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The Metabolism of Aromatic Acids by Micro-organisms

METABOLIC PATHWAYS IN THE FUNGI

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1. The metabolic pathways of aromatic-ring fission were examined in a range of fungal genera that utilize several compounds related to lignin. 2. Most of the genera, after growth on *p*-hydroxybenzoate, protocatechuate or compounds that are degraded to the latter (e.g. caffeate, ferulate or vanillate), rapidly oxidized these compounds, but not catechol. 3. Such genera possessed a protocatechuate 3,4-oxygenase and accumulated β-carboxymuconate as the product of protocatechuate oxidation. This enzyme had a high pH optimum in most organisms; the *Rhodotorula* enzyme was competitively inhibited by catechol. 4. β-Carboxymuconate was converted by all competent fungi into β-carboxymuconolactone, which was isolated and characterized. None of the fungi produced or utilized at significant rates the corresponding bacterial intermediate γ-carboxymuconolactone. 5. The lactonizing enzymes of *Rhodotorula* and *Neurospora crassa* had a pH optimum near 5·5 and approximate molecular weights of 19000 and 190000 respectively. 6. The fungi did not degrade the isomeric (+)-muconolactone, γ-carboxymethylenebutanolide or β-oxoadipate enol lactone at significant rates, and thus differ radically from bacteria, where β-oxoadipate enol lactone is the precursor of β-oxoadipate in all strains examined. 7. The end product of β-carboxymuconolactone metabolism by extracts was β-oxoadipate. 8. Evidence for a coenzyme A derivative of β-oxoadipate was found during further metabolism of this keto acid. 9. A few anomalous fungi, after growth on *p*-hydroxybenzoate, had no protocatechuate 3,4-oxygenase, but possessed all the enzymes of the catechol pathway. Catechol was detected in the growth medium in one instance. 10. A strain of *Penicillium* sp. formed pyruvate but no β-oxoadipate from protocatechuate, suggesting the existence also of a '*meta*' type of ring cleavage among fungi.

The diverse metabolic routes for the dissimilation of many aromatic compounds by bacteria are now fairly well characterized (Hayaishi, 1962; Dagley, 1967; Huddleston & Allred, 1967) and recent attention has turned to the regulation and control of the enzymes effecting such breakdown (Mandelstam & Jacoby, 1965; Ornston, 1966*c*; Cánovas & Stanier, 1967*a*,*b*). Fungi, however, have been largely neglected in studies of benzenoid catabolism. Though Henderson (1961, 1963) established that catechol, protocatechuate and *cis*–*cis*-muconate were converted into β-oxoadipate by extracts of the 'black yeast' *Aureobasidium* (*Pullularia*) *pullulans*, other work (Henderson & Farmer, 1955; Henderson, 1957, 1960; Jones & Farmer, 1967) has been concerned with interconversions by fungi of the aromatic compounds related to lignin rather than with ring-cleavage mechanisms. The metabolism of protocatechuate by *Neurospora crassa* (Gross, Gafford & Tatum, 1956), in which β-carboxymuconolactone was first identified as an intermediate, remains the only thorough investigation of the enzymology of the route adopted by fungi for cleaving the benzene ring. Much of the work now reported was performed with yeasts or yeast-like organisms because of their ease of cultivation and subsequent handling. We have, however, established that all fungi so far examined resemble *Neurospora crassa* in degrading protocatechuate through β-carboxymuconolactone, whereas those bacteria using an '*ortho*' cleavage mechanism degrade it through the corresponding γ-substituted lactone (Ornston & Stanier, 1964, 1966; Cain, 1966*a*; Cánovas & Stanier, 1967*a*). A preliminary account with a limited number of yeasts has been presented (Bilton & Cain, 1965).

MATERIALS AND METHODS

Organisms. The yeasts and fungi used during the investigations, and their origin, are shown in Table 1. In addition

Table 1. *Identity and origin of the fungi examined*

Organism	How obtained	Origin	Identified by	Special comments
Yeasts				
Rhodotorula mucilaginosa I	Aerial contaminant on *p*-hydroxybenzoate medium	Dust, Newcastle	N.C.Y.C.	—
Rhodotorula mucilaginosa II	Aerial contaminant on *p*-hydroxybenzoate medium	Dust, Leeds	R.B.C. and R.F.B.	—
Rhodotorula macerans VC4	Enrichment on vanillin medium	Dr D. Wild, University of Leeds	N.C.Y.C.	An atypical strain forming the pink pigment only in carbon assimilation tests; also forms abundant pseudomycelium, unlike the type strain
Debaryomyces subglobosus N.C.Y.C. 459	provided by National Collection of Yeast Cultures, Nutfield (N.C.Y.C.)			Inconsistent growth with protocatechuate as sole carbon source, particularly from a small inoculum
Debaryomyces hansenii N.C.Y.C. 9				
Debaryomyces kloeckeri N.C.Y.C. 8				
Candida tropicalis N.C.Y.C.4				The *Candida tropicalis* strains grow well on catechol, resorcinol and quinol as carbon sources, but will not utilize aromatic acids
Candida tropicalis N.C.Y.C. 470				
Sporobolomyces sp.	Departmental collection of Basidiomycetes	Fallen leaves in early autumn	Professor J. H. Burnett	
Debaryomyces hansenii IC	Enrichment with *p*-hydroxybenzoate	Soil from arctic Sweden collected by Miss J. Farrell	N.C.Y.C.	Tentative identification. Forms ascospores with difficulty; spores frequently not seen
'Yeast XY'	Enrichment with *p*-hydroxybenzoate	Soil from arctic Sweden collected by Miss J. Farrell	Unidentified	Culture lost since enzymic studies performed
Yeast-like fungi				
Endomycopsis sp. A	Enrichment with vanillate in kaolin pellets	Soil, near Aberdeen	N.C.Y.C.	'Mycelial yeasts A and B' isolated by Dr M. E. K. Henderson (1965) and given by Dr D. Jones, Macaulay Institute, Aberdeen. These strains showed quite different growth characteristics in liquid medium and B did not assimilate nitrate
Endomycopsis sp. B	Enrichment with vanillate in kaolin pellets	Soil, near Aberdeen	N.C.Y.C.	
Aureobasidium (*Pullularia*) *pullulans*	Aerial contaminant on *p*-hydroxybenzoate medium	Dust	R.B.C. and J.A.D.	Will not grow with catechol as carbon source

Mycelia-forming fungi				
Penicillium spinulosum P6	Isolated on Czapek–Dox medium + tannic acid; formed brown stain in medium	Woodland soil, Northumberland	Mr B. Halsall and Dr C. Dickinson	
Penicillium sp. II	Aerial contaminant on *p*-hydroxybenzoate medium	Dust, Leeds	R.B.C.	Grows poorly when aromatic compound is the sole source of carbon
Aspergillus niger L6	Isolated on Czapek–Dox medium + tannic acid; formed brown stain in medium	Soil, parkland in Newcastle	Mr B. Halsall	Grows profusely in 0·2% (w/v) *p*-hydroxybenzoate medium
Fusarium oxysporum	Enrichment with vanillate	Soil from arctic Sweden collected by Miss J. Farrell	R.B.C., R.F.B. and Dr C. Dickinson	
Neurospora crassa SY4	—	Professor W. C. Evans	—	Original wild-type strain used by Gross *et al.* (1956)
Cephalosporium acremonium	Enrichment with vanillate in kaolin pellets	Soil, Aberdeen	Dr M. E. K. Henderson and Dr C. Dickinson	Isolated by Dr M. E. K. Henderson and given by Dr D. Jones, Macaulay Institute, Aberdeen
Cylindrocephalum sp.	Enrichment with vanillate in kaolin pellets	Soil, Aberdeen	Dr M. E. K. Henderson	
Polystictus versicolor	Departmental collection of Basidiomycetes	Decaying wood	Professor J. H. Burnett	Produces high yields of extracellular laccase when induced with *o*-diphenols
Phoma sp.	Departmental collection of fungi	Acid moorland soil, Waldridge Fell, Co. Durham	Dr K. G. Singh	
Vararia granulosa	Departmental collection of Basidiomycetes	Dead wood	Dr M. K. Nobles, Plant Research Institute, Ottawa	A monokaryotic isolate from a uninucleate conidium after mitosis in a dikaryotic strain
Sphaerobolus stellatus	Departmental collection of Basidiomycetes	Dead twig, Gosforth Park, Northumberland	Professor J. H. Burnett	
Schizophyllum commune	Departmental collection of Basidiomycetes	Staging in warm humid greenhouse, University of Newcastle	Professor J. H. Burnett	
Mucor rammanianus	Departmental collection of Phycomycetes	—	Professor J. H. Burnett	
Fusarium sp. II	Aerial contaminant of *Rhizopus nigricans* culture	Dust	R.B.C. and J.A.D.	Luxuriant growth with *p*-hydroxybenzoate as sole carbon source
Mucor hiemalis	Departmental collection of Phycomycetes	—	Professor J. H. Burnett	

bacteria such as *Pseudomonas putida* A3.12 (Ornston & Stanier, 1966) and a mutant of it devoid of the enzyme β-oxoadipate enol lactone hydrolase (ELH⁻ mutant) (Ornston, 1966c), *Nocardia erythropolis* CA4 (Cartwright & Cain, 1959; Cain, Tranter & Darrah, 1968) and an unidentified organism, *Pseudomonas* C (a departmental isolate), were used to prepare purified enzymes or specific intermediates or were used in comparative experiments.

Culture of the organisms. The yeasts were maintained by monthly subculture on malt–yeast extract–glucose–peptone slopes comprising (g./l.): malt extract, 3; yeast extract (Marmite), 3; glucose, 10; peptone (Oxoid), 5. This medium was adjusted to pH 6·0, solidified with 2% (w/v) agar and sterilized by autoclaving. Fungi were maintained on potato–glucose–agar slopes; rose bengal (0·07 g./l.) was occasionally added to ensure absence of bacterial contamination.

Growth experiments for examining the spectrum of carbon compounds utilized by the yeasts were carried out in 500 ml. conical flasks containing 100 ml. of the defined media (see below) with the carbon source generally at 0·1% (w/v); with some phenols and syringate this was decreased to 0·03% (w/v). After inoculation with approx. 5×10^5 cells (0·1 ml. of a washed suspension derived from a malt–yeast extract–glucose–peptone slope) the flasks were incubated at 25° for up to 14 days in a Gyrotory shaker–incubator (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) operated at 180 rev./min. Fungi were grown similarly but in 250 ml. flasks containing 100 ml. of medium. A spore inoculum (approx. 10^5 spores from a potato–glucose–agar slope) was used where possible; alternatively two plugs of mycelium (5 mm. diam.) taken from the growing edge of a colony on solid potato–glucose–agar medium were used as an inoculum for those species that were slow to sporulate.

For bulk production of cells for manometric experiments and preparation of cell-free extracts the organisms were grown at 25° in 10 l. or 20 l. vessels containing 9 l. and 18 l. respectively of the defined medium supplemented with 0·01% (w/v) yeast extract and fitted with devices for sterile sampling and forced aeration. The aeration rate was controlled at 3 l./min. as recorded with a calibrated air-flow meter (Rotameter; Griffin and George Ltd., Manchester). The bulk medium, prewarmed to 25°, was inoculated with a 500 ml. culture and then incubated at 25° for 18–30 hr. In certain experiments concerned with following changes in enzyme activities, samples (1–3 l.) were removed at intervals starting at 6 hr. after inoculation, the aeration rate being adjusted to maintain 3 l. of air/min. with the decreased liquid volume. Yeasts were harvested in a continuous centrifuge. The pellets or diffuse mycelium of the fungi were collected by filtering through cheesecloth, washing with several volumes of water and squeezing the bulk of the water from the mycelial mass.

A few fungi would not give sufficient mycelium for enzyme studies when grown on an aromatic compound as the sole carbon source; in these cases the fungi were grown in shake culture on glucose medium, the mycelia being harvested aseptically on a Millipore filter washed with sterile water and transferred to the equivalent volume of defined medium containing the aromatic compound. Incubation was continued for 2–5 days or until test samples showed that the substrate was being degraded. We obtained much higher mycelial yields (and thus extract) by using shaken or aerated cultures than by employing the stationary replacement technique (Henderson, 1956), where the felts frequently began to sporulate in 2–3 days.

Growth medium. The defined medium consisted of Difco Yeast Nitrogen Base (Wickerham, 1951) to which was added the aromatic carbon source at 0·1% (w/v). Yeast Nitrogen Base comprises a mineral salts medium with the addition of an amino acid and vitamin supplement of known composition, available in the manufacturer's literature (*Difco Manual*, 1965, p. 251). The medium was adjusted to pH 5·5 and sterilized by autoclaving. Glucose (1%, w/v) or succinate (0·1%, w/v) replaced the aromatic carbon source when unadapted cells were required. When large culture volumes were used in bulk cell production for enzyme extraction, the expensive Yeast Nitrogen Base was replaced with a mineral salts medium, pH 5·5, of the following composition: $(NH_4)_2SO_4$, 0·5 g./l.; KH_2PO_4, 0·5 g./l.; $MgSO_4$, 0·05 g./l.; trace-element solution (Barnett & Ingram, 1955), 1 ml./l. Growth of the fungi was generally improved by adding 0·01% (w/v) yeast extract to this medium.

Preparation and treatment of cell-free extracts. The wet cell paste obtained from large cultures was stored frozen (−20°) until required. Most of the yeasts and fungi lost little enzyme activity over a 3-month period when stored in this way, whereas frozen crude extracts progressively lost activity over a few weeks.

The fungi were crushed by vigorously grinding the mycelium under liquid N_2 with a pestle and mortar for 5–10 min., liquid N_2 being added periodically to keep the material frozen. The ground material was then allowed to thaw and extracted with 1–2 ml. of buffer or water/g. wet wt. of mycelium for 15 min. and the insoluble material was removed by centrifuging at 50000*g* for 20 min. Extracts containing 2–10 mg. of protein/ml. were obtained.

Small amounts of yeast cells were ruptured in the Hughes (1951) press at −20° without abrasive, and the crushed material was extracted and centrifuged as above. These

Table 2. *Separation of the enzymes of aromatic-ring catabolism by ammonium sulphate fractionation of extracts of* Rhodotorula mucilaginosa

	Enzyme activity (units)					
		% saturation with $(NH_4)_2SO_4$				
Enzyme	Crude extracts	0–30	30–40	40–50	50–60	60–100
Protocatechuate 3,4-oxygenase	443	31·5	27·1	141·5	21·3	30
β-Carboxymuconate-lactonizing enzyme	633	26·7	18·7	335	126	76
β-Carboxymuconolactone-delactonizing enzyme	667	9·3	1·7	19	29	170
Protein (mg.)	1400	39·4	87	264	108	495

extracts did not have the glutinous character of nucleic acid-rich bacterial extracts similarly produced, so protein concentrations could be kept fairly high by minimizing the addition of water or buffer.

Bacterial extracts, used for preparing certain intermediates or enzymes (see below), were made by subjecting thick suspensions, cooled to 0°, to ultrasonic disintegration for 5–15min. at 20kcyc./sec. with the MSE–Mullard instrument, and removing broken cell debris and particulate material by ultracentrifugation at 100000*g* for 30min.

When large amounts of yeast cells were to be crushed a 50% (w/w) suspension of cells and small Ballotini beads (total volume approx. 40ml.) was placed in stainless-steel tubes (100ml. capacity) sealed with rubber bungs at each end and the tubes were placed vertically in the eccentric shaker head of a Shottman shaker attached to a refrigerated International centrifuge. The intense vertical vibration caused 90–95% cell disintegration in 10–15min. to yield extracts containing 20–30mg. of protein/ml.

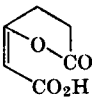
Some of the enzymes of aromatic-ring metabolism in extracts of certain of the yeasts could be partly separated by fractionation with $(NH_4)_2SO_4$. The protocatechuate oxygenase and lactonizing enzyme of *Rhodotorula mucilaginosa*, for instance, which occur in the 40–50%-saturated fraction, were separated from delactonizing activity, which was precipiated predominantly in the 60–100%-saturated fraction (Table 2). Preliminary separations of certain of the enzymes from other organisms were carried out in this way, further purification techniques varying with the enzyme required and the organism under examination (see the Results section).

To produce an enzymic preparation of *Rhodotorula mucilaginosa* that converted protocatechuate into β-carboxymuconolactone, the cell-free extract derived from *p*-hydroxybenzoate-grown cells in 0·05M-tris–HCl buffer, pH7·0, was treated with neutral saturated $(NH_4)_2SO_4$ solution in the cold and the fraction that was precipitated between 30% and 50% saturation was collected. The precipitate was dissolved in 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH7·0, and treated with 0·1vol. of aged calcium phosphate gel (gel:protein ratio 2:1) with gentle shaking for 30min. and the gel was removed by centrifugation. All the enzymic activity was adsorbed on the gel. The gel was washed for 30min. at 0° with gentle shaking with 1vol. of 0·01M-Na_2HPO_4–KH_2PO_4 buffer, pH6·5, which eluted all the delactonizing activity. The protocatechuate oxygenase and β-carboxymuconate-lactonizing enzyme were recovered in the protein eluted with 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH7·1. This fraction was dialysed to remove phosphate, which inhibits lactonizing enzyme, and then concentrated by dialysis for 6–10hr. against a 10% (w/v) solution of Carbowax 4000 (British Drug Houses Ltd., Poole, Dorset). The protein concentration was finally adjusted to approx. 15mg./ml. with 0·1M-tris–HCl buffer, pH7·0.

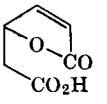
Chemical syntheses. All melting points in this paper are uncorrected for emergent thermometer stem. β-Oxoadipic acid, m.p. 117°, was synthesized via α-acetyl-β-oxoadipic ester (Bardhan, 1936); some preparations were also made enzymically (Darrah & Cain, 1967). *cis–cis*-Muconic acid was prepared by peracetic acid oxidation of redistilled phenol (Elvidge *et al.* 1950) and recrystallized from ethanol to give white crystals, m.p. 195°.

