; O (direct), 44.7. Calc. for $C_8H_6O_4$: C, 50.7; H, 4.2; O, 45.1%]. The isolated lactone was optically active and gave a value $[\alpha]_D^{20} + 48.5 \pm 2^\circ$ (mean of 10 separate determinations) in water (c, 4.36). The unnatural (–)-isomer gave $[\alpha]_D^{20} - 50.5 \pm 2^\circ$ in water (c, 4.40). These values are essentially the same as those recorded for muconolactone by Sistrom & Stanier (1954). The isolated lactone ran with synthetic muconolactone in solvents *C* and *D* when chromatographed; equal concentrations of natural and synthetic lactone gave equal colour intensities in the hydroxamate test and synthetic, unnatural and isolated lactones all had identical u.v. spectra ($\epsilon_{\max}$, at 207 m μ). There is little doubt therefore that the isolated lactone is (+)-muconolactone.

*Conversion of protocatechuic acid and
β-carboxymuconic acid into muconolactone*

The degradation of either protocatechuic acid or β-carboxymuconic acid to (+)-muconolactone involves the loss of one carbon atom. The identification of (+)-muconolactone as an intermediate in protocatechuic degradation was thus surprising, since it had previously been found only as a product of catechol metabolism (Evans, Smith, Linstead & Elvidge, 1951; Sistrom & Stanier, 1954) and it was not attacked by extracts of either fully induced *Neurospora crassa* or *Pseudomonas* (Gross *et al.* 1956). The possibility remained that the lactone produced by the vibrio was indeed β-carboxymuconolactone and that decarboxylation occurred during isolation. The following evidence indicated that this was unlikely.

First, the product of the action of heat-treated extracts upon protocatechuic acid or β-carboxymuconic acid, without any manipulations, had the same spectrum as muconolactone and ran as this compound on chromatograms. β-Carboxymuconolactone is readily distinguished by both these methods. Secondly, the results of manometric experiments showed that the conversion of either protocatechuic or β-carboxymuconic acid into (+)-muconolactone by heat-treated extracts involved the evolution of CO₂ which accounted for the carbon atom which was lost. The loss of CO₂ is thus enzymic and not the result of isolation procedure. Table 3 and Fig. 6 show the metabolism of these related substrates by heated extracts of induced cells to (+)-muconolactone, and their

Table 3. *Conversion of protocatechuic acid, β-carboxymuconic acid and β-carboxymuconolactone into (+)-muconolactone and β-oxoadipic acid*

Each Warburg flask contained: phosphate buffer, 150 μmoles, pH 7.0; substrates as shown; 2 N-H₂SO₄ in side arm, or 20% KOH in centre well, 0.2 ml.; enzyme, 0.2–0.5 ml. (1.37 mg. of protein in Expt. 1; 2.5 mg. of protein in Expt. 2; 3.4 mg. of protein in Expt. 3; 3.0 mg. of protein in Expts. 4 and 5); water to a total volume of 3.0 ml. Temp., 30°. Gas phase was air when protocatechuic acid was substrate; in all other cases, incubation was performed under O₂-free N₂. (+)-Muconolactone was determined from samples of the flask contents at completion of experiments, i.e. when no further change in gas volumes occurred. Values are given as μmoles.

Expt. no.	Enzyme	Substrate	Substrate used	O ₂ uptake	CO ₂ evolved	β-Oxo-adipate	(+)-Muconolactone
1	Heat-treated	β-Carboxymuconic acid	2.9	—	2.9	0	2.3
		β-Carboxymuconic acid	3.6	—	3.2	0.2	2.3
		Protocatechuic acid	3.0	2.9	3.0	0.1	2.7
2	Crude	β-Carboxymuconic acid	4.8	—	4.6	4.7	0
		β-Carboxymuconic acid	8.0	—	7.5	7.4	0
		β-Carboxymuconic acid	10.3	—	10.2	9.6	0
3	Heat-treated	β-Carboxymuconic acid	4.8	—	4.6	0.3	4.0
		β-Carboxymuconic acid	8.0	—	7.7	0.6	7.4
		Protocatechuic acid	5.0	4.9	4.9	0	4.3
		Protocatechuic acid	8.0	7.8	8.0	—	7.4
4	Heat-treated	β-Carboxymuconolactone	3.0	—	0.7	0	—
		β-Carboxymuconolactone	5.0	—	0.3	0	—
		β-Carboxymuconolactone	8.0	—	0.2	0	—
5	Crude	β-Carboxymuconolactone	3.0	—	0.1	0	—
		β-Carboxymuconolactone	5.0	—	0.3	—	—

conversion into β -oxoadipic acid if the heat-treatment is omitted. Thirdly, the absence of any activity towards biologically prepared β -carboxymuconolactone by heat-treated or crude extracts of the vibrio grown on *p*-hydroxybenzoate (Fig. 7)

