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Oxidative Metabolism of Phthalic Acid by Soil Pseudomonads

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Phthalic acid is removed from chemical waste effluents by biological purification through filter beds. This has led to an investigation of the oxidative metabolism of this aromatic compound by the micro-organisms responsible for this action industrially, and from the soil.

In mammals, e.g. dog and man, phthalic acid is largely excreted unchanged, and does not appear to form glycine conjugates (Shemyakin & Schukina, 1944). Jacobs (1931), on very slender evidence, suggested that phthalic acid was an intermediate in the dissimilation of naphthalene by soil micro-organisms; Fernley & Evans (1958) have shown this view to be incorrect.

From young liquid cultures of a soil pseudomonad growing on phthalate, Evans (1955) identified, by chromatography, the presence of 4:5-dihydroxyphthalate. Application of the technique of sequential induction revealed that this acid and protocatechuic acid were immediately metabolized

by phthalate-grown cells. The adaptation to protocatechuic acid was not conclusive evidence of its being an intermediate, since it might have arisen from *p*-hydroxybenzoic acid, a known anabolic product of most bacteria. Further, a hitherto undescribed enzyme reaction (a non-oxidative aromatic decarboxylation) would have been implied. We have extended this study, a preliminary report of which has been presented (Ribbons & Evans, 1959a, b).

EXPERIMENTAL

Isolation of organism. Microbes isolated from filter beds actively detoxicating phthalic acid at Monsanto Chemicals, Ruabon, were kindly made available through the courtesy of Dr G. Wilson and Miss M. Harrison. Our own isolates were obtained by the elective-culture method from garden soil, manure and coniferous litter. The enrichment medium, adjusted to pH 7 with NaOH, contained the following (g./l.): phthalic acid, 1; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5;

NaCl, 0.25; CaCl_2 , 0.25; FeCl_3 , 0.02; MnSO_4 , 0.002; ZnSO_4 , 0.002; CuSO_4 , 0.002. Pure cultures were obtained after a few transplants on phthalate-mineral salts-agar plates. Most of the isolates were Gram-negative motile organisms with characteristics placing them in the family *Pseudomonadaceae*; one strain, isolated from conifer litter, was selected for metabolic work. The organism was maintained by serial subculture on phthalate-mineral salts-agar slopes.

Cultural methods and harvesting of cells. Liquid cultures were grown in 10 l. Pyrex bottles, medium of the above composition being used with forced aeration with sterile air, either at room temperature or in a bath at 25°. Maximum growth, ascertained by turbidity measurements, was obtained within 4–5 days. Cells were harvested by centrifuging at 13 000 g in a bench-model Sharples super-centrifuge, furnished with a cellophan lining to the rotor; they were washed twice with 0.1 M-phosphate buffer and stored at –15° until required. Unadapted cells were grown on 0.1 % glucose-mineral salts medium, the mineral base being identical with that used in the phthalate medium.

Preparation of cell-free extracts. Washed cells (1 g. wet wt.) were suspended in 5 ml. of water or of buffer of choice, and disintegrated either in a Mickle apparatus for 15 min. with Ballotini glass beads, grade 12 (English Glass Co., Leicester), or sonically with no abrasive in the M.S.E.-Mullard Ultrasonic Disintegrator operating between 16 and 24 keyc./sec. at 0–4°; or with the Hughes press (Hughes, 1951) without abrasive. The suspension was centrifuged at 1000 g for 10 min. to remove unbroken cells and debris, and the supernatant was again spun at 20 000 g in the super-speed angle head of the M.S.E. refrigerated centrifuge for 20–30 min. to remove smaller particulate matter in the extract.

Materials. 4:5-Dihydroxyphthalic acid was synthesized by the demethylation of *m*-hemipinic acid [obtained by the method of Perkin, Edwards & Stoye (1925) from veratraldehyde] with hydriodic acid (sp.gr. 1.73).