4-Hydroxyhex-4-enedioic (1→4)-lactone (γ-carboxymethylenebutanolide, I) was synthesized by treating β-oxoadipic acid (0·5g.) with acetyl chloride (4ml., unredistilled and containing HCl) at room temperature for 24–36hr. (Eisner, Elvidge & Linstead, 1950). The excess of reagent was then evaporated with a stream of cold air and the residue extracted twice with boiling chloroform to remove unchanged β-oxoadipic acid, which is more soluble in this solvent than the lactone. The lactone was then recrystallized twice from ethyl acetate–light petroleum (b.p. 60–80°) to give white needles, m.p. 177°. This preparation differs in its purification stages from the original method of Eisner *et al.* (1950), the results of which we were not able to reproduce in several attempts with different preparations of acetyl chloride. Minor variations in the preparative method lead to the formation of the anhydride of the lactone, compound (IV), which has a similar m.p. to that of crude preparations of the lactone itself and gives analyses for C and H near to those for the lactone; treatment with cold water converts the anhydride into the lactone (Eisner *et al.* 1950, p. 2227), so the identity of the crude product was frequently doubtful. Rigorous identification was based on the following data. (1) The recrystallized product analysed C, 50·0; H, 4·4 (Calc. for $C_6H_6O_4$: C, 50·7; H, 4·2%). Microtitration with cold alkali showed only one inflexion of the titration curve and gave an equivalent of 139; $C_6H_6O_4$ (monobasic) requires 142. (2) The u.v. spectrum of a freshly made solution at pH7·0 gave $E_{max.}$ at 219mμ. The spectrum exhibited a shift in $E_{max.}$ from 228mμ at pH3·0 to 206mμ when the pH was raised to 9·0. This suggested that the chromophore varied with the ionization state of the carboxyl group and that the double bond was therefore exocyclic. When the solution was kept at pH9·0 or higher, the extinction fell rapidly as the lactone was hydrolysed to β-oxoadipic acid ($\epsilon < 100$ at 216mμ). Consequently γ-carboxymethylenebutanolide always gave a positive Rothera (1908) test. (3) The i.r. spectrum (KBr disk) exhibited intense absorption at 1765cm.$^{-1}$ (C=O stretching vibrations of a saturated γ-lactone), but no absorption at 1400cm.$^{-1}$ due to the grouping $-CH_2-CO-$ (present, and shown as a strong band, in the spectrum of the isomeric (+)-muconolactone, II). There was also a moderately intense band at 1705cm.$^{-1}$ (C=O stretching vibrations of an αβ-unsaturated acid) and strong bands at 1645 and 835cm.$^{-1}$ due to the stretching vibration of a carbonyl-conjugated C=C and the C–H stretch and deformation vibration in $>C{=}CH-$. A weak band at 1812cm.$^{-1}$ indicated a little anhydride impurity in this preparation. (4) The proton nuclear-magnetic-resonance spectrum of a 10% (w/v) solution of the synthetic material in deuterated acetone [99·9% $CD_3{\cdot}CO{\cdot}CD_3$; Ciba (A.I.R.), Duxford, Cambs.] differed radically from that of (+)-muconolactone and β-oxoadipate enol lactone (III) (Ornston & Stanier, 1966). γ-Carboxymethylenebutanolide is the only isomer of compounds (I), (II) or (III) that should exhibit an A_2B_2-type spectrum, owing to the 4 protons of the saturated lactone ring. This spectrum was observed in the suspected material with two triplet peaks centred at 3·02 and 3·57p.p.m. A singlet peak at 5·82p.p.m. in the spectrum was attributed to the shift due to the single proton on the α-carbon atom of the side chain.

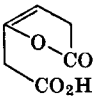
Succinyl-CoA was prepared from CoA (85% pure; Sigma Chemical Co., St Louis, Mo., U.S.A.) and succinic anhydride by the method of Simon & Shemin (1953). Synthetic (±)-muconolactone, m.p.109°, and 2,6-dioxa-3,7-



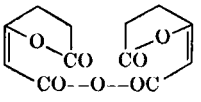
γ-Carboxymethylenebutanolide (I)



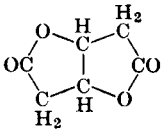
(+)-Muconolactone (II)



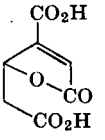
β-Oxoadipate enol lactone (III)



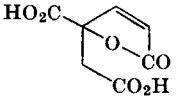
Anhydride of compound (I) (IV)



Dilactone (V)



β-Carboxymuconolactone (VI)



γ-Carboxymuconolactone (VII)

dioxobicyclo-[3,3,0]octane (dilactone, V), m.p. 127°, were provided by Professor W. C. Evans. Other chemicals were commercial products, recrystallized where necessary.

Enzymic syntheses. (+)-4-Hydroxyhex-2-enedioic (1→4)-lactone [(+)-muconolactone, II], m.p. 74°, was prepared as described by Cain (1961) and also by the method of Sistrom & Stanier (1954) by using a partially purified extract of benzoate-grown *Pseudomonas putida* A 3.12.

β-Carboxy-*cis*–*cis*-muconic acid was synthesized from protocatechuate by treatment with extracts of *Pseudomonas* C2 that had been grown with *p*-hydroxybenzoate. Crude cell-free extracts, after simple dialysis for 48 hr. against water, accumulated β-carboxymuconate quantitatively. The reaction mixture contained in a total volume of 350 ml.: Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, 30 m-moles; potassium protocatechuate, 2 m-moles; $FeSO_4$, 10 μmoles; extract, 200 mg. of protein. The substrate was added in 200 μmole portions to the enzyme solution, which was maintained at 30° and magnetically stirred sufficiently rapidly to produce a vortex to the base of the vessel. The pH of the reaction mixture was maintained at 7·0 by periodic addition of N-NaOH and the disappearance of substrate was followed by the Evans (1947) method. After protocatechuate had been utilized, incubation was continued for 1 hr. and the product isolated as the trisodium salt as described by McDonald, Stanier & Ingraham (1954). Several similar preparations were pooled and portions recrystallized from aqueous methanol–propan-2-ol as described by McDonald *et al.* (1954). The best preparation of trisodium β-carboxymuconate contained 93% of active isomer as determined by spectrophotometric and enzymic analysis (see below). Crude preparations contained approx. 65–70% of active isomer and were used without further purification for enzymic syntheses.

3-Carboxy-4-hydroxyhex-2-enedioic (1→4)-lactone (β-carboxymuconolactone, VI), m.p. 164°, was synthesized from crude β-carboxymuconate by using the purified lactonizing enzyme from vanillate-induced cultures of *Neurospora crassa* SY 4 (Gross *et al.* 1956; Cain, 1961).

4-Carboxy-4-hydroxyhex-2-enedioic (1→4)-lactone (γ-carboxymuconolactone, VII) was reported to be very unstable (half-life approx. 30 min. at pH 6·0 at 30°; Ornston & Stanier, 1966) and so was generated *in situ* from β-carboxymuconate by using the partially purified lactonizing enzyme (step 5 in Table 4 of the method of Ornston, 1966*a*) from *p*-hydroxybenzoate-grown cells of *Pseudomonas putida* A 3.12. A typical reaction mixture contained in a total volume of 5 ml.: tris–HCl buffer, pH 7·0, 500 μmoles; β-carboxymuconate, 5 μmoles; purified lactonizing enzyme, 2 units (defined below) (approx. 40 μg. of protein). After 5 min., when lactonization was completed, the tube containing this reaction mixture was kept at 0° on cracked ice during assays.

Determinations. Growth of those yeasts that formed uniformly turbid cultures was measured spectrophotometrically at 450 mμ and these values were related to a cell count. The course of growth in such cultures was followed nephelometrically in flasks into which had been sealed a side arm suitable for insertion into the nephelometer. Approximations of growth of one or two of the fungi that grew in a finely divided mycelial state in shake culture or when supplied with forced aeration (e.g. *Fusarium* and *Cephalosporium*) could also be made spectrophotometrically (Fig. 3), but the growth yields of fungi on the various carbon sources were measured by determination of the dry weights of the washed mycelia.

Protein in crude extracts was determined by the biuret method of Gornall, Bardawill & David (1949). During enzyme purifications where low protein readings were expected in the final stages the Folin method of Lowry, Rosebrough, Farr & Randall (1951) was used. Lactones were determined by the alkaline and acid hydroxamate methods described by Cain (1961). Keto acids were measured by the method of Friedmann & Haugen (1943), with ethyl acetate as the final extractant. β-Oxoadipate was detected by the Rothera (1908) test and determined by catalytic decarboxylation at pH 4·0 with 0·1 M-4-aminoantipyrine (Sistrom & Stanier, 1953). Succinic acid, after

extraction from the reaction mixture with ether, was determined manometrically at 37° with a succinoxidase preparation from ox heart muscle (Umbreit, Burris & Stauffer, 1957). Acetyl-CoA was assayed by the change in E_{232} (ϵ 4500) brought about by arsenolysis in the presence of phosphotransacetylase (Stadtman, 1957), and succinyl-CoA as its hydroxamate derivative by the method of Lipmann & Tuttle (1945).

o-Diphenols were determined by a minor modification of the method of Evans (1947). If sodium molybdate was replaced by sodium tungstate as one of the reagents of this test, catechol still reacted in the cold but protocatechuate only developed its full colour after the reaction tubes were immersed for 15min. in a boiling-water bath. This difference permitted catechol and protocatechuate to be determined in each other's presence, individual calibration curves being made with authentic materials. Where two *o*-diphenols were suspected by this test, they were always confirmed by chromatography.

Infrared measurements were made with KBr disks or Nujol mulls with a Hilger and Watt Infrascan spectrophotometer, and visible and u.v. measurements with a Unicam SP.500 spectrophotometer with cuvettes of 10mm. light-path.

Enzyme assays. All enzymes were assayed spectrophotometrically at pH7·0 in quartz cuvettes of 10mm. light-path with a Unicam SP.800 recording spectrophotometer fitted with the SP.820 constant-wavelength attachment and a constant-temperature cuvette holder through which water at 30° was circulated with a Circotherm unit (Braun, W. Germany; available from Shandon Scientific Co., London, N.W.10).

Protocatechuate 3,4-oxygenase (EC 1.13.1.3) was measured by following the disappearance of substrate at 290mμ in crude preparations that did not accumulate β-carboxymuconate, or by measuring the rate of change of E_{270} and E_{290} in purified preparations (McDonald *et al.* 1954) from which the concentrations of substrate and product can be calculated. At pH7·0 in 0·1M-tris–HCl buffer, protocatechuate has ϵ 2730 and 3890 and β-carboxymuconate has ϵ 6400 and 1590 at 270mμ and 290mμ respectively.

Catechol 1,2-oxygenase (EC 1.13.1.1) was assayed as described by Hegemen (1966).

β-Carboxymuconate-lactonizing enzyme was measured by the rate of decrease of E_{260} due to the disappearance of β-carboxymuconate (ϵ8150); the two possible products (β- or γ-carboxymuconolactone) do not absorb appreciably at this wavelength.

γ-Carboxymuconolactone decarboxylase and β-carboxymuconolactone-delactonizing enzyme were assayed by measurement of the rate of decrease of substrate at 230mμ with crude extracts. At this wavelength γ-carboxymuconolactone has ϵ 4200 and β-carboxymuconolactone has ϵ 8500. Cuvettes for all the above assays contained: tris–HCl buffer, pH7·0, 200μmoles; substrate, 0·5μmole; enzyme extract; water to a total volume of 3·0ml. Blank cuvettes contained no substrate. γ-Carboxymuconolactone decomposes spontaneously and measurements of its enzymic decomposition were corrected for values obtained without extract under otherwise identical conditions.

cis–cis-Muconate-lactonizing enzyme (EC 5.5.1.1) was assayed by following the decrease in E_{260} due to the disappearance of *cis–cis*-muconate (ϵ 17500). Cuvettes contained 0·3μmole of substrate and the other contents described above.

(+)-Muconolactone isomerase catalyses the formation of β-oxoadipic enol lactone from (+)-muconolactone, both of which absorb equally at 230mμ. The enol lactone can be removed, however, by adding a partially purified extract of *p*-hydroxybenzoate-grown *Pseudomonas putida* (step 3 in Table 7 of the procedure of Ornston, 1966*b*), which does not attack (+)-muconolactone (ϵ 1650) but rapidly hydrolyses accumulated enol lactone to β-oxoadipate, this end product having negligible u.v. absorption (Ornston & Stanier, 1966). (+)-Muconolactone isomerase in the fungal extracts was therefore measured in the presence of excess of β-oxoadipate enol lactone hydrolase provided by *Pseudomonas putida* extract in a reaction mixture containing: tris–HCl buffer, pH7·0, 200μmoles; (+)-muconolactone, 1μmole; extract of *p*-hydroxybenzoate-grown *Pseudomonas putida*, 0·1mg. of protein (0·5unit of β-oxoadipate enol lactone hydrolase); fungal extract; water to a total volume of 3·0ml. Blank cuvettes contained no substrate.

β-Oxoadipate enol lactone hydrolase was preferably assayed by measuring the rate of disappearance of β-oxoadipate enol lactone (ϵ 1600), but in the absence of this material an indirect assay was carried out on the fungal extracts. The mutant strain (A14) of *Pseudomonas putida* is devoid of β-oxoadipate enol lactone hydrolase (ELH$^-$ mutant) and cannot grow with benzoate or *p*-hydroxybenzoate as carbon source, though the wild-type utilizes each equally well. After growth with benzoate+succinate, however, the ELH$^-$ mutant has normal muconolactone isomerase activity (Ornston, 1966*b*). The hydrolase in the extract under test could therefore be assayed approximately by measuring the decrease in E_{230} due to the disappearance of (+)-muconolactone in the presence of excess of muconolactone isomerase provided by extracts of the (benzoate+succinate)-grown ELH$^-$ mutant. Cuvettes therefore contained: tris–HCl buffer, pH7·0, 200μmoles; (+)-muconolactone, 1μmole; extract of ELH$^-$ mutant, approx. 40μg. of protein (containing 0·5unit of muconolactone

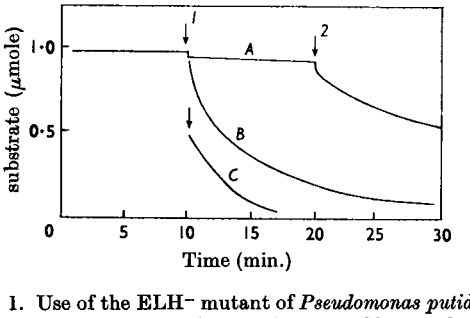


Fig. 1. Use of the ELH$^-$ mutant of *Pseudomonas putida* to determine the presence of β-oxoadipate enol lactone hydrolase in extracts of *p*-hydroxybenzoate-grown microorganisms. Cuvettes contained in a total volume of 2·8ml.: tris–HCl buffer, 250μmoles; (+)-muconolactone, 1μmole; extract of ELH$^-$ mutant. At arrow *1*, extract of either *Rhodotorula mucilaginosa* (0·39mg. of protein) (curve *A*) or *Nocardia erythropolis* CA4 (0·27mg. of protein) (curve *B*) was added. At arrow *2*, wild-type *Pseudomonas putida* extract (0·10mg. of protein) was added to the *Rhodotorula* system. Curve *C* shows the degradation of β-carboxymuconolactone (0·5μmole) effected by the same amount of *Rhodotorula* extract as used to obtain curve *A*.

isomerase); fungal extract; water to a total volume of 3·0ml. The use of this assay method is illustrated in Fig. 1. This method depended on the absence of any activity for (+)-muconolactone from the extract under test. Where this was significant, β-oxoadipate enol lactone was generated *in situ* from β-carboxymuconate (1·0μmole) with purified β-carboxymuconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase (0·2unit of each) from *p*-hydroxybenzoate-grown *Pseudomonas putida* A3.12.

Crude bacterial extracts and extracts of many of the fungi absorbed strongly at 230mμ. Wild-type and ELH⁻ mutant *Pseudomonas* extracts were therefore partially purified to concentrate the enzyme activities required and remove extraneous protein and nucleic acid contributing to this absorption. The extracts of the ELH⁻ mutant were purified to step 5 in Table 3 of the procedure of Ornston (1966*b*) for crystallizing muconolactone isomerase or to step 5 in Table 6 of the procedure of Ornston (1966*a*) for purifying γ-carboxymuconolactone decarboxylase.

A few fungi after growth with protocatechuate, or one of its precursors, appeared to have the ability to degrade intermediates of both the protocatechuate and catechol pathways. In these instances the disappearance of (+)-muconolactone (1μmole) was followed by addition of fungal extract without supplementary bacterial enzymes, giving an overall measure of the activity of both muconolactone isomerase and β-oxoadipate enol lactone hydrolase.

The disappearance of γ-carboxymethylenebutanolide was followed by the decrease in E_{230} as the substrate (ϵ 3520) was hydrolysed to β-oxoadipate. The significant hydrolysis of this lactone at neutral or alkaline pH made it necessary to prepare fresh solutions daily; values for hydrolysis in the presence of boiled extract were subtracted from results obtained in the following test system: tris–HCl buffer, pH 7·0, 200μmoles; γ-carboxymethylenebutanolide, 0·5μmole; extract; water to a total volume of 3ml.

p-Hydroxybenzoate hydroxylase activity was measured by following the substrate-dependent oxidation of reduced nicotinamide nucleotide at 340mμ (ϵ 6220) (Hosokawa & Stanier, 1966). Most of the yeasts and moulds examined were specific for NADPH, but a few mycelial-forming fungi gave higher activity with NADH. The standard assay system contained in a total volume of 3ml.: Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, 200μmoles; reduced nicotinamide nucleotide, 0·5μmole; FAD, 0·01μmole; *p*-hydroxybenzoate, 1μmole; extract; water to a total volume of 3ml. The endogenous rate of oxidation of reduced nicotinamide nucleotide was first determined (usually negligible for NADPH with extracts from which all the particulate material had been removed by ultracentrifugation) and subtracted from the rate obtained on addition of *p*-hydroxybenzoate. Control cuvettes contained all components except reduced nicotinamide nucleotide. The unit of enzyme activity in all these assays is defined as the change of 1μmole of substrate/min.