Fig. 6. Synthesis of (+)-muconolactone from some precursors by heat-treated extracts. The reaction mixtures contained: phosphate buffer, 800 μ moles, pH 7.0; substrate, 75 μ moles; enzyme, 1.0 ml. (2.65 mg. of protein); total vol., 10 ml. Temp., 30°. Incubations were performed under conditions identical with those described in Table 4. Control tubes contained boiled enzyme or no substrate. Samples were estimated for lactone at 15 and 30 min., and then at 30-min. intervals. ●, Protocatechuic acid; ○, β -carboxymuconic acid as substrates.

Fig. 7. Synthesis of β -carboxymuconolactone by the resolved lactonizing enzyme of *Neurospora crassa* and the lack of activity towards this substrate shown by *Vibrio* extracts. Cuvettes originally contained: tris-HCl buffer, 100 μ moles, pH 7.4; β -carboxymuconic acid, 0.4 μ mole; resolved *Neurospora* lactonizing enzyme, 0.05 ml. Synthesis of β -carboxymuconolactone and disappearance of the acid were followed by extinction measurements at 225 and 270 $m\mu$. At the completion of this reaction, 0.02 ml. (0.15 mg. of protein) of crude *Vibrio* extract was added ($\downarrow$), and the extinction at 225 $m\mu$ followed for a further 1 hr. At this time, 0.05 ml. (0.15 mg. of protein) of crude *Neurospora* extract (containing the specific delactonizing enzyme) was added ($\downarrow\downarrow$), and disappearance of the accumulated lactone followed. ●, β -Carboxymuconic acid; ○, β -carboxymuconolactone. Temp., 25°.

confirmed the belief that this lactone was not an intermediate in protocatechuic acid degradation by this organism. Manometric experiments confirmed the absence of significant CO_2 evolution from β -carboxymuconolactone by heat-treated extracts, and also the failure of crude extracts to form β -oxoadipic acid from this substrate (Table 3).

After it was established that CO_2 was lost in conversion of β -carboxymuconic acid into (+)-muconolactone and that β -carboxymuconolactone was not an intermediate, the six-carbon analogue *cis-cis*-muconic acid was investigated as a likely intermediate. It was considered possible that active β -carboxymuconic acid was decarboxylated first and the resulting *cis-cis*-muconic acid lactonized to (+)-muconolactone; [Gross *et al.* (1956) and McDonald *et al.* (1954) discuss the stereo-configuration of these compounds.] Crude extracts, however, did not attack *cis-cis*-muconic acid nor did heat-treated extracts synthesize any lactone from this substrate.

Lactonizing activity in unadapted cells

Protocatechuic acid oxidase is a typical adaptive enzyme formed only in response to its specific substrate or a metabolic precursor (Sleeper & Stanier, 1950; Stanier, 1950; Gross *et al.* 1956; Cartwright & Cain, 1959; Ribbons & Evans, 1960). Only in the 'aromaticless mutant' Y7655a of *Neurospora crassa* is the enzyme constitutive, and even here it might be more accurately described as auto-induced. In the vibrio, there was no attack on protocatechuic acid by extracts of cells grown with succinate as the carbon source or in peptone media, as measured by β -oxoadipate production from the substrate. Extracts from cultures of unadapted cells inoculated from slopes containing aromatic substrates often give some activity towards protocatechuic acid (0.5–2.0% of that of fully induced cultures), but, in the present experiments, large-scale cultures of unadapted cells were inoculated from a nutrient-agar slope derived by serial subculture from the original N.C.I.B. dried culture; the cells produced had thus never been in contact with an aromatic substrate. Under these conditions, there was complete absence of protocatechuic acid oxidase and with protocatechuic acid as substrate, no lactone was formed by heat-treated non-induced extracts.