3:4-Dihydroxyphthalic acid was prepared by demethylation of hemipinic acid (L. Light and Co. Ltd.) with PCl_5 at 180°, and boiling the resulting 3:4-bis(chloromethoxy)phthalic anhydride with water (Freund & Horst, 1894).

Protocatechuic acid was synthesized by the method of Pearl (1949).

3-Hydroxyphthalic acid was prepared by the method of Pratt & Perkins (1918).

4-Hydroxyphthalic acid was prepared by the method of Bentley, Robinson & Weizmann (1907).

2:4:5-Trihydroxybenzoic acid was synthesized from 2:4-dihydroxyacetophenone by treatment with iodine in pyridine and subsequent treatment with alkali (Thiele & Jaeger, 1907).

cis-cis-Muconic acid and γ -carboxymethyl- Δ^2 -butenolide were prepared by the methods of Elvidge *et al.* (1950).

Oxythiamine diphosphate was synthesized by the method of Cerecedo & Eusebi (1950).

Other chemicals were available commercially; all melting points are uncorrected.

Estimations. Phenols were determined by the Folin-Ciocalteu reagent and *o*-dihydroxyphenols by the Evans (1947) reagent.

Spectrophotometric determinations were carried out in the Unicam SP. 500 in 1 cm. light-path silica cuvettes. Protein was estimated by the method of Warburg &

Christian (1942). Protocatechuic acid was estimated spectrophotometrically (Stanier & Ingraham, 1954).

Manometric experiments were made in the conventional Warburg constant-volume apparatus at 30°. Oxygen uptake and evolution of CO_2 were estimated by the direct method (Dixon, 1952). 3-Oxoadipic acid was estimated by catalytic decarboxylation with 4-aminoantipyrine (Sistrom & Stanier, 1953). The enzyme system that decarboxylates 4:5-dihydroxyphthalate to protocatechuate (4:5-dihydroxyphthalate decarboxylase) was conveniently assayed by evolution of CO_2 under anaerobic conditions. The standard reaction mixture for the assay was: 4:5-dihydroxyphthalate (10 μ moles in a volume of 0.2 ml. in the side arm), 0.1 M-phosphate buffer, pH 6.5 (2 ml.), enzyme solution (concentration being such that between 5 and 50 μ l. of CO_2 was liberated in 5 min.) and water to make a final volume of 3 ml. The flasks were equilibrated for 10 min., during which they were flushed with $\text{CO}_2 + \text{N}_2$ (5:95) gas mixture. The last traces of O_2 were removed from the manometer arm by manipulation of the manometer fluid. The reactions were initiated by tipping the substrate into the main compartment and initial velocities taken as the measure of activity. Enzyme activity is expressed as μ moles of CO_2 liberated/mg. of protein/hr.

Chromatography. Both ascending and descending techniques were used with Whatman no. 4 paper; all chromatograms were run at room temperature. The two solvents used were benzene-acetic acid-water (80:20, saturated) and butanol-acetic acid-water (40:50:10). The R_F values given in the former solvent have been published (Coulson & Evans, 1958); better separation of the dihydroxyphthalic acids and protocatechuic acid was obtained with the latter solvent, the R_F values being 0.52, 0.48 and 0.73 for 3:4-dihydroxyphthalic acid, 4:5-dihydroxyphthalic acid and protocatechuic acid respectively.

Detection reagents. Phenolic substances were detected by spraying the air-dried chromatograms with the following reagents: (a) diazotized *p*-nitroaniline or sulphanilic acid, followed by sodium carbonate solution (Bray & Thorpe, 1954), or (b) Folin-Ciocalteu reagent, followed by exposure of the chromatogram to NH_3 fumes (Mitchell & Davies, 1954).

o-Dihydroxyphenolic substances were detected by either ammoniacal silver nitrate or the molybdate-nitrite sprays (Coulson & Evans, 1958).