β-Oxoadipate CoA-transferase (succinyl-CoA–3-oxoadipate CoA-transferase, EC 2.8.3.6) and β-ketothiolase (acyl-CoA–acetyl-CoA *C*-acyltransferase, EC 2.3.1.16) were measured as described by Katagiri & Hayaishi (1957) but with 50μmoles of potassium β-oxoadipate in a final volume of 1·5ml. The pen trace of the recording spectrophotometer for these assays was expanded tenfold vertically (extinction scale) with the SP.840 expansion unit and a Honeywell Electronik 15 slave recorder because the extinction increase was less than 0·2 at 305mμ. The unit of activity for this enzyme is defined as that amount of enzyme producing an increase in E_{305} of 1·0/min. (Cánovas & Stanier, 1967*a*).

Manometric procedures. The O_2 uptake and CO_2 production were followed by the usual manometric method with the Warburg respirometer (Umbreit *et al.* 1957). Gas exchanges were measured at 30°. Flask contents for examining the respiratory activity of the fungi on aromatic compounds comprised: Na_2HPO_4–KH_2PO_4 buffer, pH 5·6 200μmoles; substrate, 5μmoles; washed suspension of cells or homogenized mycelium, 0·5ml. (dry wt. equivalent given in the text); 20% (w/v) KOH in centre well, 0·2ml.; water to a total volume of 3ml.

Isolation and identification of protocatechuate from fungal cultures. The identity of the diphenol appearing in fungal cultures with protocatechuate was confirmed by isolation as follows. *p*-Hydroxybenzoate medium (9l.) at 30° was inoculated with a 500ml. culture of *Sporobolomyces* sp. and aerated with 5l. of air/min. for 6hr. or until exponential growth was evident. The aeration rate was then decreased to 1l./min. and incubation continued for a further 12hr., samples of the medium being periodically tested for protocatechuate. When this reached a maximum, the cells were removed by centrifugation, the supernatant medium was concentrated to 1l. *in vacuo* at 45° with a circulatory cyclone evaporator and the protocatechuate was isolated as described by Cartwright & Cain (1959). The isolated material after recrystallization twice from cold water had m.p. 195–197°; the m.p. of authentic protocatechuic acid (197°) was not depressed on admixture. The i.r. and u.v. spectra of the natural product and protocatechuic acid were virtually identical.

Isolation and characterization of β-oxoadipic acid. Protocatechuate (3m-moles) was oxidized by an extract of *Sporobolomyces* or *Aspergillus niger* L6 (300mg. or 160mg. of protein respectively) in 300ml. of 0·1M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, in a glass vessel maintained at 30° and fitted with a magnetic stirrer moving sufficiently rapidly to cause a vortex through the whole depth of the solution. The substrate was added in 50μmole batches and the reaction followed by removing samples and testing them for residual protocatechuate by the Evans (1947) method. When all the substrate had been utilized incubation was continued for a further 1hr. and the product was isolated by deproteinization, 'salting out' and continuous ether extraction as described by Darrah & Cain (1967). The material (188mg. from *Sporobolomyces*; 127mg. from *Aspergillus*), after two recrystallizations from ethyl acetate–benzene, had m.p. 117° (decomp.). Authentic β-oxoadipic acid prepared by chemical synthesis (Bardhan, 1936) and a mixture of the natural and synthetic compounds melted at 118°.

The natural product formed a 2,4-dinitrophenylhydrazone on addition of excess of 2,4-dinitrophenylhydrazine and boiling for 5min. to complete decarboxylation. On recrystallizing from aq. ethanol, this derivative had m.p. 205–206°, undepressed by admixture with authentic laevulate 2,4-dinitrophenylhydrazone (m.p. 206°). The derivative of the natural product and laevulate 2,4-dinitrophenylhydrazone co-chromatographed in solvents *D* and *F* (see below.)

Isolation of β-carboxymuconolactone. Extract of *p*-hydroxybenzoate-grown *R. mucilaginosa* I (750mg. of protein) containing protocatechuate 3,4-oxygenase (680 units) and lactonizing enzyme (56 units) was added in step-

wise portions of 250mg. (10ml.) to a solution of protocatechuate (3m-moles) and $FeSO_4$ (10μmoles) in 0·1M-tris–HCl buffer, pH 7·5 (100ml.), maintained at 30° and stirred magnetically. The reaction was followed spectrophotometrically at 230 and 270mμ and the reaction mixture maintained at pH 7·5 by adjusting with 0·1N-HCl. When no further extinction changes occurred, the pH of the solution was adjusted to 2·0, the precipitated protein centrifuged off and the solution extracted once with diethyl ether (0·5vol.) to remove residual β-carboxymuconic acid, which was much more ether-soluble than the lactone. The aqueous phase was concentrated to 30ml. *in vacuo* and then continuously extracted with ether for 24hr. with the distillation temperature of the solvent kept no higher than at 40–42°. The ethereal solution was dried over anhydrous Na_2SO_4 and distilled, leaving a pale-yellow residue that was recrystallized from ethyl acetate–benzene. White crystals of carboxymuconolactone (184mg.), m.p. 165°, were obtained (Found: C, 44·8; H, 3·0. $C_7H_6O_6$ requires: C, 45·2; H, 3·2%. Equivalent by titration with cold alkali, 90·5; a dicarboxylic acid, $C_7H_6O_6$, requires 93).

Chromatography. Paper chromatography was carried out on Whatman no. 1 paper in the descending or ascending direction. Authentic markers were always chromatographed on the same sheets as test substances. For organic acids and phenolic compounds, the following solvent systems were used: *A*, ethanol–aq. NH_3 (sp.gr. 0·88)–water (20:1:4, by vol.); *B*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *C*, benzene–acetic acid–water (2:2:1, by vol.). Organic acids were detected by spraying with ethanolic bromothymol blue (0·05%, w/v) made just alkaline with NH_3; acids with unsaturated double bond (e.g. *cis–cis*-muconate) bleached a 0·1% (w/v) solution of $KMnO_4$ in aq. 50% (v/v) acetone when this was used as a spray reagent. Phenolic compounds were detected by their fluorescence under u.v. light, by spraying with diazotized 4-aminophenyl 2-diethylaminoethyl sulphone (I.C.I. 5091) [1% (w/v) in 10% (w/v) Na_2CO_3] or by spraying with $FeCl_3$ [0·5% (w/v) in aq. 50% (v/v) ethanol made acid with dil. HCl].

2,4-Dinitrophenylhydrazone derivatives of keto acids were chromatographed in the following solvents: *D*, 2-methylbutan-2-ol–propan-1-ol–aq. NH_3 (sp.gr. 0·88) (13:1:6, by vol.); *E*, butan-1-ol–0·5N-NH_3–ethanol (7:1:2, by vol.); *F*, propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (20:1:2, by vol.). They were detected by their quenching of u.v. light or by spraying with 0·1N-NaOH.

Lactones were run in the following solvents: *G*, pyridine–ethyl acetate–water (1:1:1, by vol.); *H*, diethyl ether–benzene–formic acid (98%, w/v)–water (35:15:7:5, by vol.). They were detected by forming the hydroxamate derivative on the paper followed by a $FeCl_3$ spray (Cain, 1961).

Determination of molecular weights by Sephadex G-200 chromatography. Molecular-weight determinations were performed essentially as described by Cánovas & Stanier (1967*a*) but with a 90cm.-long column. The following markers were used (mol.wt. in parentheses): blue dextran (2000000), obtained from Pharmacia (G.B.) Ltd., London, W.13; glutamate dehydrogenase (1000000), fumarase (220000), hog heart malate dehydrogenase (40000) and cytochrome *c* (22000 by Sephadex G-200), all obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; muscle lactate dehydrogenase (135000), the Sigma 340–10 preparation. Blue dextran was measured by E_{620} and cytochrome *c* by E_{405}. Glutamate dehydrogenase was deter-

Table 3. *Growth of some yeasts on aromatic carbon sources*

An extinction of 1·0 represents approx. $1·02 \times 10^8$ cells/ml. —, Not tested.

Substrate	$+\Delta E_{450}$ after 7 days										
	Rhodotorula mucilaginosa I	*Rhodotorula macerans* VC4	*Debaryomyces hansenii* IC	*Debaryomyces hansenii* N.C.Y.C. 9	*Debaryomyces subglobosus* N.C.Y.C.459	*Debaryomyces kloeckeri* N.C.Y.C.8	'Yeast XY'	*Sporobolomyces* sp.	*Aureobasidium pullulans*	*Candida tropicalis* N.C.Y.C.470
None	0·012	0·015	0·020	0·010	0·002	0·005	0·008	0·015	0·015	0·011
p-Hydroxybenzoate	1·008	1·112	0·850	0·015	0·935	0·750	0·935	0·825	1·14	0·010
Protocatechuate	0·928	1·00	0·890	0·913	0·895	0·338	0·815	0·944	1·02	0·015
Ferulate	0·988	0·460	0·250	—	0·010	—	—	0·848	0·555	—
Vanillate	0·930	1·010	0·810	0·015	0·010	0·700	—	—	0·705	0·010
Veratrate	0·051	0·100	0·055	—	—	—	—	0·026	0·985	—
p-Coumarate	1·021	—	0·800	0·010	—	0·045	0·455	0·944	—	—
Chlorogenate	0·688	—	0·120	0·015	—	0·755	—	0·654	—	—
Salicylate	0·025	—	0·015	0	—	0·005	—	0·038	—	—
Quinate	0·870	0·660	0·770	—	0·955	0·852	—	0·864	0·865	—
Catechol	0·020	0·020	0·310	1·093	1·097	0·047	0·905	0·020	0	0·734
Resorcinol	—	0·020	0·800	0·005	0·975	0·855	—	0·034	0	0·634

Table 4. *Utilization of aromatic compounds as carbon and energy source by* Fusarium oxysporum, Aspergillus niger *L*6 *and* Penicillium spinulosum *P*6

Cultures (100ml.) were grown for 5 days from a standard inoculum of 10^5 spores, the mycelium was filtered through tared Millipore disks and washed with 5vol. of water and the disks and mycelium were dried at 105° to constant weight. Results are the means of duplicate vessels. All carbon sources at 0·1% (w/v) except phenol and *p*-cresol (0·02%, w/v) and syringate (0·05%, w/v). —, Not tested.

	Mycelial dry wt. (mg.)		
Substrate	*Fusarium oxysporum*	*Aspergillus niger* L6	*Penicillium spinulosum* P6
None	1·3	0·6	0·4
Benzoate	3·0	29·9	31·8
p-Hydroxybenzoate	38·4	36·5	27·3
p-Methoxybenzoate	—	30·0	0·2
m-Methoxybenzoate	0·3	1·4	0·2
Vanillate	34·4	20·5	7·3
Veratrate	4·4	3·2	1·5
Protocatechuate	33·7	27·2	26·1
Ferulate	33·5	33·5	25·5
Caffeate	—	34·0	21·8
p-Coumarate	32·2	35·9	32·2
Chlorogenate	13·3	—	—
Syringate	3·6	0·7	1·0
Salicylate	0·4	—	—
Catechol	23·6	41·0	31·5
Resorcinol	9·5	—	—
Phenol	1·8	—	—
p-Cresol	0·3	0·8	0·2
Vanillin	0·2	0·6	0·1
p-Hydroxybenzaldehyde	5·3	—	—
Syringaldehyde	1·1	0·6	0·3
Quinate	31·8	28·8	24·6

mined by the method of Cooper, Srere, Tabachnick & Racker (1958), fumarase by the method of Racker (1950), malate dehydrogenase by the method of Mehler, Kornberg, Grisolia & Ochoa (1948) and lactate dehydrogenase by the method of Kubowitz & Ott (1943).

Nomenclature. The terms '*ortho*' and '*meta*' when applied to a metabolic sequence of aromatic-ring breakdown refer to those pathways initiated by an oxygenase cleavage of an *o*-dihydroxylated aromatic substrate. The '*ortho*' cleavage occurs at the bond between two carbon atoms each carrying a hydroxyl group to form a dicarboxylic acid (usually a muconate derivative); the '*meta*' cleavage occurs at the bond between two carbon atoms only one of which carries a hydroxyl group and results in the formation of a 2-hydroxymuconic semialdehyde derivative.

RESULTS

Utilization of aromatic compounds by fungi

Several aromatic compounds related to oxidation and breakdown products of lignin were tested for their ability to support growth of the yeasts and a selection of the mycelia-forming fungi. The results (Tables 3 and 4) showed that the ability to utilize *p*-hydroxybenzoate, protocatechuate and monomethoxylated derivatives of these compounds was widespread. The utilization of phenols and dimethoxy compounds was much more restricted. Nearly all the organisms examined, for instance, utilized protocatechuate and vanillate (4-hydroxy-3-methoxybenzoate), but only *Aureobasidium pullulans* grew significantly in veratrate (3,4-dimethoxybenzoate). Those compounds believed to be metabolized through protocatechuate (e.g. ferulate, caffeate and *p*-coumarate) showed a remarkably consistent growth yield of about 33mg. dry wt. of mycelium/100ml. of culture in the three moulds examined in detail (Table 4). Aromatic aldehydes, however, were poor substrates for growth.

Certain species showed unusual features; *Debaryomyces hansenii* N.C.Y.C. 9 and *Debaryomyces kloeckeri* N.C.Y.C. 8 appeared to lack the ability to hydroxylate or assimilate monophenols, utilizing only protocatechuate and catechol as carbon sources. *Candida tropicalis* strains grew well on the three isomeric dihydric phenols (Harris & Ricketts, 1962), but not when the molecule contained an additional carboxyl group.

All the organisms grew readily on the cyclic (non-aromatic) plant acid, quinate, which is widely

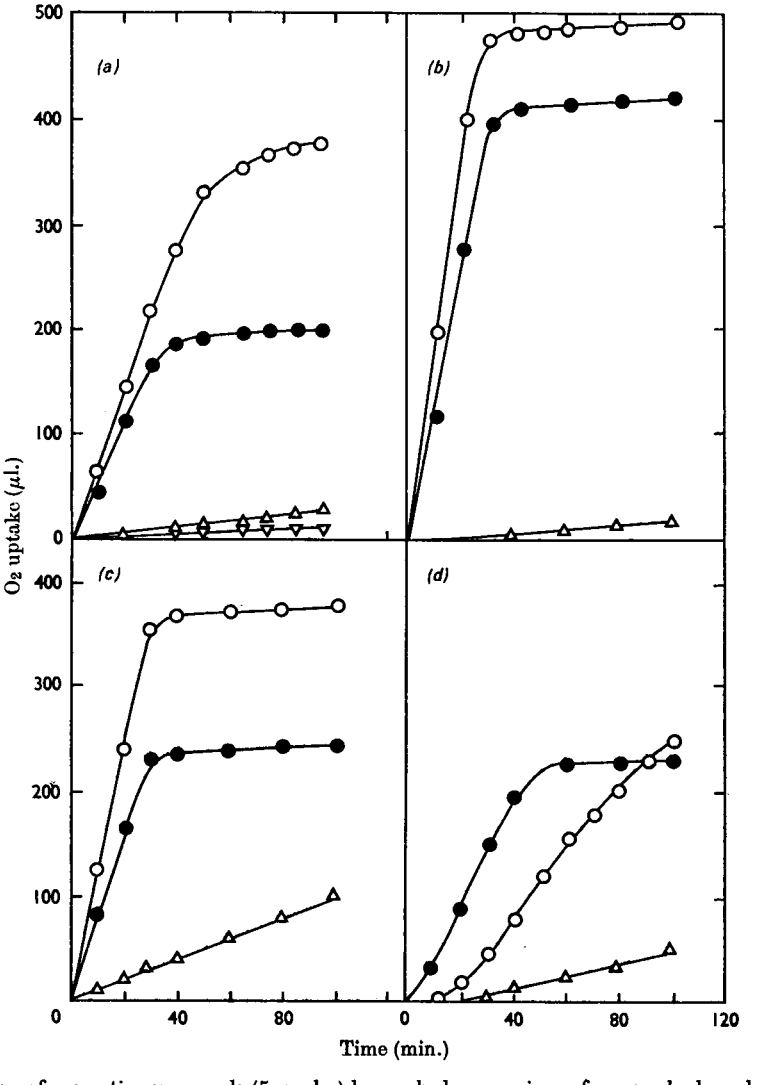


Fig. 2. Oxidation of aromatic compounds (5 μmoles) by washed suspensions of some *p*-hydroxybenzoate-grown 'typical' (group I) organisms. Flasks contained: (*a*) 21·0 mg. dry wt. for 'yeast XY'; (*b*) 10·3 mg. dry wt. for *Rhodotorula mucilaginosa*; (*c*) 9·8 mg. dry wt. for *Sporobolomyces* sp.; (*d*) 17·5 mg. dry wt. for *Debaryomyces hansenii* N.C.Y.C. 9 (the last organism was grown with protocatechuate as carbon source; it did not grow in *p*-hydroxybenzoate media). ○, *p*-Hydroxybenzoate; ●, protocatechuate; △, catechol; ▽, *p*-cresol.

distributed in plants both in the free state and particularly as a constituent of chlorogenic acid (3,4-dihydroxycinnamoylquinate) (Pridham, 1965).

Metabolism of aromatic compounds by 'typical' organisms

Oxidation by washed-cell suspensions. From chemical and enzymic evidence, our fungi could be divided into two groups depending on the pathways by which they degraded *p*-hydroxybenzoate and protocatechuate. Group I comprised all the fungi that oxidized *p*-hydroxybenzoate through protocatechuate to β-carboxymuconate, lactonized this substrate to β-carboxymuconolactone and converted it ultimately into β-oxoadipate. Most of the fungi examined fell into this category; the atypical forms are described separately (see below).