Gross *et al.* (1956) detected lactonizing and delactonizing enzymes of *Neurospora crassa* in non-induced cultures of the mould. The lactonizing enzyme of *Vibrio* O1 was also present in extracts from cells grown with succinic acid but the amount of enzyme was radically reduced. By using large amounts of extract, however, with longer incubation times, measurable amounts of lactone were produced (Table 4).

Table 4. *Lactonizing activity in induced and non-induced cells*

Induced cells were grown with *p*-hydroxybenzoic acid as carbon source from inocula grown on the same medium. Non-induced cells were grown with either succinic acid or peptone as carbon source from inocula derived from the original N.C.I.B. dried culture by serial subculture on nutrient-agar slopes; such cells had never been in contact with the aromatic carbon source. Extracts of both types of cells were made with a Hughes press, and the extracts heat-treated. Incubation mixtures (in 6 in. boiling tubes) contained: phosphate buffer, 700 μ -moles, pH 7.0; β -carboxymuconic acid (active isomer) or protocatechuic acid, 50 μ -moles; heat-treated extract, 1.0 ml. (16.4 mg. of protein for succinate-grown cells; 15.5 mg. of protein for peptone-grown cells, 2.7 mg. of protein for *p*-hydroxybenzoate-grown cells); total volume, 10 ml. Control tubes contained boiled enzyme. The boiling tubes were incubated in a water bath at 30°, and were vigorously shaken on a reciprocating device at 280 strokes/min. with a 5 cm. throw. Samples were estimated for lactone every 15 min. for induced extracts, every hour for non-induced extracts and the rate of synthesis was calculated from the linear response found.

Carbon source for cells	Substrate	(+)-Muconolactone formed (μ moles/hr./mg. of protein)
Succinic acid	Protocatechuic acid	0
Succinic acid	β -Carboxymuconic acid	0.04
Peptone	β -Carboxymuconic acid	0.05
<i>p</i> -Hydroxybenzoic acid	Protocatechuic acid	1.00
<i>p</i> -Hydroxybenzoic acid	β -Carboxymuconic acid	3.25

Table 5. *Stimulation of lactonizing activity in heat-treated extracts by some bivalent metal ions*

Lactonizing enzyme was assayed by decrease in extinction at 260 $m\mu$ due to disappearance of β -carboxymuconic acid (see Experimental section for details). Cuvettes contained 0.06 mg. of enzyme protein. Metal ions were added to a final concentration of 1 mM in form of the chloride or sulphate. The decrement in extinction was followed for 5 min. and calculated per min. over the initial part of the curve.

Salt added	ΔE /min.	Stimulation (+) or inhibition (-) (% of control)
None	0.072	
CoCl ₂	0.060	-17
NiCl ₂	0.044	-39
MnSO ₄	0.120	+67
ZnSO ₄	0.030	-54
MgSO ₄	0.150	+108
CaCl ₂	0.120	+67
FeSO ₄ *	0.130	+82
HgCl ₂	0	-100
{None†	0.110	-45 }
{FeCl ₃	0.060	

* Fe(OH)₃ precipitation occurred at pH 7.4; this may be an unreliable result.

† Separate experiment with fresh enzyme.

Table 6. *Effect of some metal-chelating agents on the lactonizing activity of heat-treated extracts*

Assay and other conditions were as for Table 5. Inhibitors, and metal ions, were added to a concn. of 1 mM.

Additions	ΔE /min.	Stimulation (+) or inhibition (-) (% of control)
NaN ₃	0.046	-58
1:10-Phenanthroline*	0.080	-27
EDTA†	0.124	+13
EDTA*	0.076	-31
EDTA* + MnSO ₄	0.193	+75
None	0.110	

* Incubated with enzyme for 20 min. before addition of substrate.

† Added after substrate.

Some properties of the lactonizing enzyme

Experiments with both the *cis-cis*-muconic acid-lactonizing enzyme of *Pseudomonas* (Sistrom & Stanier, 1954) and the 'decarboxylase complex' of *Nocardia* (Cain & Cartwright, 1960) demonstrated a metal requirement; for *Nocardia* this requirement was believed to be associated with a lactonizing reaction, for which tentative evidence was produced. In the present study, a similar metal requirement has been shown in the vibrio to be associated with the lactonizing enzyme converting β -carboxymuconic acid into (+)-muconolactone. Table 5 demonstrates the stimulation of the activity of heat-treated extracts by some bivalent metal ions and inhibition by heavy-metal ions (Ni²⁺, Co²⁺, Zn²⁺ and Hg²⁺). The metal requirement was emphasized by the complete reversal of EDTA inhibition by Mn²⁺ ions (Table 6). The lactonizing activity was also associated with sulphhydryl groups as indicated by the inhibition due to *p*-chloromercuribenzoic acid and sodium arsenate and the partial reversal of the former with cysteine. Cysteine and cysteine + Mn²⁺ ions markedly stimulated the enzyme (Table 7); hydroxylamine was strongly inhibitory. The lactonizing enzyme had a pH optimum at 8.0; phosphate buffer appeared inhibitory (Fig. 8). The enzymic activity in these experiments was determined by following the disappearance of substrate. In all cases, except the determination of a pH optimum, such results have been checked by measurement of lactone production in confirmatory experiments; this ensured that the properties described were indeed associated with the lactonizing stage.

The lactonizing enzyme of the vibrio in impure extracts, prepared with the Hughes press, showed remarkable heat-stability; only at temperatures

above 70° did the activity decrease sharply. Protocatechuic acid oxidase had a similar though less-pronounced property, its activity falling off rapidly above 55° (Fig. 9).

Equilibrium between β -carboxymuconic acid and (+)-muconolactone

At pH 7.0, the conversion of β -carboxymuconic acid into the (+)-lactone was essentially irreversible

Table 7. *Effect of some inhibitors and sulphhydryl reagents on the lactonizing activity in heat-treated Vibrio extracts*

Conditions were as for Tables 5 and 6. Expt. 1, 0.06 mg. of enzyme protein; Expt. 2, 0.08 mg. of protein; Expt. 3, 0.4 mg. of protein, dialysed for 24 hr. against several changes of distilled water. Each experiment was performed with fresh extract.

Expt. no.	Inhibitor	Concn. (mm)	$\Delta E/\text{min.}$	Stimulation (+) or inhibition (-) (% of control)
1	None		0.110	
	NaF	5	0.056	-49
	Hydroxylamine	1	0.020	-82
	<i>p</i> -Chloromercuribenzoic acid	0.1	0	-100
	<i>p</i> -Chloromercuribenzoic acid	0.01	0.005	-95
	<i>p</i> -Chloromercuribenzoic acid	0.01		
	+ cysteine	2	0.053	-52
	Na_3AsO_4	1	0.039	-65
			0.136	
2	None		0.061	-55
	KAsO_2	1	0.176	+29
	Cysteine	10	0.114	-9
	Sodium thioglycollate	1	0.480	
3	None		0.530	+12
	Cysteine	1		
	Cysteine	1	0.750	+56
	+ MnSO_4	1

Fig. 8. pH optimum for the lactonizing enzyme in heat-treated extracts. Conditions of assay were as described in Table 5; 0.06 mg. of enzyme protein was used per cuvette. x, Phosphate buffer; o, tris-HCl buffer; ●, KCl-borate buffer. The pH was determined at the end of the reaction with a glass electrode.

Fig. 9. Heat-stability of protocatechuic acid oxidase and the lactonizing enzyme in *Vibrio* extracts. The extracts were rapidly heated in tubes to the given temperature in a water bath, held at that temperature for 5 min., and quickly cooled by placing them in melting ice where they were held till assayed. The assay mixture per cuvette contained: tris-HCl buffer, 200 μ moles, pH 7.4; substrate, 0.5 μ mole; cell extract, 0.1 ml. (1.5 mg. of protein). Assay temp., 30°. The disappearance of substrate/min. was calculated from the initial portion of the response-time curve for each batch of enzyme. ●, Lactonizing enzyme; ▲, protocatechuic acid oxidase.

in favour of lactone synthesis. This was confirmed spectrophotometrically, for the amounts of acid in mixtures of acid and lactone can be calculated from extinction measurements at 260 $m\mu$ where absorption due to the lactone is negligible. The ratio of acid to lactone at equilibrium is markedly affected by pH, since H^+ is a reactant:

The ratios of lactone: acid at equilibrium decreased rapidly with increasing pH, probably as a result of changes in the reacting species (Table 8).