Organisms of group I, after growth on *p*-hydroxybenzoate, rapidly oxidized this substrate and protocatechuate (29–116 μl. of oxygen/hr./mg. dry wt. of cells depending on the organism) at pH 5·6 (Fig. 2).

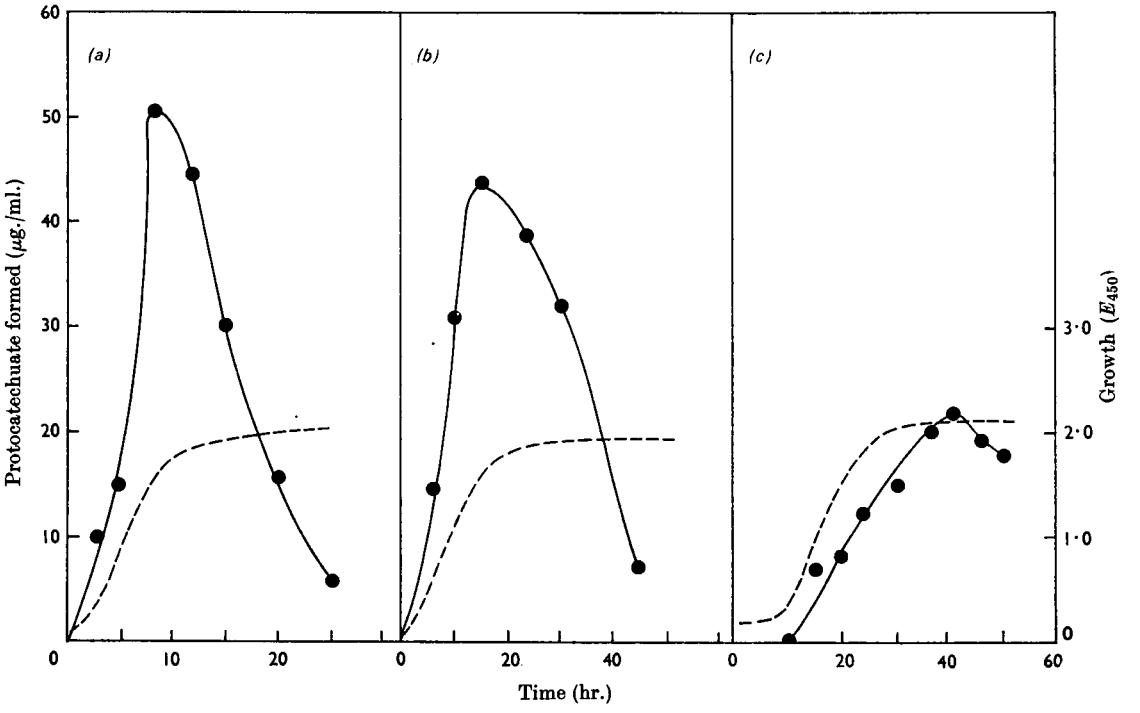


Fig. 3. Formation of protocatechuate during growth of some fungi at 25° in *p*-hydroxybenzoate medium (9l. batches aerated at approx. 3l. of air/min.): (*a*) *Rhodotorula mucilaginosa* I; (*b*) *Sporobolomyces* sp.; (*c*) *Fusarium oxysporum*. ----, Growth measured turbidimetrically; ●, protocatechuate formed.

Catechol, phenol and the three cresol isomers were oxidized by *p*-hydroxybenzoate-grown cells only at very low rates, but, on transfer to media containing catechol, induction of the enzymes effecting dissimilation of this substrate occurred after a lag of about 1hr. *Debaryomyces hansenii* N.C.Y.C. 9, after growth with protocatechuate, oxidized *p*-hydroxybenzoate after a short lag (10min.) even though this substrate would not support growth of the organism. Endogenous respiration was consistently high in this particular organism under all growth conditions.

Group I fungi all produced abundant growth with glucose as carbon source, but washed cells of *Rhodotorula mucilaginosa* or *Sporobolomyces* adapted to *p*-hydroxybenzoate or catechol oxidation within 30min. The presence of a methoxy group did not hinder oxidation; *p*-hydroxybenzoate-grown *Sporobolomyces* oxidized both *m*- and *p*-methoxybenzoate immediately, but vanillate only after a lag period. Oxidation of the aromatic substrates by washed-cell suspensions never approached the theoretical value; it was usually about 65–70% of the theoretical value, but not infrequently fell to 40% for protocatechuate oxidation by *Debaryomyces hansenii* N.C.Y.C. 9. 2,4-Dinitrophenol (0·01–0·5mM) and azide (0·05–3mM) did not increase the extent of substrate oxidation by the yeasts.

Conversion of p*-hydroxybenzoate and other aromatic acids into protocatechuate.* During the growth of group I organisms on *p*-hydroxybenzoate or *p*-methoxybenzoate, an *o*-dihydric phenol was detected in the growth medium by the Evans (1947) test, reaching maximum concentrations at approx. 20hr. and thereafter rapidly disappearing in well-aerated cultures (Fig. 3). Much higher concentrations of this *o*-dihydric phenol accumulated and persisted under conditions of restricted aeration. This material gave a positive reaction only after heating in the tungstate-modified Evans test, and on paper chromatography in solvents *A*, *B* and *C* it moved with the same R_F and gave the same colour reactions with ferric chloride and alkaline diazotized I.C.I. 5091 as authentic protocatechuate.

Protocatechuate was isolated and characterized from *p*-hydroxybenzoate-grown cultures of *Sporobolomyces* (see the Materials and Methods section), from vanillate-grown cultures of *Rhodotorula mucilaginosa* I and identified chromatographically in *p*-hydroxybenzoate-, ferulate- or *p*-coumarate-grown cultures of *Rhodotorula macerans*, *Rhodotorula*

Table 5. p-*Hydroxybenzoate hydroxylase in extracts of fungi*

Results are corrected for the rate of oxidation of reduced nicotinamide nucleotide in the absence of *p*-hydroxybenzoate. Results in parentheses are for NADH oxidation with the same extract.

Organism	Growth substrate	Hydroxylase activity (mμmoles of NADPH oxidized/min./mg. of protein)
Rhodotorula mucilaginosa I	*p*-Hydroxybenzoate	20·2
	Glucose	1·7
Sporobolomyces sp.	*p*-Hydroxybenzoate	20·0
Debaryomyces hansenii IC	*p*-Hydroxybenzoate	40·8
	Vanillate	0·3
Debaryomyces subglobosus N.C.Y.C. 459	*p*-Hydroxybenzoate	0·6 (11·4)
	Catechol	0 (0)
Endomycopsis sp. A	*p*-Hydroxybenzoate	31·0
Aureobasidium pullulans	*p*-Hydroxybenzoate	0·5 (11·6)
	Glucose	0 (0)
Cylindrocephalum sp.	*p*-Hydroxybenzoate	31·5
Fusarium oxysporum	*p*-Hydroxybenzoate	17·5
	Glucose	0
Neurospora crassa	Vanillate	0
Aspergillus niger L6	*p*-Hydroxybenzoate	3 (23·5)
Penicillium spinulosum P6	*p*-Hydroxybenzoate	6 (20)
	Glucose	0 (0)
Polystictus versicolor	Glucose, then induced with *p*-hydroxybenzoate	19
Pseudomonas putida A3.12	*p*-Hydroxybenzoate	241
Nocardia erythropolis CA4	*p*-Hydroxybenzoate	19·6

mucilaginosa, *Fusarium oxysporum*, *Cephalosporium acremonium*, *Penicillium spinulosum* and *Aureobasidium pullulans*.

p-*Hydroxybenzoate hydroxylase in the fungi*. The hydroxylating enzyme for *p*-hydroxybenzoate has been purified from *Pseudomonas putida* and *Pseudomonas acidovorans* (Hosokawa & Stanier, 1966) and from *Pseudomonas desmolytica* (Yano, Morimoto, Higashi & Arima, 1966), in each of which it has been found to be a labile FAD enzyme, completely specific for NADPH as the hydrogen donor.

This enzyme was present in extracts of most of the yeasts and moulds examined (Table 5) provided they had been grown with *p*-hydroxybenzoate or a precursor; the enzyme was absent from, or present in only small amounts in, extracts of the same fungi grown in a glucose medium. It was also absent from *Neurospora crassa* induced with vanillate. The yeast enzymes were all very active with NADPH, but some of the other fungi showed higher activity when NADH was used as the hydrogen donor. *Aspergillus niger* and *Penicillium* gave low values for this hydroxylase in cell-free extracts even when prepared from freshly harvested mycelium and examined within 30min. Stoicheiometric oxidation of NADPH (0·57μmole) was effected by the addition of *p*-hydroxybenzoate (0·6μmole) to cuvettes containing: reduced nucleotide, 1μmole; tris–hydrochloric acid buffer, pH7·0, 150μmoles; extract of induced *Rhodotorula mucilaginosa* I (0·6mg. of protein); water to a total volume of 3ml. Activity of crude extracts decreased by some 60–70% over 3–4 days at 4°.

The product of *p*-hydroxybenzoate hydroxylation by NADPH and a partially purified preparation of *Rhodotorula mucilaginosa* was identified as protocatechuate by chromatography and a comparison of the u.v. spectra of the enzyme product at pH3·0 and 7·0 with those of authentic protocatechuate ($E_{max.}$ at pH3·0, 263 and 294mμ; $E_{max.}$ at pH7·0, 250 and 288mμ). In the presence of NADPH, crude extracts of this organism and *Fusarium*, *Sporobolomyces* or *Endomycopsis* formed β-oxoadipate from *p*-hydroxybenzoate.

Oxidation of protocatechuate and catechol by cell-free extracts. Extracts of all the micro-organisms, after growth with the appropriate carbon source, rapidly oxidized protocatechuate with the uptake of 1mole of oxygen, the release of 1mole of carbon dioxide and the formation of approx. 1mole of a β-oxo acid/mole of substrate (Table 6). The β-oxo acid gave a strong Rothera (1908) test and was isolated and identified as β-oxoadipate as described in the Materials and Methods section.

Table 6. *Oxidation of protocatechuate and catechol by cell-free extracts of selected fungi*

Warburg flasks contained: Na_2HPO_4–KH_2PO_4 buffer, pH 7·2, 200 μmoles; substrate, 0–10 μmoles; 20% (w/v) KOH in centre well, 0·2 ml.; extract, 0·5 ml. The final volume was adjusted to 3·0 ml. with water. A total of 2·8 mg. of extract protein was added for *Debaryomyces subglobosus*, 0·5 mg. of protein for *Sporobolomyces* sp. and *Rhodotorula mucilaginosa* and 1·3 mg. for *Fusarium*. Flasks were incubated till O_2 uptake was complete. —, Not measured.

Organism	Growth substrate	Substrate supplied	O_2 uptake (μmoles)	CO_2 evolved (μmoles)	β-Oxoadipate formed (μmoles)
Debaryomyces subglobosus N.C.Y.C. 459	*p*-Hydroxybenzoate	Protocatechuate (2 μmoles)	1·9	—	1·65
		Protocatechuate (4 μmoles)	3·9	—	2·65
	Catechol	Catechol (4 μmoles)	3·9	0·2	3·80
Sporobolomyces sp.	*p*-Hydroxybenzoate	Protocatechuate (1 μmole)	0·95	—	0·94
		Protocatechuate (3 μmoles)	2·80	2·9	2·5
		Protocatechuate (5 μmoles)	4·8	5·1	4·4
		Protocatechuate (10 μmoles)	9·8	9·7	7·8
Rhodotorula mucilaginosa I	*p*-Hydroxybenzoate	Protocatechuate (5 μmoles)	4·8	4·6	4·3
		Catechol (5 μmoles)	0·1	—	0·3
	Catechol	Catechol (5 μmoles)	4·9	0·1	4·7
Fusarium oxysporum	*p*-Hydroxybenzoate	Protocatechuate (3 μmoles)	2·8	—	2·6
		Protocatechuate (5 μmoles)	4·7	4·6	4·3

Catechol was oxidized by the yeasts (group I) only after they had been grown or previously induced in its presence. The end product of catechol oxidation by extracts was also identified as β-oxoadipate, but there was no evolution of carbon dioxide from this substrate (Table 6).

Enzyme patterns of aromatic-ring fission in the fungi

Conversion of protocatechuate into β-carboxymuconate: protocatechuate 3,4-oxygenase. The primary product of the ring cleavage of protocatechuate by bacteria employing the '*ortho*' route (McDonald *et al.* 1954; Cain & Cartwright, 1960; Ribbons & Evans, 1960; Stanier, Palleroni & Doudoroff, 1966; Ornston & Stanier, 1966) and by *Neurospora crassa* (Gross *et al.* 1956) is β-carboxymuconate, a reaction catalysed by protocatechuate 3,4-oxygenase. This enzyme has not been unequivocally identified in fungi other than *Neurospora crassa*, though Henderson (1963) demonstrated the presence of a 'protocatechuate oxidase' in extracts of *Aureobasidium* (*Pullularia*) *pullulans*.

When the oxidation of protocatechuate (0·5 μmole) by extracts of group I organisms was followed by spectrophotometric scanning of the u.v. absorption between 220 and 320 mμ at intervals of a few minutes, an intermediate that absorbed strongly at 260 mμ usually accumulated transiently if the reaction was carried out in phosphate buffer. From the respective molar extinction coefficients (see the Materials and Methods section) the conversion of protocatechuate into this product, β-carboxymuconate, was calculated (Fig. 4). Extracts of *Sporobolomyces* produced the ring-fission product in nearly quantitative amounts, whereas crude extracts of other species accumulated no more than 20% of the theoretical yield. With *Rhodotorula macerans*, however, simple dialysis of the crude extract overnight at 4° against several changes of water permitted approximately quantitative conversion of protocatechuate into the ring-fission product.

Protocatechuate 3,4-oxygenase was partially purified from extracts of *Rhodotorula mucilaginosa* I as follows. Extract was treated with saturated ammonium sulphate solution (Table 1) and the bulk of the oxygenase activity found to lie in the 40–50%-saturated ammonium sulphate fraction. This fraction (17 mg. of protein/ml.) was chromatographed on a Sephadex G-200 column (25 cm. × 2 cm.) equilibrated with 0·1 M-phosphate buffer, pH 7·0, and 5 ml. fractions were eluted. The oxygenase was eluted in fractions 5–8 (suggesting a high molecular weight), which were pooled and dialysed against water for 12 hr. The precipitate formed in the dialysis sac contained the enzyme activity; it was washed with a saturated solution of ammonium sulphate, dissolved in 0·1 M-phosphate buffer, pH 7·0, and dialysed against this buffer to remove the salt. This preparation (approx. sixfold increase in specific activity compared with crude extracts) contained no lactonizing or delactonizing enzyme.

The product of protocatechuate oxidation by the partially purified *Rhodotorula* oxygenase was identified as β-carboxy-*cis*–*cis*-muconate on the following data. (1) Manometric experiments showed that for

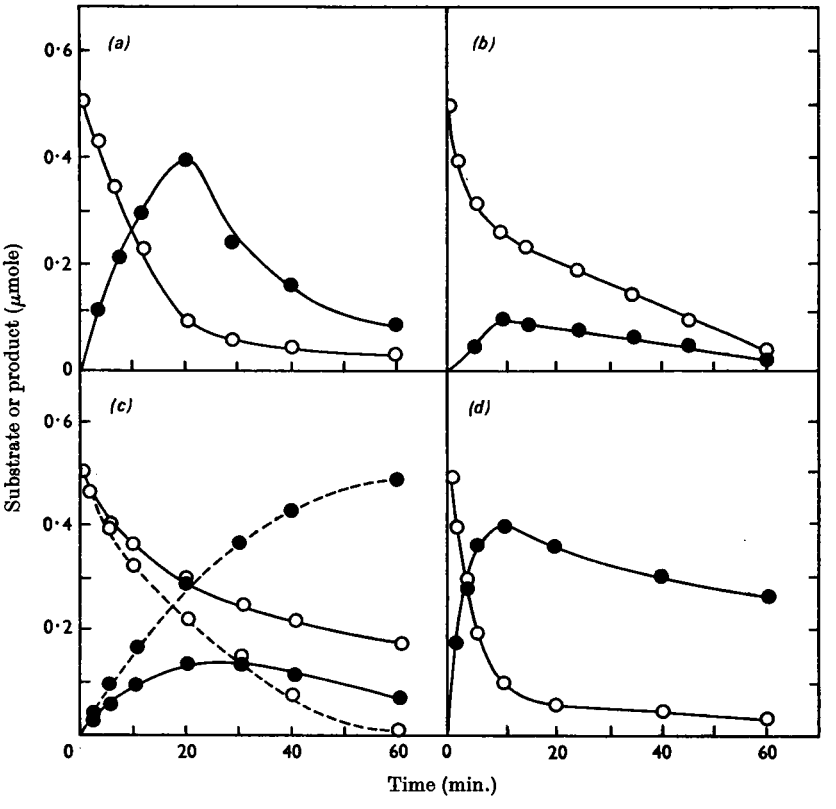


Fig. 4. Formation of β-carboxymuconate from protocatechuate by extracts of representative fungi previously grown in *p*-hydroxybenzoate media. Cuvettes contained: Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, 200μmoles; protocatechuate, 0·5μmole; cell-free extract, 0·1ml. (*a*) 0·29mg. of protein for *Rhodotorula macerans*; (*b*) 0·21mg. of protein for 'yeast XY'; (*c*) 1·10mg. of protein for *Rhodotorula mucilaginosa* I; (*d*) 0·06mg. of protein for *Fusarium oxysporum*. ——, Results obtained with crude extracts; – – – –, results obtained after dialysis of the extract against water for 24hr. ○, Protocatechuate; ●, β-carboxymuconate.

every mole of protocatechuate oxidized 1mole of oxygen was consumed, no carbon dioxide was evolved and 0·95mole of β-carboxymuconate (determined spectrophotometrically) was produced. (2) Paper chromatography of the reaction mixtures at the completion of such oxidations showed an unsaturated compound that co-chromatographed with authentic β-carboxymuconate in alkaline solvent systems *A* and *C*. In acid solvents decomposition took place. (3) The u.v. spectra of the enzymic product and authentic β-carboxymuconate (provided by Dr S. Gross) at pH 7·0 were identical. Both fungal and authentic compounds showed the typical bathochromic and hyperchromic shift in their u.v.-absorption maxima as the result of heating at 100° for 30min. (Fig. 5). (4) The i.r. spectrum of the trisodium salt, isolated by a procedure similar to that used for enzymic preparation of this material (see the Materials and Methods section) and measured in a potassium bromide disk, exhibited intense absorption bands at 1560 and 1375cm.$^{-1}$ due to the anti-symmetrical and symmetrical vibrations of the carboxylate ion. Superimposed in the former at 1605cm.$^{-1}$ was a sharp band due to the C=C stretching vibrations of a conjugated double bond. An intense band found at 817cm.$^{-1}$ and a weaker one at 702cm.$^{-1}$ are characteristic of the out-of-plane hydrogen deformations of the group –CH=CH–CO–OR (*cis*) (Bellamy, 1958) and of the disubstituted –CH=CH– (*cis*) grouping respectively. These absorption bands are consistent with the structure of the trisodium salt of 4-carboxyhexa-*cis*–*cis*-2,4-dienoic acid (β-carboxy-*cis*–*cis*-muconic acid). A weak isolated band at 1280cm.$^{-1}$ suggested the presence of some *cis*–*trans*-isomer, an impurity found in most preparations.