Delactonizing activity of Vibrio O1

The delactonizing enzyme occurred at its highest levels in cells entering the stationary phase, and was strictly adaptive; no activity was found in bacteria grown with succinate as carbon source. This enzyme catalysed the essentially irreversible hydrolysis of (+)-muconolactone to β -oxoadipic

acid. The synthetic lactone was only half-utilized, the (–)-isomer was not attacked, nor did it inhibit utilization of the (+)-isomer at equimolar concentrations (Table 9). The enzyme did not attack the dilactone. The delactonizing enzyme in crude extracts was much less heat-stable than protocatechuic acid oxidase or the lactonizing enzyme; it lost 85 % of its activity after 5 min. at 55°, and was completely inactivated after 15 min. at this temperature (Fig. 10). The delactonizing activity of crude extracts was slightly stimulated by several metal ions, Ca^{2+} ions giving the best stimulation, 20 %. Inhibition of delactonizing activity by 30–40 % has consistently been found with added 10 mM-EDTA, such inhibition being reversed by Ca^{2+} ions (Table 10). Dialysis for up to 3 days against several changes of distilled water did not reduce activity nor did dialysis against 1 mM-EDTA for 24 hr. The marked inhibition by *p*-

Table 8. *Effect of pH on the equilibrium between β -carboxymuconic acid and (+)-muconolactone*

Cuvettes contained: buffer, 100 μ moles; β -carboxymuconic acid, 0.5 μ mole; enzyme (heat-treated extract), 0.1 mg. of protein; water to a final volume of 3 ml. Extinction decrease at each pH was followed at 260 $m\mu$ until it attained a steady value from which the amount of acid remaining was calculated. (+)-Muconolactone does not absorb at this wavelength. Allowance was made in the calculations for the 7 % inactive isomer in the purest sample of β -carboxymuconic acid available.

Buffer	pH	Acid remaining (%)	Ratio: (+)-lactone acid at equilibrium
Phosphate	6.0	0	.
Phosphate	7.0	0.1	99.9
Tris	7.4	2.9	33.5
Tris	8.0	12.7	6.9
Tris	8.4	13.8	6.2
Tris	9.0	14.7	5.8

Fig. 10. Heat-stability of the delactonizing enzyme. The heat-treatment of the enzyme extracts was identical with that in Fig. 9. The assay mixture for the delactonizing activity is described in the Experimental section; flasks contained 2.25 mg. of protein. Incubation time, 20 min.; temp., 30°.

Table 9. *Conversion of the isomeric muconolactones and dilactone into β -oxoadipic acid*

Extract was derived from 48 hr.-old cells. Each Warburg flask contained: phosphate buffer, 200 μ moles, pH 7.0; substrate, 3, 5 or 6 μ moles; water to a final volume of 3 ml.; enzyme, 3.0 mg. of protein (as crude extract). Incubation time, 3 hr. β -Oxoadipic acid was determined by catalytic decarboxylation. Results are corrected for endogenous activity.

Substrate	Substrate added (μ moles)	β -Oxoadipic acid produced	
		(μ moles)	(μ moles/mole of substrate)
(+)-Muconolactone	3.0	3.30	1.10
(+)-Muconolactone	5.0	4.70	0.94
(+)-Muconolactone	6.0	5.70	0.95
(±)-Muconolactone	3.0	1.45	0.48
(±)-Muconolactone	5.0	2.55	0.51
(–)-Muconolactone	5.0	0	0
(+)-Muconolactone + (–)-muconolactone	5.0	4.90	0.98
Dilactone (synthetic)	5.0	0.30	0.06

Table 10. *Effect of some inhibitors on the delactonizing enzyme*

The assay system was that described in the Experimental section. Each flask contained 2.5 mg. of enzyme protein. After a preliminary experiment had established a linear disappearance of lactone with time, the incubation was carried out for 30 min., and the β -oxoadipic acid produced was determined by catalytic decarboxylation.

	Inhibitor	Concn. (mM)	β -Oxoadipic acid formed (μ moles/hr.)	Inhibition (%)
Expt. 1	None		10.8	
	EDTA	2.0	7.4	31
	NaN ₃	2.0	10.8	0
	NaF	2.0	11.4	0
	Na ₃ AsO ₄	1.0	11.0	0
	KAsO ₂	1.0	10.8	0
	<i>p</i> -Chloromercuribenzoic acid	0.1	7.0	36
	<i>p</i> -Chloromercuribenzoic acid + cysteine	0.01 2.0	9.8	9
	EDTA	2.0		
	+ CaCl ₂	1.0	10.6	2
Exp. 2	None		6.0	
	EDTA	10	3.4	43
	EDTA	10	5.4	10
	+ CaCl ₂	5		
	<i>p</i> -Chloromercuribenzoic acid	0.5	1.0	84

chloromercuribenzoate was again partly reversed by cysteine. The pH optimum was about 8.4.

A partial purification of the enzyme was effected as follows. The fresh, crude, Hughes-press extract was fractionated with saturated (NH₄)₂SO₄ and the 0.45 saturated fraction collected. This fraction was taken up in water and dialysed. It was then treated with 0.05 vol. of 1.0M-MnCl₂, and the precipitate discarded. The supernatant was then re-fractionated with (NH₄)₂SO₄ and the fraction precipitating between 0.4 and complete saturation collected. After dialysis to remove (NH₄)₂SO₄, this fraction was used as source of delactonizing enzyme. This procedure effected a fourfold purification based on specific activity.

A plot of increasing substrate concentrations (*S*) against the reaction velocity (*V*), with the partially purified enzyme, gave a *K_m* value of 6.2 mM. A plot of *S/V* against *V* and calculating *K_m* from both intercepts (Lineweaver & Burk, 1934) gave a mean *K_m* of 10.2 mM with a *V_{max}* of 30 μ moles/hr. Both these values of *K_m* are much higher than the figure of 78 μ M given for the *Pseudomonas* system by Sistro & Stanier (1954). Inhibition of the enzyme occurred at substrate concentrations above 30 mM. The presence of Ca²⁺ ions caused a pronounced stimulation (20–30%) of activity with the partially purified enzyme.

DISCUSSION

Pathways previously described for carbocyclic ring-fission by micro-organisms have involved either the protocatechuic acid or the catechol

sequences (Stanier, 1948; Stanier, Sleeper, Tsuchida & McDonald, 1950; Sistro & Stanier, 1953; Evans *et al.* 1951). These pathways are quite distinct up to the stage of the product, β -oxoadipic acid, from whence they enter terminal oxidation mechanisms (Kilby, 1951; Katagiri & Hayaishi, 1957; Ottey & Tatum, 1957). Gross *et al.* (1956) have shown that in *Neurospora* β -carboxymuconic acid is converted into the corresponding β -carboxymuconolactone. This mould did not attack muconolactone; direct decarboxylation of β -carboxymuconolactone does not therefore occur in this organism. On the other hand neither extracts of *Pseudomonas* grown with *p*-hydroxybenzoic acid nor those from *Nocardia* grown with *p*-nitrobenzoic acid (both of which are simultaneously adapted to metabolize protocatechuic acid) attack β -carboxymuconolactone (Gross *et al.* 1956; Cain & Cartwright, 1960). This result and those obtained with [¹⁴C]-protocatechuic acid (Gross *et al.* 1956; Tatum & Gross, 1956) show that β -carboxymuconolactone is not involved in β -carboxymuconic acid degradation in these two bacteria. Results from the present study imply a similar situation in *Vibrio* O1. The vibrio differs from *Pseudomonas*, however, in that extracts of fully induced cells produce (+)-muconolactone and carbon dioxide from β -carboxymuconic acid and rapidly and completely degrade this lactone to β -oxoadipate. Decarboxylation occurs at the β -carboxymuconic acid level, for fully induced extracts do not lactonize, nor degrade further, *cis-cis*-muconic acid, which was the sole precursor of muconolactone previously known. In addition, whereas the delactonizing

enzyme of *Vibrio* O1 is strictly adaptive, both *Neurospora* and the pseudomonad which oxidizes phthalic acid, possess a delactonizing enzyme when grown with glucose as sole carbon source (Gross *et al.* 1956; Dr D. W. Ribbons, personal communication). The adaptive nature of this specific enzyme further emphasizes the true intermediary position of muconolactone on the protocatechuic acid-degradation pathway of *Vibrio* O1. In contrast with the strictly adaptive development of the delactonizing enzyme, the lactonizing enzyme of the vibrio is detectable in cells grown with succinic acid or in peptone media; nevertheless, growth in the presence of *p*-hydroxybenzoic acid causes a 75-fold increase in the enzyme.