β-Carboxymuconate was identified as the product of protocatechuate oxidation by extracts of *Sporobolomyces* sp., *Fusarium* spp. and *Rhodotorula macerans* in a similar manner, but the presence of

Table 7. *Activities of enzymes of the protocatechuate and catechol pathways in extracts of fungi*

Activities were all measured at pH 7·0 in 0·1 M-tris–HCl buffer except where indicated by *italics*, where measurements were made in 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0. Results in parentheses were obtained in 0·1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 5·6. All values are means of at least two assays with different extract preparations. —, Not tested.

Organism	Growth substrate	Enzyme activity (mμmoles degraded/min./mg. of protein)								
		Protocatechuate*	β-Carboxymuconate	β-Carboxymuconolactone	γ-Carboxymuconolactone	Catechol	*cis–cis*-Muconate	(+)-Muconolactone	β-Oxoadipate enol lactone	γ-Carboxymethylenebutanolide
Typical (group I) organisms										
Rhodotorula mucilaginosa I	*p*-Hydroxybenzoate	226	192	295	0	0	1	0	13	8
	Glucose	9	30	17	0	0	0	0	0	—
Rhodotorula mucilaginosa II	*p*-Hydroxybenzoate	*140*	*20(208)*	*615*	*0*	*0*	*0*	*2*	—	—
Rhodotorula macerans VC4	*p*-Hydroxybenzoate	*253*	*51*	*125*	*0*	*0*	*0*	*13*	*17*	—
Sporobolomyces sp.	*p*-Hydroxybenzoate	250	372	377	0	0	0	10	30	28
'Yeast XY'	*p*-Hydroxybenzoate	*751*	*273*	*141*	*0*	*0*	*0*	*61*	—	—
Endomycopsis sp. A	*p*-Hydroxybenzoate	127	860	560	0	0	0	27	50	—
Fusarium oxysporum	*p*-Hydroxybenzoate	150	365	430	5	12	0	32	51	15
	Succinate	0	33	14	0	0	2	0	0	—
	Catechol	0	60	56	—	45	38	630	—	17
Aspergillus niger L6	*p*-Hydroxybenzoate	199	341	78	0	8	0	0	19	7
	Glucose	11	24	5	—	1	0	0	0	28
Penicillium spinulosum P6	*p*-Hydroxybenzoate	184	887	605	55	14	0	22	16	15
	Glucose	0	178	63	0	0	0	24	0	—
	Glucose, then induced with catechol	4	254	97	—	83	17	152	198	—
Cylindrocephalum sp.	*p*-Hydroxybenzoate	62	409	179	0	2	0	31	9	—
Cephalosporium acremonium	*p*-Hydroxybenzoate	63	82	296	0	5	2	13	11	16
Phoma sp.	*p*-Hydroxybenzoate	45	560	214	0	4	1	52	22	0
Neurospora crassa SY4	Glucose, then induced with vanillate	3	354	167	5	0	0	10	26	0
	Glucose, no inducer added	0	44	7	0	0	0	0	0	0

Polystictus versicolor	Glucose + vanillate (*a*)	21	12	85	0	2	5	0	0	—
	Glucose, then induced with succinate (*b*)	2	2	8	—	0	3	0	—	—
	Concentrated supernatant medium from (*a*) (extracellular enzyme protein)	0	0	0	—	—	—	—	—	—
Vararia granulosa	*p*-Hydroxybenzoate	32	71	330	0	19	0	0	0	41
Schizophyllum commune	*p*-Hydroxybenzoate	40	378	163	0	56	0	5	50	60
Sphaerobolus stellatus	*p*-Hydroxybenzoate	52	91	102	0	0	0	0	0	0
Mucor rammanianus	*p*-Hydroxybenzoate	18	113	61	0	0	0	0	0	10
Fusarium sp. II	*p*-Hydroxybenzoate	94	340	61	0	1	0	16	0	0
	Glucose	4	39	2	0	5	0	0	0	—
Mucor hiemalis	*p*-Hydroxybenzoate	390	556	410	—	70	2	35	70	0
Atypical (group II) organisms										
Debaryomyces subglobosus	*p*-Hydroxybenzoate	*10*	*0*	*0*	*0*	*160*	*64*	*92*	*180*	—
N.C.Y.C. 459	Catechol	*0*	*0*	*0*	*0*	*937*	*466*	*1700*	—	—
Debaryomyces hansenii IC	*p*-Hydroxybenzoate	45	5	0	0	32	34	106	140	—
	Catechol (+glucose, 0·5%, w/v)	0	0	4	—	34	28	55	60	61
Aureobasidium pullulans	*p*-Hydroxybenzoate	134	178	200	0	60	25	63	—	75
	Glucose, then induced with catechol	2	18	17	0	36	39	120	76	27
	Glucose	0	23	5	—	25	1	8	8	34
Candida tropicalis N.C.Y.C. 470	Catechol	0	0	0	—	10	72	441	—	8
Bacteria (for comparison)										
Pseudomonas putida A3.12	*p*-Hydroxybenzoate	2650	940	0	2905	0	0	10	1175	35
	Succinate	5	21	0	25	0	0	0	24	—
Nocardia erythropolis CA4	*p*-Hydroxybenzoate	512	381	0	625	8	1	25	688	48

* Rates for protocatechuate are a measure of its disappearance. In *Debaryomyces* species these values are not a measure of protocatechuate 3,4-oxygenase.

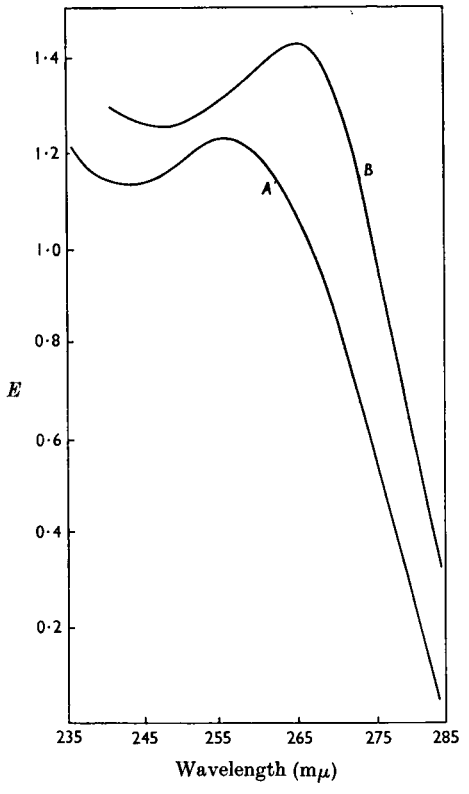


Fig. 5. Spectra in 0·1 M-phosphate buffer, pH 7·0, of the product of protocatechuate oxidation by the purified protocatechuate 3,4-oxygenase of *Rhodotorula mucilaginosa*. The spectra were recorded before (curve *A*) and after (curve *B*) boiling the product for 30 min.

protocatechuate 3,4-oxygenase was established spectrophotometrically in all *p*-hydroxybenzoate-grown organisms of group I (Table 7).

Properties of fungal protocatechuate 3,4-oxygenases. The oxygenase in extracts of *Rhodotorula mucilaginosa* resembled that of *Neurospora crassa* (Gross *et al.* 1956) and *Pseudomonas putida* (McDonald *et al.* 1954) in showing no pH optimum, but a steady rise in activity from pH 5·0 to pH 9·0, above which measurements were not taken because of increasing autoxidation (Fig. 6*a*). The oxygenases of *Fusarium oxysporum* and *Sporobolomyces*, however, had optima at pH 8·0 (Fig. 6*b*). When protocatechuate oxidation by intact washed cells of *Rhodotorula mucilaginosa* or *Sporobolomyces* was examined, a very clear pH optimum at 6·0 was found, irrespective of whether the cells had been grown at pH 5·5, 6·4 or 7·2. Further, washed cells showed appreciable oxidation of protocatechuate at pH 4·5, whereas extracts did not oxidize this substrate below pH 6·0. The mechanism for protocatechuate transport into the intact cell therefore largely controls the effectiveness of protocatechuate oxidation in *Rhodotorula* and *Sporobolomyces*.

The nature of the buffer had no effect on oxidation rates by extracts or the partially purified enzyme. The protocatechuate 3,4-oxygenase of *Rhodotorula mucilaginosa* had K_m 33 μM for protocatechuate in 0·1 M-phosphate buffer, pH 7·5. It was unaffected by chelating agents (1 mM) or by aldehyde- or quinone-trapping agents such as semicarbazide (0·1 mM). Cyanide (1 mM) and fluoride (10 mM) caused less than 10% inhibition. Vanillate, catechol and the other isomers of dihydroxybenzoate were not oxidized by the partially purified enzyme, nor did vanillate act as an inhibitor, but catechol was a strong competitive inhibitor (K_i 0·2 mM). The *Rhodotorula* enzyme was completely inactivated in 15 min. at 55°.

Conversion of β-carboxymuconate into β-carboxymuconolactone: lactonizing enzyme in the fungi. Micro-organisms lactonize β-carboxymuconate either to 3-carboxy-4-hydroxyhex-2-enedioic (1→4)-lactone (β-carboxymuconolactone, VI) or to 4-carboxy-4-hydroxyhex-2-enedioic (1→4)-lactone (γ-carboxymuconolactone, VII). The presence of β-carboxymuconate-lactonizing enzyme in extracts of group I fungi from *p*-hydroxybenzoate- or protocatechuate-grown cultures is shown in Table 7; the activity of this enzyme was absent from or radically decreased in extracts from cells grown with glucose or with catechol as the carbon source.

Purification of the lactonizing enzyme from *Rhodotorula mucilaginosa* I was effected as follows. Maximum lactonizing activity (0·11 unit/mg. of protein in crude extracts) was located in that fraction of crude extracts precipitated by ammonium sulphate between 30% and 50% saturation (Table 2). After ammonium sulphate fractionation and dialysis, the enzyme solution in 2 mM-phosphate buffer, pH 6·0 (0·18 unit/mg.; 130 mg. of enzyme protein), was gently shaken with aged calcium phosphate gel (5 ml., 120 mg. dry wt.) for 30 min. at 4°, resulting in adsorption of nearly all the protein. Residual delactonizing enzyme was eluted from the washed gel with 0·01 M-phosphate buffer, pH 6·1. Lactonizing enzyme (1·1 units/mg.) was eluted with two washings of 0·01 M-phosphate buffer, pH 7·5, and was free of delactonizing activity, but still contained some protocatechuate 3,4-oxygenase (approx. 1% compared with lactonizing enzyme). This preparation was dialysed to remove phosphate, made 0·01 M with respect to tris–hydrochloric acid buffer, pH 7·0, and concentrated by dialysis against 10% (w/v) Carbowax 4000. An overall purification of some tenfold was achieved. Further purification could be effected by heating the crude extracts at 50° for 5 min. before

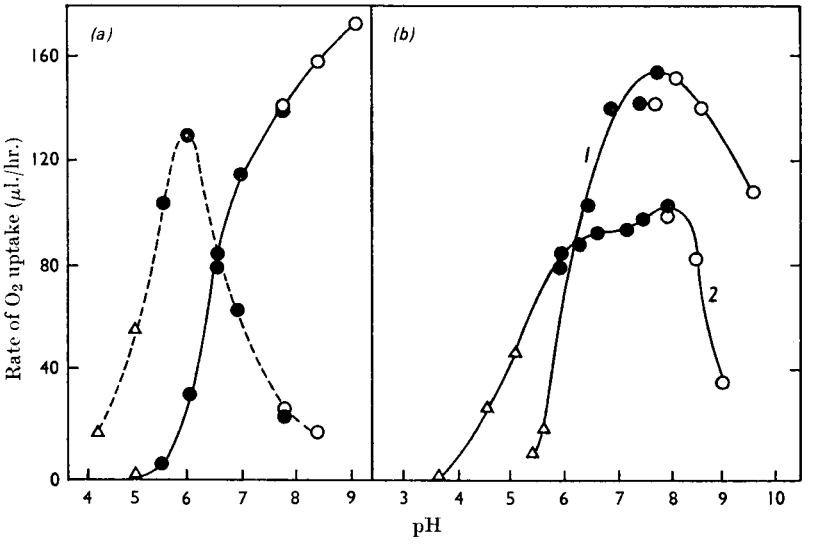


Fig. 6. pH optima for the protocatechuate 3,4-oxygenases of: (*a*) *Rhodotorula mucilaginosa* in a washed-cell suspension (16·9mg. dry wt.) (----) and a cell-free extract (0·55mg. of protein) from the same suspension (——); (*b*) extracts of *Sporobolomyces* sp. (0·25mg. of protein) (*1*) and *Fusarium oxysporum* (0·70mg. of protein) (*2*). Flasks contained in a total volume of 3ml.: the indicated buffer, 200μmoles; protocatechuate, 10μmoles; 20% KOH, 0·2ml. in centre well. Control flasks without substrate were run at each pH. The buffers were: △, acetate; ●, Na_2HPO_4–KH_2PO_4; ○, tris–HCl.

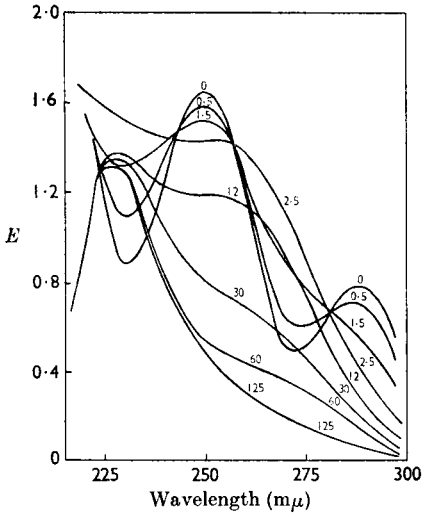


Fig. 7. Spectrophotometric demonstration with a recording spectrophotometer of the enzymic conversion of protocatechuate into β-carboxymuconolactone by a partially purified extract (see the Materials and Methods section) of *Rhodotorula mucilaginosa*. The cuvettes contained in a total volume of 3ml.: tris–HCl buffer, pH 7·2, 200μmoles; extract (0·13mg. of protein). The reaction was started by the addition of 0·6μmole of protocatechuate and the spectrum determined at the times (min.) indicated. The example demonstrates about 90% overall conversion (from extinction measurements at 290 and 240mμ).

proceeding with salt fractionation and gel treatment. This treatment completely eliminated oxygenase and delactonizing activity and gave an overall purification of some 25-fold. The lactonizing enzyme was much more heat-sensitive, however, in partially purified preparations. This preparation was used to prepare β-carboxymuconolactone in small quantities for spectrophotometric experiments, but the limited quantities of β-carboxymuconate available precluded production of the lactone from this source on a scale sufficiently large for isolation. By omitting the heat treatment, an enzyme preparation from *Rhodotorula mucilaginosa* was obtained that permitted the formation of β-carboxymuconolactone from the readily available protocatechuate on a scale large enough for chemical characterization (see the Materials and Methods section) and demonstrated the direct conversion of protocatechuate into this key intermediate via β-carboxymuconate (Fig. 7). The product from *Rhodotorula mucilaginosa* was identified as β-carboxymuconolactone as follows. (1) It readily formed a hydroxamate under alkaline conditions (Cain, 1961); the free lactone had the same R_F as the authentic compound in solvents *A*, *G* and *H*. (2) The formation of the lactone from β-carboxymuconate (10μmoles) by purified *Rhodotorula* lactonizing enzyme in unbuffered aqueous solution was followed with an automatic titrator and required the addition of 9·7μequiv. of hydrochloric

acid to neutralize the effect of lactonizing the carboxyl group. (3) The u.v. spectrum of the *Rhodotorula* product showed a sharp peak at 217mμ (indicating a conjugated double-bond system) and had ϵ 10300; the bacterial γ-carboxymuconolactone, besides being too unstable for isolation and analysis, had $E_{max.}$ at 230mμ and ϵ 4100 (Ornston & Stanier, 1966). (4) The i.r. spectrum of the isolated product showed absorption bands at 3400, 1700 and 1440cm.$^{-1}$, characteristic of carboxylic acid groups. Absorption bands observed at 1735 and 1635cm.$^{-1}$ were due to an $\alpha\beta$-unsaturated γ-lactone and the absorption of a C=C–C=O conjugated system respectively. (+)-Muconolactone had bands at 3400, 1700 and 1440cm.$^{-1}$ for carboxyl groups, 1740cm.$^{-1}$ for a γ-lactone and 1600 and 685cm.$^{-1}$ for a *cis*-double bond; the absorption of light at shorter wavelengths (greater wave number) by the double bond of β-carboxymuconolactone suggested the substitution of a carboxyl group on one of the carbon atoms of the double bond. The natural product and synthetic β-carboxymuconolactone (McDonald *et al.* 1954) had almost identical i.r. spectra. (5) Crude extracts or the delactonizing enzyme of *Neurospora crassa* rapidly converted the isolated product or authentic β-carboxymuconolactone (10μmoles) into β-oxoadipate (9·65μmoles). Synthetic β-carboxymuconolactone was also attacked by extracts of *Rhodotorula* or *Neurospora crassa*, but the reaction terminated when only half the substrate had been metabolized and the β-oxoadipate yield was only 4·3μmoles. γ-Carboxymuconolactone was attacked neither by *Rhodotorula* nor by *Neurospora crassa* (Fig. 8*a*). Conversely, crude extracts of *Pseudomonas putida* A 3.12 or *Nocardia erythropolis* would not attack the isolated product or authentic β-carboxymuconolactone (Fig. 8*b*), but they rapidly degraded γ-carboxymuconolactone, synthesized *in situ* from β-carboxymuconate with purified *Pseudomonas putida* lactonizing enzyme.