The protocatechuic acid oxidases from *Vibrio* (this study), *Pseudomonas* (Stanier & Ingraham, 1954), *Nocardia erythropolis* (Cain & Cartwright, 1960) and the phthalate-oxidizing pseudomonad (Ribbons & Evans, 1960) are little affected by dialysis and show no requirement for Fe^{2+} ions. These contrast with the findings in *Neurospora crassa* (Ottey & Tatum, 1957) and in another *Pseudomonas* (Dagley & Patel, 1957), although the enzyme in the latter organism differs from all the others in that β -carboxymuconic acid is not the oxidation product. At the other end of the scale even crude extracts of some soil bacteria, recently examined in this laboratory, require supplementation with Fe^{2+} ions before protocatechuic acid was oxidized at an appreciable rate. If it is assumed that Fe^{2+} ions are involved in all protocatechuic acid oxidases (Mason, 1957), then in some bacteria the iron must be very tightly bound to the apoenzyme, whereas in others it is equally labile.

The properties of the lactonizing enzyme of *Vibrio* are very similar to those described for the analogous enzyme of *Pseudomonas* which uses *cis-cis*-muconic acid as substrate (Sistrom & Stanier, 1954) and to those recorded for the enzyme

complex degrading β -carboxymuconic acid in *Nocardia* (Cain & Cartwright, 1960). Each has a slightly alkaline pH optimum, each shows a requirement for a bivalent metal, and the *Vibrio* and *Nocardia* enzymes have reactive sulphhydryl groups associated with their activity. The *Vibrio* enzyme is distinguished from those of the other bacteria by its remarkable heat-stability and by the simultaneous decarboxylation-lactonizing activity. Enzyme-fractionation attempts have so far failed to separate the two processes.

Sistrom & Stanier (1954) briefly described some properties of the *Pseudomonas* delactonizing enzyme; the *Vibrio* enzyme closely resembles it in specificity. A consistent inhibition by chelating agents has been found in the *Vibrio* system, whereas no such effect was recorded for the *Pseudomonas* enzyme. The stimulation by Ca^{2+} ions and the reversal of EDTA inhibition by this ion also imply a metal requirement for the delactonizing step.

The results of this study present a pathway of aromatic breakdown involving intermediates previously thought to be on quite distinct sequences (Fig. 11). There has indeed been a tendency to regard the known mechanism of fission of the carbocyclic ring (i.e. opening between two hydroxyl groups *ortho* to each other) as ubiquitous and to assume that the ring-opened (aliphatic) products must be metabolized through either, *cis-cis*-muconic acid and its lactone on the catechol pathway, or β -carboxymuconic acid on the protocatechuic pathway. In the second pathway, some organisms form β -carboxymuconolactone; the majority of those at present known do not. Apart from the results in this paper which show that *Vibrio* O1 uses parts of both sequences, other recent work has cast some doubt on the supposed biochemical unity in this field. Dagley & Stopher (1959) have proposed a new mode of ring-fission of catechol in which 2-hydroxymuconic semialde-

Fig. 11. Metabolic pathway for degradation of protocatechuic acid by *Vibrio* O1. (a) Protocatechuic acid; (b), β -carboxymuconic acid; (c), *cis-cis*-muconic acid; (d), β -carboxymuconolactone; (e), (+)-muconolactone; (f), β -oxoadipic acid (enol form); (g), β -oxoadipic acid. $\longrightarrow$, Pathway in *Vibrio*; $-\cdots\|-\rightarrow$, pathway not involved in *Vibrio*.

hyde is formed instead of muconic acid. Trippett *et al.* (1960) have shown that protocatechuic acid undergoes a similar enzymic ring-cleavage to give an intermediate that reacts spontaneously with ammonia to give a pyridine derivative, 2:4-lutidinic acid, which has been isolated and characterized. The conversion of the dilactone of *cis-cis*-muconic acid into β -oxoadipic acid by extracts of the phthalate-oxidizing pseudomonad (Ribbons & Evans, 1959) offers the possibility of yet another pathway, although the observation that cells grown with glucose also perform this reaction renders its status as an intermediate in the reaction sequence uncertain at present. Nevertheless, the isotopic data of Gross *et al.* (1956) require a symmetrical intermediate between β -carboxymuconic acid and β -oxoadipic acid in the *Pseudomonas* system; the dilactone or $\beta\gamma$ -dihydroxyadipic acid fulfil this requirement.