β-Carboxymuconolactone was identified as the product of lactonization of β-carboxymuconate in *Fusarium oxysporum*, *Aspergillus niger*, *Sporobolomyces* sp. and *Rhodotorula macerans*. The presence of the enzymes to attack β-carboxymuconolactone in extracts of all group I fungi grown with *p*-hydroxybenzoate or protocatechuate and the absence of enzymic attack by the same extracts on γ-carboxymuconolactone strongly suggests, however, that β-carboxymuconolactone is always the product of β-carboxymuconate lactonization in the fungi.

Properties of fungal lactonizing enzymes. The enzymes from *Rhodotorula mucilaginosa*, *Rhodotorula macerans* and *Neurospora crassa* had optimum pH 5·5–6·0 measured in histidine–hydrochloric acid buffer. Phosphate and citrate strongly inhibited

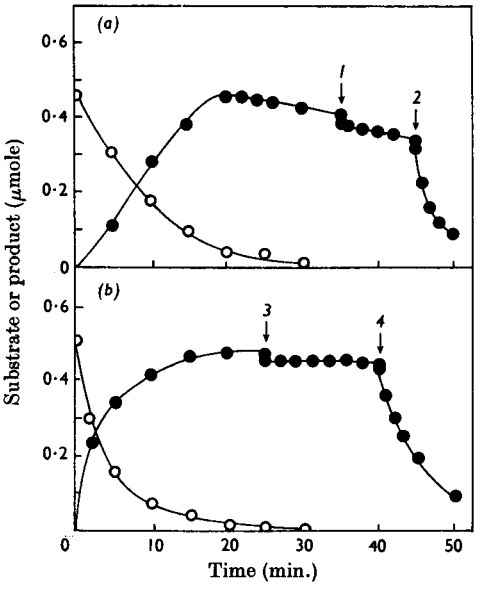


Fig. 8. Distinctive utilization of the two carboxymuconolactones by bacteria and fungi. (*a*) Formation of γ-carboxymuconolactone (●) from β-carboxymuconate (○) by purified lactonizing enzyme from wild-type *Pseudomonas putida*. Cuvettes contained in a total volume of 3ml.: tris–HCl buffer, pH 7·5, 250μmoles; purified lactonizing enzyme, 96μg. of protein. The reaction was started by the addition of β-carboxymuconate (0·5μmole) to the reaction cuvette; at arrow *1* extract of vanillate-induced *Neurospora crassa* (1·74mg. of protein) and at arrow *2* crude extract of *p*-hydroxybenzoate-grown *Pseudomonas putida* (0·12mg. of protein) were added to both cuvettes. (*b*) Formation of β-carboxymuconolactone (●) from β-carboxymuconate (○) by purified lactonizing enzyme from *Neurospora crassa*. Cuvettes contained in a total volume of 3ml.: tris–HCl buffer, pH 7·2, 250μmoles; purified lactonizing enzyme, 46μg. of protein. The reaction was started by the addition of β-carboxymuconate (0·5μmole). At arrow *3* a crude extract of *p*-hydroxybenzoate-grown wild-type *Pseudomonas putida* (0·15mg. of protein) and at arrow *4* crude extract of *p*-hydroxybenzoate-grown *Fusarium oxysporum* (0·13mg. of protein), were added to both cuvettes.

this enzyme both in the yeasts and all the other fungi tested (Table 7), though the inhibition was less pronounced for the same inhibitor concentration at low pH values than at pH 7·0 or higher. The activity of the enzyme from *Rhodotorula mucilaginosa* was not significantly affected by 1mM-EDTA, nor was it enhanced by the addition of bivalent metal ions (0·1–1mM); Co^{2+} and Cu^{2+} (0·1mM) were slightly inhibitory. The enzyme from *Rhodotorula mucilaginosa* had K_m 67μM in tris–hydrochloric acid buffer, pH 6·0; the K_m of the *Neurospora* enzyme under the same conditions was 18μM. These values are within the same order of magnitude as that

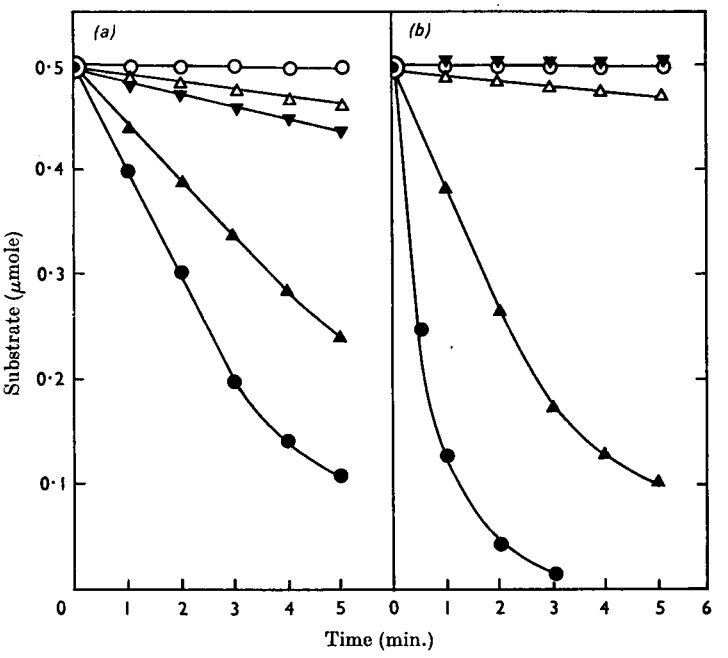


Fig. 9. Comparison of the rates of degradation by extracts of (*a*) *Schizophyllum commune* and (*b*) *Aspergillus niger* of the lactones possibly involved in aromatic-ring metabolism. Cuvettes contained in a total volume of 3 ml.: tris–HCl buffer, pH 7·0, 250 μmoles; cell-free extract, 0·1 ml. (0·39 mg. of protein for *Schizophyllum*; 0·54 mg. of protein for *Aspergillus*); substrate, 0·5 μmole. The degradation of β-oxoadipate enol lactone was followed by preincubating (10 min.) (+)-muconolactone and 0·02 ml. of a partially purified extract of ELH⁻ mutant of *Pseudomonas putida* originally grown in succinate + benzoate media (0·22 mg. of protein, 0·7 unit of muconolactone isomerase) followed by addition of the fungal extract. ○, (+)-Muconolactone; △, β-oxoadipate enol lactone; ▼, γ-carboxymethylenebutanolide (corrected for spontaneous decomposition); ▲, β-carboxymuconolactone; ●, β-carboxymuconate.

recorded for the *Pseudomonas putida* lactonizing enzyme (Ornston, 1966*a*). A molecular-weight determination on Sephadex G-200 gave a value for the *Neurospora* lactonizing enzyme of 190000, but the enzyme from *Rhodotorula mucilaginosa* had an unusually low value of 19000 (mean of three separate preparations); the molecular weight of *Pseudomonas putida* lactonizing enzyme was also 190000 (Ornston, 1966*a*).

The enzymes from *Rhodotorula mucilaginosa*, *Sporobolomyces* and *Fusarium oxysporum* did not catalyse the lactonization of *cis–cis*-muconate, but this substrate acted as a strong competitive inhibitor of β-carboxymuconate lactonization in each case.

Conversion of β-carboxymuconolactone into β-oxoadipate. All the fungi that metabolized β-carboxymuconate also degraded β-carboxymuconolactone at high rates (Fig. 9 and Table 7). With fungal extracts containing enzymes of the catechol pathway this enzyme pattern was, with a few exceptions, absent (Table 7). The end product of β-carboxymuconolactone breakdown by competent extracts was β-oxoadipate; this reaction was accompanied by almost stoicheiometric release of carbon dioxide (Table 8). γ-Carboxymuconolactone, produced *in situ* by purified lactonizing enzyme from *Pseudomonas putida*, was not degraded at a rate exceeding that of its spontaneous decomposition by extracts of any of the fungi examined except *Penicillium spinulosum* P6, but even here no β-oxoadipate was formed.

β-Carboxymuconolactone metabolism by extracts of *Rhodotorula mucilaginosa* I, *Sporobolomyces* and *Fusarium oxysporum* was inhibited, in a competitive manner, both by (+)-muconolactone and by γ-carboxymuconolactone when the analogues were preincubated for 10 min. with enzyme. The presence of (+)-muconolactone (1·0 μmole) caused 30% inhibition of β-carboxymuconolactone (0·5 μmole) breakdown by *Sporobolomyces* extract (0·09 mg. of protein) and by *Rhodotorula mucilaginosa* extract (0·04 mg. of protein). The delactonizing enzyme from *Rhodotorula mucilaginosa*, partially purified (tenfold) by ammonium sulphate fractionation (Table 2) and chromatography on a Sephadex G-200 column

Table 8. *β-Oxoadipate formation from lactones and their precursors by extracts of* p*-hydroxybenzoate-grown* Rhodotorula mucilaginosa *I*, Fusarium oxysporum *and* Aspergillus niger *L6*

Duplicate sets of Warburg flasks contained: tris–HCl buffer, pH 7·0, 200 μmoles; substrate, in one side arm, 3 μmoles; 4N-H_2SO_4 in second side arm (in first set of flasks only), 0·2 ml.; extract, 0·5 ml. (*Rhodotorula*, 3·7 mg. of protein; *Fusarium*, 1·78 mg. of protein; *Aspergillus*, 3·5 mg. of protein); water to a total volume of 3·0 ml. At the end of incubation (1 hr. for *Rhodotorula* and *Fusarium*; 30 min. for *Aspergillus*), the flask contents of the second set of flasks were adjusted to pH 3·5 with acetic acid (0·1 ml.) and 0·1M-4-aminoantipyrine (0·4 ml.) was added to the side arm. After equilibration, the catalyst and flask contents were mixed and CO_2 evolution was determined. Results are corrected for CO_2 and β-oxoadipate formed in control flasks without substrate or without enzyme.

Organism	Substrate	CO_2 evolved (μmoles)	β-Oxoadipate formed (μmoles)
Rhodotorula mucilaginosa I	β-Carboxymuconate	3·0	2·8
	β-Carboxymuconolactone	2·6	2·5
	cis–cis-Muconate	0	0·2
	(+)-Muconolactone	0·1	0·15
Fusarium oxysporum	β-Carboxymuconate	2·7	3·1
	β-Carboxymuconolactone	2·3	2·8
	cis–cis-Muconate	0·1	0·1
	(+)-Muconolactone	0	0·7
	γ-Carboxymethylenebutanolide	—	0·5
	Dilactone	0	1·1
Aspergillus niger L6	β-Carboxymuconate	2·5	2·9
	β-Carboxymuconolactone	2·3	3·0
	(+)-Muconolactone	0·1	0·5
	(+)-Muconolactone and 1 unit of muconolactone isomerase	0·2	0·1
	γ-Carboxymethylenebutanolide	—	0·3

(25 cm. × 2 cm.), had a sharp pH optimum at 9·1 (Fig. 10); the same enzyme from *Neurospora crassa*, *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium spinulosum* also had an optimum within the range pH 8·5–9·3.

The K_m of the *Rhodotorula* enzyme, determined in 0·1M-tris–hydrochloric acid buffer, pH 8·6, was 0·4mM. The *Neurospora* enzyme, under similar conditions, had K_m 0·33mM. The delactonizing enzyme in crude extracts of *Rhodotorula mucilaginosa* was very heat-labile, the half-life at 50° being approx. 90sec. Partially purified preparations of the enzyme in some fungal extracts were even more sensitive to heat, 2min. at 45° inactivating the *Penicillium spinulosum* delactonizing enzyme and 10min. at 45° inactivating the *Aspergillus* delactonizing enzyme.

Evidence for a C_6 *lactone between β-carboxymuconolactone and β-oxoadipate.* Extracts of *Neurospora crassa* that degraded β-carboxymuconolactone to β-oxoadipate did not attack (+)-muconolactone (II) (Gross *et al.* 1956; Table 7 of this paper), nor did Gross *et al.* (1956) find evidence for a C_6 intermediate between β-carboxymuconolactone and β-oxoadipate. In more recent studies Ornston & Stanier (1966) and Cánovas & Stanier (1967*a*) identified β-oxoadipate enol lactone (III) as the first intermediate common to both the catechol and protocatechuate pathways in several Gram-negative bacteria, and R. Y. Stanier (personal communication) has suggested that this or a similar compound might be similarly involved in the fungal metabolism of these aromatic compounds. The third possible isomer, γ-carboxymethylenebutanolide (I), was not utilized by extracts of several bacteria examined by Evans, Smith, Linstead & Elvidge (1951), but it was synthesized and its activity checked with the fungal extracts. The results for these three isomers are shown in the last three columns of Table 7. (+)-Muconolactone was negligibly utilized by most group I organisms grown on *p*-hydroxybenzoate medium, but after growth or induction with catechol much higher amounts of muconolactone isomerase were found. Utilization of γ-carboxymethylenebutanolide was also low in *p*-hydroxybenzoate-grown fungi (Fig. 9); the highest rate observed was in extracts of *Aureobasidium pullulans*, where it was approx. 35% of that for β-carboxymuconolactone metabolism. Appreciable amounts were also observed in *Schizophyllum commune* and *Vararia granulosa*.

β-Oxoadipate enol lactone itself was not available, so enol lactone hydrolase activity was tested with partially purified preparations of the ELH$^-$ mutant of *Pseudomonas putida* (see the Materials and Methods section). Negligible activity was found in all group I organisms that had been grown with protocatechuate. Activity was high, however, in

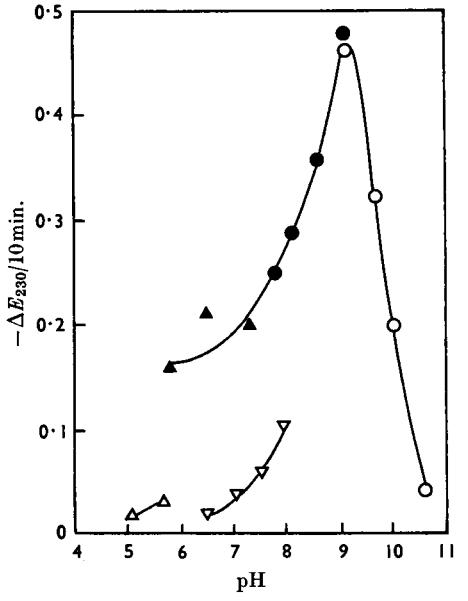


Fig. 10. pH optimum of the β-carboxymuconolactone-delactonizing enzyme from *Rhodotorula mucilaginosa* I. Cuvettes contained: buffer, 250μmoles; β-carboxymuconolactone, 0·5μmole; partially purified extract (see the text) of *p*-hydroxybenzoate-grown *Rhodotorula mucilaginosa* (76μg. of protein). Activities were measured in: ○, glycine–KOH buffer; ●, tris–HCl buffer; ▲, Na_2HPO_4–KH_2PO_4 buffer; ▽, ethylenediamine–HCl buffer; △, histidine–HCl buffer.

extracts of the same organisms grown with catechol and containing the other enzymes of the catechol pathway (Table 7). Neither the dilactone (V) (Landa & Eliasek, 1956) nor ββ-dihydroxyadipate (Cain, Ribbons & Evans, 1961) was attacked at rates in excess of their non-enzymic conversion into β-oxoadipate.

Metabolism of p-*hydroxybenzoate by atypical fungi*

Certain of the fungi examined (group II organisms), when grown in *p*-hydroxybenzoate medium, rapidly oxidized catechol as well as *p*-hydroxybenzoate and protocatechuate (Fig. 11). Further, when the formation of *o*-diphenols was followed in *p*-hydroxybenzoate cultures of *Debaryomyces subglobosus* N.C.Y.C. 459, both protocatechuate and catechol were observed by the modified Evans test (Fig. 12). Catechol was confirmed by extraction with ether from the neutral supernatant after centrifuging off the cells and chromatography with authentic material (R_F 0·76 and 0·31 respectively in solvents *A* and *C*). The sequence of appearance of these two intermediates suggested that catechol was produced from the protocatechuate.

Further evidence for conversion of protocatechuate into catechol by *Debaryomyces subglobosus* N.C.Y.C. 459 or *Debaryomyces hansenii* IC was afforded by an examination of the enzyme pattern in extracts of cells grown with either *p*-hydroxybenzoate or catechol as carbon source. *p*-Hydroxybenzoate-grown cells had no protocatechuate 3,4-oxygenase activity (though protocatechuate disappeared) and had only small or negligible activities of β-carboxymuconate-lactonizing enzyme and β-carboxymuconolactone-delactonizing enzyme. These extracts catalysed β-oxoadipate production from catechol, *cis–cis*-muconate and (+)-muconolactone when tested manometrically, and spectrophotometric examination showed the presence of catechol 1,2-oxygenase, *cis–cis*-muconate-lactonizing enzyme and muconolactone isomerase. These two *Debaryomyces* species were also the only yeasts showing significant enol lactone hydrolase activity in extracts from *p*-hydroxybenzoate-grown cells (Table 7). The specific activity of these enzymes in crude extracts was generally higher, however, if catechol itself was the carbon source. These results strongly suggest that the anomalous pathway diverges at protocatechuate, where decarboxylation to catechol occurs, but we have so far been unable to demonstrate the decarboxylase in extracts.

In many of the mycelia-forming moulds examined (e.g. *Aspergillus niger*, *Cephalosporum acremonium*, *Fusarium oxysporum* and *Penicillium spinulosum* P 6) the immediate oxidation of catechol by washed mycelial suspensions from *p*-hydroxybenzoate cultures was not paralleled by the appearance of the whole sequence of enzymes of the catechol pathway in extracts of these mycelia. Though catechol 1,2-oxygenase was detectable in small amounts, *cis–cis*-muconate-lactonizing enzyme was usually completely absent (Table 7), and on addition of catechol to cell-free extracts *cis–cis*-muconate, but no β-oxoadipate, accumulated quantitatively. *cis–cis*-Muconate was identified positively by isolation as follows. Catechol (1m-mole) was added in 10μmole portions to *Penicillium spinulosum* extract (0·53g. of protein) and phosphate buffer, pH 7·0 (20m-moles), in a magnetically stirred and well aerated vessel maintained at 30°. When no further reaction for catechol was noted, the product was isolated as described by Cain (1966*b*). It had m.p. 193°, not depressed by admixture with synthetic *cis–cis*-muconic acid (m.p. 195°). The u.v. spectra of both natural and synthetic products were identical.

If the same fungi were grown in catechol medium, however, all the enzymes of the catechol pathway were detected in mycelial extracts, *cis–cis*-muconate appeared only transiently and in small amounts and

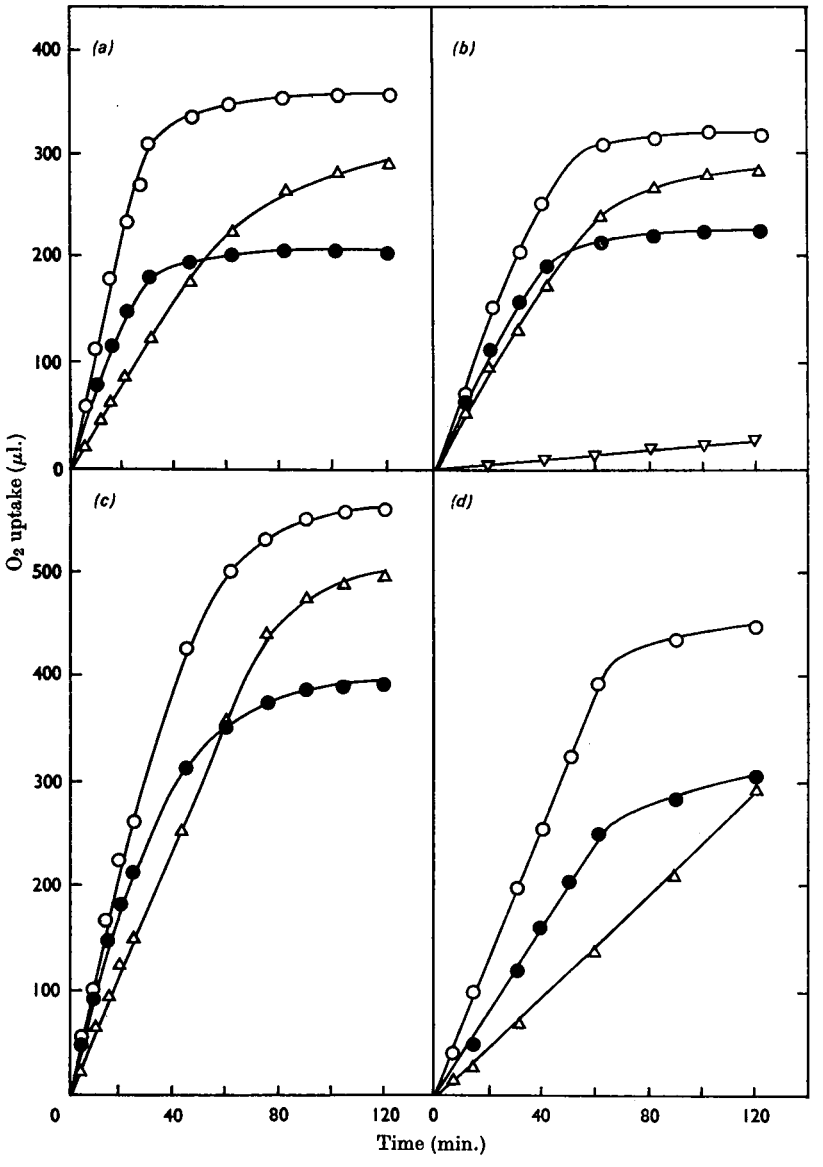


Fig. 11. Oxidation of aromatic compounds (5 µmoles) by washed suspensions of *Fusarium oxysporum* and some 'atypical' (group II) organisms after growth in *p*-hydroxybenzoate medium. Flasks contained: (*a*) 10·0 mg. dry wt. for *Fusarium oxysporum*; (*b*) 8·4 mg. dry wt. for *Aureobasidium pullulans*; (*c*) 22·0 mg. dry wt. for *Debaryomyces subglobosus* N.C.Y.C. 409; (*d*) 4·0 mg. dry wt. for *Debaryomyces hansenii* IC. ○, *p*-Hydroxybenzoate; ●, protocatechuate; △, catechol; ▽, phenol.

β-oxoadipate was consistently formed, as indicated by a positive Rothera test, when extracts were incubated with catechol (5 µmoles).

Further metabolism of β-oxoadipate

Extracts of *Pseudomonas* (Katagiri & Hayaishi, 1957) and *Moraxella calcoacetica* (Cánovas & Stanier, 1967*a*) converted β-oxoadipate into succinate and acetyl-CoA in a reaction requiring succinyl-CoA and CoA and catalysed by a transferase and a thiolase. The two enzymes of this sequence have also been demonstrated in *Nocardia erythropolis* (Cain *et al.* 1968), but Ottey & Tatum (1957) found no requirement for succinyl-CoA nor any evidence for the formation of β-oxoadipyl-CoA

during metabolism of β-oxoadipate by *Neurospora crassa*. They concluded that a hydrolytic reaction was responsible for β-oxoadipate cleavage in this fungus.

In *Aspergillus niger*, however, a requirement for succinyl-CoA was clearly indicated by incubating freshly prepared extracts (2·7mg. of protein) at 30° in a total volume of 3·0ml. with 10μmoles of β-oxoadipate, 20μmoles of magnesium chloride, 40μmoles of GSH, 3μmoles of CoA, 100μmoles of tris–hydrochloric acid buffer, pH 8·0, and, in

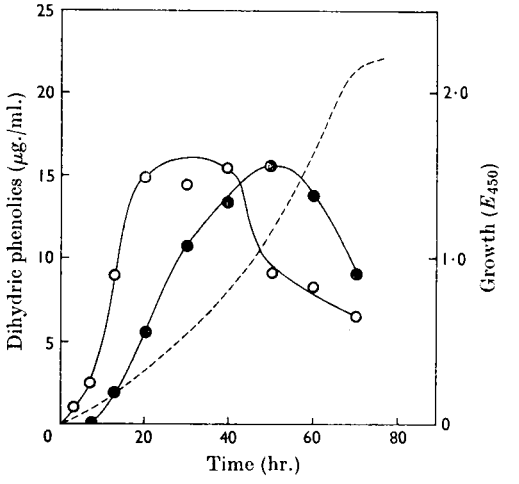


Fig. 12. Formation of dihydric phenolic compounds during growth of *Debaryomyces subglobosus* at 25° in *p*-hydroxybenzoate media (9l. of culture aerated at 3l. of air/min.). ○, Protocatechuate; ●, catechol; ----, growth.

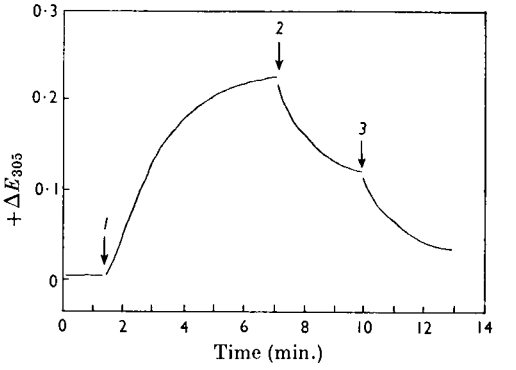


Fig. 13. Reversal of the β-oxoadipate CoA-transferase reaction in extracts of *Aspergillus niger* by succinate. Reaction cuvettes contained in a total volume of 1·2ml.: tris–HCl buffer, pH 8·0, 50μmoles; $MgCl_2$, 10μmoles; succinyl-CoA, 0·4μmole; potassium β-oxoadipate, 50μmoles. The reaction was started (arrow *1*) by the addition of extract (0·04mg. of protein). At arrow *2*, potassium succinate, 10μmoles, was added followed by a second 10μmoles at arrow *3*. The Figure shows a recording-spectrophotometer tracing.

Table 9. *β-Oxoadipate CoA-transferase in extracts of fungi*

Cuvettes contained in a total of 1·5ml.: tris–HCl buffer, pH 8·0, 100μmoles; $MgCl_2$, 10μmoles; succinyl-CoA, 0·4μmole; potassium β-oxoadipate, 50μmoles; cell-free extract, 0·02ml.

Organism	Growth substrate	Sp. activity of CoA-transferase (units/mg. of protein)
Rhodotorula mucilaginosa I	*p*-Hydroxybenzoate	0·63
	Glucose	0·005
Debaryomyces hansenii IC	*p*-Hydroxybenzoate	0·55
Candida tropicalis N.C.Y.C. 4	Catechol	0·58
Aureobasidium pullulans	*p*-Hydroxybenzoate	0·19
Fusarium oxysporum	*p*-Hydroxybenzoate	0·56
	Succinate	0·01
Aspergillus niger L6	*p*-Hydroxybenzoate	0·21
Penicillium spinulosum P6	*p*-Hydroxybenzoate	0·80
Schizophyllum commune	*p*-Hydroxybenzoate	0·71
Sphaerobolus stellatus	*p*-Hydroxybenzoate	0·45
Vararia granulosa	*p*-Hydroxybenzoate	0·73
Neurospora crassa SY4	Glucose + vanillate	0
Pseudomonas putida A3.12	*p*-Hydroxybenzoate	0·41
Nocardia erythropolis CA4	*p*-Nitrobenzoate	0·19

Table 10. *Incorporation of* [^{14}C]*succinate into β-oxoadipate by extracts of* Aspergillus niger *and* Rhodotorula mucilaginosa

Reaction mixtures contained in a total volume of 2ml.: β-oxoadipate, 10μmoles; succinyl-CoA, 0·5μmole; CoA, 3μmoles; GSH, 20μmoles; $MgCl_2$, 10μmoles; tris–HCl buffer, pH 8·0, 100μmoles; [2,3-$^{14}C_2$]succinate, 1μmole (containing $1·25 \times 10^6$ counts/min.); extract of *Aspergillus niger* or *Rhodotorula mucilaginosa*, 2·7mg. of protein. They were incubated at 30° for 3hr. (*Aspergillus*) or 1·5hr. (*Rhodotorula*). The reaction mixtures were boiled for 5min., the coagulated protein was centrifuged off and excess of 2,4-dinitrophenylhydrazine (0·2%, w/v, in 0·1N-HCl) was added to the supernatants, which were then boiled for 30min. to convert β-oxoadipate dinitrophenylhydrazone into the laevulate derivative. After storage overnight at 4°, the acidic dinitrophenylhydrazones (and succinate) were extracted into ethyl acetate and then 10% (w/v) Na_2CO_3. After acidification, the carbonate-soluble products were re-extracted into ethyl acetate, concentrated and chromatographed as a band on Whatman no. 1 paper in solvent *D*. Radioactive succinate was left at the origin whereas laevulate dinitrophenylhydrazone moved with R_F 0·5. The latter band was cut out, the product was eluted with ethyl acetate, the eluate was concentrated and samples were assayed for radioactivity in a Packard Tri-Carb scintillation spectrometer (49·4% efficiency) and for laevulate dinitrophenylhydrazone by the method of Friedmann & Haugen (1943). A minimum of 5000 counts was measured.

	Sp. radioactivity (counts/min./μmole) of laevulate 2,4-dinitrophenylhydrazone from incubations with extracts of	
	Aspergillus niger	*Rhodotorula mucilaginosa* I
Complete reaction mixture	1950	1640
Mixture without succinyl-CoA	420	750
Complete, but with boiled extracts	0	320

different flasks, the following amounts of succinyl-CoA: (1) 0·5μmole; (2) 0·2μmole; (3) nil. Flask (4) had the same contents as flask (1), but with boiled extract. After 2hr. the following amounts of β-oxoadipate had disappeared: flask (1) 5·6μmoles; flask (2) 3·5μmoles; flask (3) 2·1μmoles; flask (4) nil.

These results were confirmed by demonstrating the presence of succinyl-CoA–3-oxoadipate CoA-transferase in extracts of all aromatic-grown fungi examined except vanillate-induced wild-type *Neurospora crassa* (Table 9). The reversal of this enzyme by succinate was demonstrated spectrophotometrically with extracts of *Aspergillus niger* (Fig. 13) and confirmed by demonstrating the ability of *Aspergillus niger* and *Rhodotorula mucilaginosa* extracts to catalyse the incorporation of ^{14}C-labelled succinate into β-oxoadipate in the presence of succinyl-CoA (Table 10).

Protocatechuate metabolism by possible 'meta' *ring cleavage*

A single isolate, *Penicillium* II, obtained as an aerial contaminant, grew very poorly on media containing the aromatic acid as sole carbon source. It was therefore grown in stationary incubations in glucose medium and adapted to *p*-hydroxybenzoate by the replacement technique (Henderson, 1957). After 3 days the incubation medium gave strong positive reactions for protocatechuate, and washed mycelial suspensions from the cultures oxidized both *p*-hydroxybenzoate and protocatechuate without a lag. Control suspensions from unadapted cultures had negligible activity.

Extracts prepared from *p*-hydroxybenzoate-adapted mycelium oxidized protocatechuate (5μmoles) with an oxygen uptake of 4·2μmoles, but no Rothera reaction was given by the flask contents. Chromatography of the carbonate-soluble 2,4-dinitrophenylhydrazones prepared from the incubation mixtures showed a component with the same R_F as pyruvate 2,4-dinitrophenylhydrazone in solvents *D*, *E* and *F*. Pyruvate was confirmed by eluting the pyruvate 2,4-dinitrophenylhydrazone spots with xylene, concentrating this solution and extracting it with 0·2vol. of 10% (w/v) sodium carbonate. This solution (1ml.) was mixed with 1·5N-sodium hydroxide (2ml.) and the spectrum of the 2,4-dinitrophenylhydrazone compared with that of a similar solution of authentic material; they were identical.

The amount of pyruvate formed from protocatechuate was always below the theoretical value. In a typical experiment, protocatechuate (5μmoles) was oxidized for 2·5hr. in Warburg flasks with 3·8mg. of *Penicillium* II extract. The substrate took up 4·3μmoles of oxygen and produced 1·40μmoles of pyruvate (corrected for control flasks without substrate). β-Oxoadipate was not oxidized by this extract, nor did it give rise to pyruvate.

Spectrophotometric studies in phosphate buffer at pH 7·0 showed that the extracts rapidly degraded protocatechuate, a reaction that was lost after dialysis for 18hr. against water but regained on addition of Fe^{2+}; maximum activation of crude extracts (fourfold) occurred at 0·14mM-Fe^{2+}. No β-carboxymuconate accumulated, but neither did the cuvette contents take on the characteristic yellow colour due to the formation of the keto form of a 2-hydroxymuconic semialdehyde derivative,

typical of the '*meta*' pathway in many bacteria (Dagley, Chapman, Gibson & Wood, 1964). The extracts were inactive towards β-carboxymuconolactone and (+)-muconolactone, and lactonizing activity was also very low (13mμmoles/min./mg. of protein).