A further possibility arises from the present study. Although β -carboxymuconolactone is not metabolized, a double lactonization of β -carboxymuconic acid (V) would yield a carboxydilactone (IV). Decarboxylation and non-enzymic hydrolysis of the carboxydilactone (IV) to muconolactone (I) is thus possible (Scheme I).

Decarboxylation and hydrolysis in Scheme I would be required to occur simultaneously, without the appearance of the decarboxylation product [i.e. the dilactone, (II)] dissociated from the enzyme, because (II) is not attacked by the vibrio. Nevertheless (IV) would satisfy a requirement for a symmetrical intermediate in this organism should this be required to interpret isotopic data with [2- ^{14}C]protocatechuate. As yet there is no evidence for it.

SUMMARY

1. Protocatechuic acid is oxidized by crude extracts of *Vibrio* O1 to β -oxoadipic acid; partially purified extracts yield stoichiometric amounts of β -carboxymuconic acid.

2. Heat-treated extracts of the vibrio accumu-

late a lactone from either protocatechuic acid or β -carboxymuconic acid.

3. The lactone has been isolated and identified as (+)-4-hydroxyhex-2-enedioic 1 $\rightarrow$ 4-lactone [(+)-muconolactone].

4. Enzymic synthesis of (+)-muconolactone from β -carboxymuconic acid is accompanied by loss of carbon dioxide. Neither *cis-cis*-muconic acid nor β -carboxymuconolactone is attacked by vibrio extracts grown with *p*-hydroxybenzoic acid as carbon source, so the lactonization and decarboxylation appear to occur simultaneously.

5. Some properties of the lactonizing and de-lactonizing enzymes are described and compared with similar enzymes from other micro-organisms. In particular, a metal requirement and sulphydryl groups are associated with the lactonizing enzyme of *Vibrio*, the amount of which in cells is markedly increased by growth on *p*-hydroxybenzoic acid.

6. The significance of symmetric and asymmetric lactones is discussed in relation to new and distinct modes of aromatic ring fission.

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The Metabolism of Protocatechuic Acid by Certain Micro-organisms

A REASSESSMENT OF THE EVIDENCE FOR THE PARTICIPATION OF 2:6-DIOXA-3:7-DIOXOBICYCLO[3:3:0]OCTANE AS AN INTERMEDIATE

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A variety of microbial species are capable of the aerobic metabolism of protocatechuic acid through the β -oxoadipate pathway. Gross, Gafford & Tatum (1956), using [2:6- ^{14}C]protocatechuic acid, showed that the carbonyl-C of β -oxoadipate produced by the *Neurospora crassa* enzyme system originated solely from C-6, whereas with certain *Pseudomonas* enzymes (strain, A3.12, Stanier) it was randomly derived from C-1 and C-6 of protocatechuic acid. The latter pathway clearly diverges from the former route after the *cis-cis*- β -carboxy-muconic acid stage. Table 1 summarizes results showing the ability of cell-free systems of various micro-organisms, induced to protocatechuate, to form β -oxoadipate from likely precursors.

Elsden & Peel (1958) pointed out that the isotopic results of Gross *et al.* (1956) with the *Pseudomonas* system could be explained if an intermediate possessing a symmetrical structure was formed

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before β -oxoadipate, and suggested this might be 2:6-dioxa-3:7-dioxobicyclo[3:3:0]octane (I). This dilactone was isolated by Landa & Eliasek (1956) from *Oospora* (*Geotrichum*?) cultures growing with catechol as carbon source, and had previously been synthesized by Elvidge *et al.* (1950). Ribbons & Evans (1959) repeated this synthesis, and found that both the synthetic dilactone and the compound isolated from the culture (kindly provided by Professor Landa) were converted into β -oxoadipate by crude extracts of a phthalic acid-oxidizing *Pseudomonas* sp., which also converted protocatechuate into the same product. The rate of production of β -oxoadipate from the dilactone under the conditions of this experiment, with extracts of cells grown with phthalate, was as rapid as that from protocatechuate, β -carboxy-muconate or muconolactone; only the dilactone and muconolactone were rapidly attacked if the cells were grown with glucose as carbon source. This evidence appeared to support the contention that the dilactone or its open-chain counterpart, β -