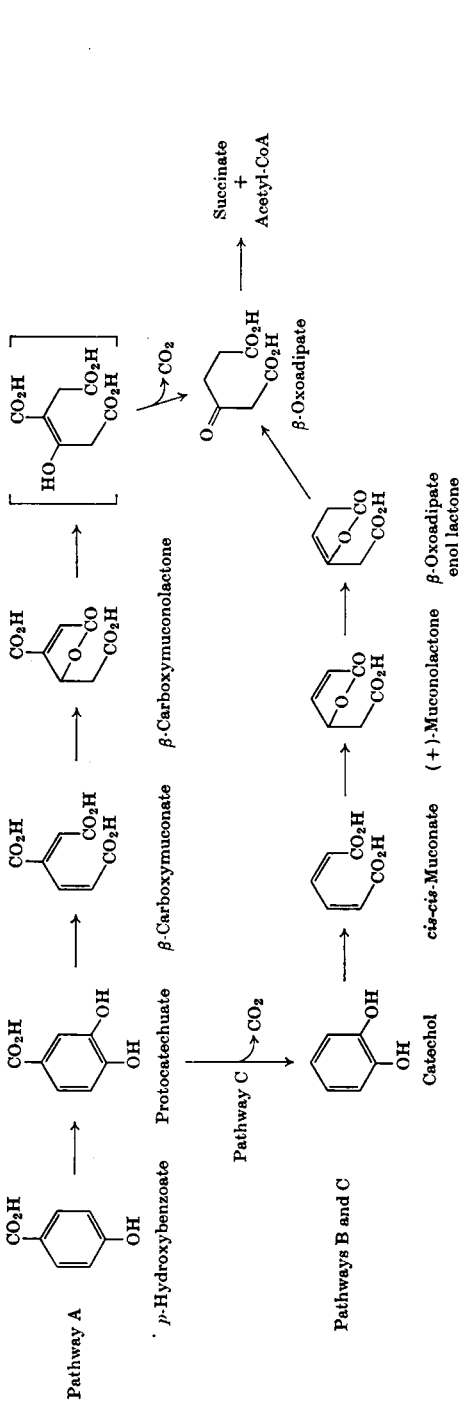
DISCUSSION

Lignin represents one of the principal sources of the aromatic ring in Nature. This insoluble polymer is known to be attacked both by bacteria (Sørensen, 1962) and particularly by fungi (Campbell, 1931; Fahraeus, Nilsson & Nilsson, 1949), probably by means of extracellular enzymes, with the release of monomeric and substituted ring compounds. The 'white-rot' fungi of the genera *Polyporus*, *Poria*, *Fomes* and *Trametes* are particularly effective agents for depolymerizing lignin. Identified among the products of beech, pine and spruce lignin breakdown by these fungi were *p*-hydroxybenzoate, *p*-coumarate, vanillate, ferulate, 4-hydroxy-3-methoxyphenylpyruvate and the aldehydes vanillin, dehydrodivanillin, syringaldehyde and coniferaldehyde (Fukuzumi, 1960; Ishikawa, Schubert & Nord, 1963). Some of these methoxylated compounds were also recognized among the products of the chemical oxidation of humic acids in soil (Morrison, 1958). Hydroxybenzoates and their methoxylated and reduced analogues have also been detected in most higher-plant tissues (Pridham, 1965), where *p*-hydroxybenzoate, protocatechuate and vanillate occur frequently.

At leaf-fall or death of the plant these aromatic units are returned to the soil, where they are metabolized by a variety of bacteria and soil fungi (Moreau & Augier, 1962). The routes by which such compounds are degraded by bacteria are now well defined. Demethylation of vanillate by a *Pseudomonas* has been shown to yield protocatechuate, with the methyl group undergoing oxidation to formaldehyde and then formate (Cartwright & Smith, 1967; Cartwright & Buswell, 1967). Ornston & Stanier (1966) found that *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Hydrogenomonas eutropha* oxidized protocatechuate to β-carboxymuconate and lactonized this acid to the unstable γ-carboxymuconolactone. The two enzymes for the conversion of this intermediate into β-oxoadipate were also found in these pseudomonads, in *Moraxella calcoacetica* (Cánovas & Stanier, 1967*a*) and in about 100 other bacteria examined by Cain (1966*a*) including species of *Mycobacterium*, *Nocardia*, *Streptomyces*, *Flavobacterium*, *Achromobacter* and *Pseudomonas ovalis*, *Pseudomonas fluorescens* and *Pseudomonas cuneatus*. There is therefore strong evidence that γ-carboxymuconolactone is typical of bacterial metabolism of protocatechuate by the '*ortho*' route.

Fungi, in contrast, have received little detailed attention at the enzyme level, despite the fact that they are probably the principal agents of lignin decomposition, particularly in acid soils. Henderson & Farmer (1955) and Jones & Farmer (1967) have recorded the utilization of many hydroxylated and methoxylated aromatic aldehydes and acids as sole carbon source by soil fungi. Henderson (1956, 1957) was able to clarify several of the interconversions that such compounds underwent before ring cleavage by using the replacement technique of Kluyver & van Zijp (1951) and sequential induction methods with intact mycelia. Among these conversions were: (1) oxidation of aromatic aldehydes to the corresponding acids; (2) oxidation of side chains (e.g. ferulate → vanillate); (3) demethylation (anisate → *p*-hydroxybenzoate; vanillate → protocatechuate; veratrate → vanillate); (4) ring hydroxylation (benzoate → *p*-hydroxybenzoate; *p*-hydroxybenzoate → protocatechuate). Flaig & Haider (1961) observed an enzyme complex converting protocatechuate into β-oxoadipate in *Polystictus versicolor* and separated it from the extracellular laccase by chromatography on calcium phosphate. β-Oxoadipate formation from protocatechuate, catechol and *cis–cis*-muconate was also catalysed by extracts of the yeast-like *Aureobasidium* (*Pullularia*) *pullulans* (Henderson, 1961). In neither of these reports were intermediates identified, so the more detailed study with *Neurospora crassa* (Gross *et al.* 1956) remained the only instance where the nature of these intermediates had been clarified.

The general features of microbial degradation of the aromatic ring by the catechol and protocatechuate pathways have now been shown to be of wide occurrence in the fungi; in catechol degradation, the fungal and bacterial pathways are metabolically identical, though their regulation may well differ. *Neurospora crassa*, however, is not an isolated case of an atypical organism lactonizing β-carboxymuconate to β-carboxymuconolactone instead of the γ-substituted isomer characteristic of bacterial pathways. This reaction was typical of all *p*-hydroxybenzoate-grown fungi of group I that we have examined. Similarly, this pathway is not confined to a narrow group of related fungi, for we have demonstrated the enzymes for metabolism of β-carboxymuconate via β-carboxymuconolactone in genera from the Basidiomycetes (*Polystictus*, *Vararia*, *Schizophyllum*, *Sphaerobolus*, *Sporobolomyces* and *Aureobasidium*), the Ascomycetes (*Neurospora*, *Fusarium*, *Phoma* and the yeasts) the Phycomycetes (*Mucor*) and the Fungi Imperfecti (*Penicillium*, *Aspergillus*, *Cylindrocephalum* and *Cephalosporium*). Though the absolute number of fungi so far examined is small compared with the many studies in which bacteria have been used, the number of different genera investigated (Table 7) is

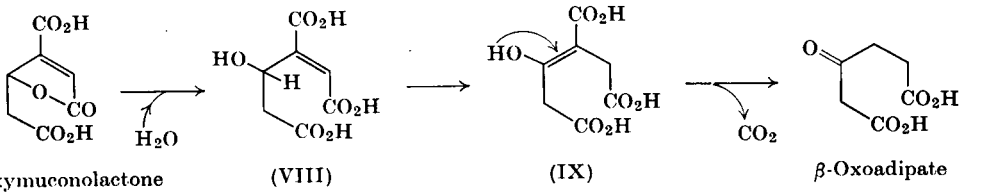


Scheme 1. Metabolic pathways of aromatic-ring fission in the fungi. Pathway A is followed by all group I fungi grown with *p*-hydroxybenzoate or a protocatechuate precursor. Pathway B is followed by groups I and II after growth with catechol, but pathway C is followed by group II fungi grown also with *p*-hydroxybenzoate.

similar. There is therefore strong evidence for suggesting that a ring cleavage via β-carboxymuconolactone is as typical of the fungal mechanism for protocatechuate breakdown as cleavage through γ-carboxymuconolactone is of the bacterial pathway. None of the bacteria examined by Cain (1966*a*) attacked β-carboxymuconolactone, and the inability of fungi to degrade the γ-isomer to β-oxoadipate is just as absolute. Though *Penicillium spinulosum* P 6 utilized γ-carboxymuconolactone at low rates, no β-oxoadipate was produced; the identity of the natural intermediate in both this organism and *Aspergillus niger* with β-carboxymuconolactone was confirmed by accumulation and isolation of this isomer from β-carboxymuconate in experiments with purified extracts (B. Halsall, unpublished work).

The metabolic pathways of aromatic-ring fission in fungi are outlined in Scheme 1. It is noteworthy that those micro-organisms that have a (sometimes transient) mycelial form of growth, e.g. *Mycobacterium*, *Nocardia* and *Streptomyces*, but that on other evidence [staining characteristics and cell size (Bisset, 1955); susceptibility to phage and acid media (Waksman, 1957); general sensitivity to antibiotics active against bacteria but not fungi; absence of chitin from the cell substance (Avery & Blank, 1954); cell-wall analysis (Hoare & Work, 1957; Cummins & Harris, 1958)] are still regarded as true bacteria rather than fungi, also appear typically bacterial in their ring-cleavage mechanism. Extracts of *p*-hydroxybenzoate-grown *Mycobacterium rhodochrous*, *Nocardia erythropolis* CA 4 and a *Streptomyces* sp., among others, synthesized and degraded γ-carboxymuconolactone, but could not convert β-carboxymuconolactone into β-oxoadipate (Cain, 1966*a*).

Though the point of divergence of the bacterial and fungal pathways occurs at the lactonization stage, the general properties of fungal and bacterial β-carboxymuconate-lactonizing enzymes have several features in common. The enzymes from *Pseudomonas putida* and *Neurospora crassa* had similar molecular weights; the enzymes from these two organisms and *Rhodotorula mucilaginosa* had similar Michaelis constants and were inhibited by phosphate and citrate buffers. The inhibition (95%) of the enzyme from these three genera by heavy-metal ions such as Hg^{2+} (0·1mM) was significantly reversed (to 25–30% inhibition) by dithiothreitol or cysteine (0·01M) and less efficiently by GSH. The only marked difference in properties of the three enzymes so far discernible is the pH optimum, which in the bacterial enzyme lies near pH 7·0 but in the fungal enzymes is about pH 6·0, a not unexpected feature considering the acid habitat and ecology of many of the organisms (Jones & Farmer, 1967).



Scheme 2. Possible route for conversion of β-carboxymuconolactone into β-oxoadipate by fungi.

A second feature in which *p*-hydroxybenzoate-grown fungi differ from bacteria is the inability of extracts to utilize any of the three isomeric C_6 lactones, at least one of which, on grounds of comparative biochemistry, might be expected to be an intermediate between β-carboxymuconate and β-oxoadipate. We have not in these initial studies carried out a rigorous purification of the delactonizing enzyme(s) from a yeast or a mould, but during partial purification of the delactonizing fraction from extracts of *Rhodotorula mucilaginosa* the ratio of decarboxylase activity to the rate of formation of β-oxoadipate remained unchanged. Therefore, though on present evidence the fungi we have examined resemble *Neurospora crassa* in their apparently simultaneous decarboxylation and delactonization, we suspect that the corresponding hydroxy acid, 3-carboxy-2-hydroxyhex-2-enoic acid (VIII), may be formed followed by migration of the double bond (IX) and enzymic (or spontaneous) decarboxylation to form β-oxoadipate (Scheme 2). The high pH optimum of the delactonizing enzyme(s) in the fungi would certainly favour the latter stages of such a scheme.

In contrast with the results obtained with species of *Pseudomonas* (Ornston, 1966c) basal activities of some of the enzymes in extracts of unadapted (glucose- or succinate-grown) fungi were fairly high; this was particularly true for β-carboxymuconate-lactonizing enzyme and the delactonizing enzyme, where specific activities of over 10% of that found in fully induced extracts were common (Table 7). These high activities cannot be attributed to incomplete loss of enzyme after removal from an environment containing an aromatic inducer; both glucose-grown and aromatic-grown cultures were invariably started from malt–yeast extract–glucose–peptone or potato–glucose–agar stock cultures where the media contain no inducer. This phenomenon of high basal enzyme activities of the lactonizing enzyme in *Neurospora crassa* was also noted by Gross *et al.* (1956). Because the metabolic pathway for protocatechuate metabolism in the fungi differs from that found in bacteria, the regulation of enzyme induction will also probably differ in many respects, but from our initial survey one feature of similarity with the *Pseudomonas putida* system was evident, namely that growth or induction of a group I fungus with catechol led to increase in specific activity above basal levels not only of the four enzymes of the catechol pathway (Scheme 1) but also of β-carboxymuconate-lactonizing enzyme and β-carboxymuconolactone-delactonizing enzyme, even though these two enzymes have no function in the catechol pathway. The only exception to this general response was *Candida tropicalis*, but this organism cannot in any case grow on *p*-hydroxybenzoate or protocatechuate.

Contrary to the results obtained by Ottey & Tatum (1957) with *Neurospora crassa*, most of the fungi we have examined possess a β-oxoadipate CoA-transferase and show a requirement for succinyl-CoA for the degradation of β-oxoadipate to tricarboxylic acid-cycle compounds; they therefore parallel the bacteria in this metabolic step. In the wild-type strain of *Neurospora crassa* used here, as well as in the 'aromaticless' mutant (Ottey & Tatum, 1957), no CoA-transferase could be detected with several variations of reactant and extract concentration.

Hough & Wase (1965) noted that the adaptive patterns of fungi grown on aromatic carboxylic acids were by no means so clearly defined as those of bacteria, where the benzoate and *p*-hydroxybenzoate pathways are, with a few exceptions (see, e.g., Proctor & Scher, 1960; Wheelis, Palleroni & Stanier, 1967), distinct. Germinating conidia of *Aspergillus nidulans* and intact mycelia of *Aspergillus niger* and *Schizophyllum commune*, for instance, hydroxylate benzoate to *p*-hydroxybenzoate and protocatechuate without the involvement of catechol (Shepherd & Villanueva, 1959; Bocks, 1967; Moore & Towers, 1967), but Hough & Wase (1965) found that certain yeasts after growth on *p*-hydroxybenzoate were sequentially induced for catechol oxidation, a feature we have confirmed with washed-cell or mycelial suspensions of our group II yeasts and with several moulds. There was, however, a clear enzymic difference between these moulds and the anomalous (group II) yeasts. In the moulds, small amounts of catechol 1,2-oxygenase alone accounted for the ability of the intact

mycelium to oxidize catechol and such extracts slowly accumulated *cis–cis*-muconate from catechol; the yeasts, on the other hand, possessed all four enzymes of the catechol pathway after growth on *p*-hydroxybenzoate and showed only minimal amounts of the enzymes of the fungal protocatechuate pathway as a result of the probable decarboxylation of protocatechuate to catechol. A similar decarboxylation probably accounts for the conversion of 2,3-dihydroxybenzoate into catechol by *Aspergillus nidulans* conidia (Shepherd & Villanueva, 1959). Whether, in the moulds, the low catechol 1,2-oxygenase activity can be attributed to a protocatechuate 3,4-oxygenase of low specificity, or to a co-ordinate induction of both enzymes by protocatechuate, it is impossible at present to say; in those few yeasts where the protocatechuate oxygenase has been partially purified, however, the enzyme is highly specific for protocatechuate. Some of the higher fungi themselves have low protocatechuate oxygenase activities even after growth with *p*-hydroxybenzoate (Table 7), a feature noticed also by Gross *et al.* (1956) in their original work with vanillate-induced *Neurospora crassa*, where maximum specific activities of approx. 2mμmoles/min./mg. of protein (calculated from their data) were obtained.

A '*meta*' type of ring cleavage (defined in the Materials and Methods section) has now been reported widely in bacteria (Dagley *et al.* 1964; Bayly, Dagley & Gibson, 1966; Cain & Farr, 1968), in animals (Mehler, 1958) and probably in higher plants (Centrospermae) (Senoh & Sakan, 1966). Fungi remain the only major group in which this type of ring-fission mechanism is still not described, so the tentative evidence for '*meta*' ring cleavage of protocatechuate by *Penicillium* II is of some interest. The failure to detect the typical yellow colour of a 2-hydroxymuconic semialdehyde derivative does not necessarily rule out this pathway, because Ribbons (1966) has also found a *Pseudomonas* extracts of which did not accumulate the yellow ring-fission product from 3-methylcatechol oxidations. Five pieces of evidence point to a '*meta*' cleavage mechanism: (1) protocatechuate, though rapidly oxidized by extracts at nearly 1mole of oxygen consumed/mole of substrate, was not attacked by a protocatechuate 3,4-oxygenase; (2) the oxygenase was stimulated, even in crude extracts, by addition of Fe^{2+} ions; this feature is characteristic of '*meta*' oxygenases (Kojima, Itada & Hayaishi, 1961; Cain, 1962; Ribbons & Evans, 1962); (3) β-carboxymuconate was attacked only very slowly by extracts, at rates well below that of protocatechuate disappearance; (4) β-oxoadipate was neither formed from protocatechuate nor metabolized to pyruvate by extracts; (5) pyruvate, a characteristic end product of the '*meta*' cleavage of *o*-diphenols, was found and identified in incubation mixtures of protocatechuate and *Penicillium* extract at the end of oxygen uptake.

There is considerable evidence therefore that fungi are no less diverse than bacteria in their ability to degrade the aromatic ring and utilize carbon derived from metabolically inert lignin polymers.

Note added in proof. The activities of protocatechuate 3,4-oxygenase recorded for cultures of *Neurospora crassa* SY4, grown as described by Gross *et al.* (1956), have been approx. 3mμmoles/min./mg. of protein, values very much lower than those found in any of the other fungi examined.

D. R. Thatcher & G. K. Watson in this Department have recently examined the enzymes of aromatic metabolism in *Neurospora crassa* SY4 (wild-type) in some detail, and, by raising the concentration of the vanillate inducer to 4mM in shaken cultures, have obtained protocatechuate 3,4-oxygenase activities as high as 72mμmoles/min./mg. of protein, a value comparable with those found in other Ascomycetes. In the absence of inducer no oxygenase activity was detected. The activities of the other enzymes of the protocatechuate pathway were little altered from those shown in Table 7 by this increase in inducer concentration.

The activity of the oxygenase has also been examined in *Neurospora crassa* Y7655–10575, an aromaticless indoleless double mutant, which requires tyrosine, phenylalanine, *p*-aminobenzoate and tryptophan for growth (Gross *et al.* 1956). Stationary cultures of this mutant strain accumulated protocatechuate (14μg./ml.) from glucose after 2 days, when about half of the initial glucose (10g./l.) had disappeared. The protocatechuate 3,4-oxygenase activity reached a maximum of 16mμmoles/min./mg. of protein in extracts obtained from mycelia harvested after 2½-days' growth. In the absence of exogenous inducer, oxygenase activities in this mutant did not approach those of induced wild-type *Neurospora crassa*.

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