Acids were detected by spraying with bromocresol green (Lugg & Overall, 1948).

Ultraviolet-absorbing materials were detected with a Hg lamp (253.7 m μ).

Buffers. Incubations were carried out in either Na_2HPO_4 - KH_2PO_4 buffer or 2-amino-2-hydroxymethylpropane-1:3-diol-HCl (tris-HCl) buffer. The proportions of each constituent were varied according to the pH required and the molarity of the resulting buffer was as indicated in the text.

RESULTS

Sequential-induction experiments

Washed cells harvested in the logarithmic phase of growth on phthalate or glucose were used. Cells grown upon phthalate immediately oxidized phthalate, 4:5-dihydroxyphthalate and protocatechuate without any appreciable induction period

Fig. 1. Oxidation of various compounds by whole cells grown (a) with phthalate and (b) with glucose. Each Warburg flask contained: bacterial suspension (approx. 10 mg. dry wt. in 0.065M-phosphate buffer, pH 7) 1 ml.; 0.067M-phosphate buffer, 1.8 ml. Side arms contained 5 μmoles of the following substrates dissolved in 0.2 ml. of the same buffer. The centre well contained 0.2 ml. of 20% KOH. Temperature, 30°. Atmosphere, air. (1) Phthalate; (2) 4:5-dihydroxyphthalate; (3) protocatechuic acid; (4) succinate; (5) 3:4-dihydroxyphthalate; (6) 3-hydroxyphthalate; (7) 4-hydroxyphthalate; (8) glucose; (9) no substrate.

(Fig. 1). Succinate was also oxidized, being included in these experiments as a typical member of the tricarboxylic acid cycle. These cells were not sequentially induced to the following substrates: 3-hydroxyphthalate, 4-hydroxyphthalate, *o*-, *m*- and *p*-hydroxybenzoate, phenol, catechol, the dihydroxybenzoates (except protocatechuic acid), 3:4-dihydroxyphthalate, 2:4:5-trihydroxybenzoate, *cis-cis*-muconate or γ -carboxymethyl- Δ^2 -butenolide.

Glucose-grown cells did not metabolize any of these aromatic compounds immediately, indicating that the enzymes responsible for their dissimilation are induced (Fig. 1).

Detection of phenolic intermediate in growing cultures

Early in the logarithmic phase of growth, some phthalate-grown cultures gave transiently an *o*-dihydroxyphenol reaction, detected by the Evans reagent. This is illustrated for a typical culture in Fig. 2.

When the *o*-dihydroxyphenol reaction was at a maximum, a 10 l. culture was centrifuged, acidified to Congo red with 3N-HCl, concentrated under reduced pressure to 400 ml. and extracted with ether (three times with 200 ml. portions). The combined ether extracts were dried over Na₂SO₄; evaporation of the ether at 35°/760 mm. Hg yielded 1.5 g. of yellow crystals. Chromatography of this material revealed two components detected by u.v. light and by bromocresol-green indicator. Their *R_F* values were identical with protocatechuic acid and phthalic acid in both solvent systems. Further support for the identity of the phenolic component was obtained by the application of the phenolic and *o*-dihydroxyphenolic sprays. Determination of the u.v.-absorption spectra of the

Fig. 2. Accumulation of phenolic and *o*-dihydroxy materials in a typical phthalate culture (10 l.). ○, Growth (measured by measurements of *E* at 400 mμ); ●, phenolic materials produced; ▲, *o*-dihydroxyphenolic materials produced.

eluted spots showed them to be identical with authentic samples of phthalic acid and protocatechuic acid (Fig. 3).

*Oxidation with acetone-dried powders
and cell-free extracts*

4:5-Dihydroxyphthalic acid and protocatechuic acid were rapidly oxidized by acetone-dried powders of washed cells grown on phthalate. Phthalic acid oxidation is much reduced by this treatment and none of the other aromatic substrates mentioned was metabolized (Fig. 4a).

Cell-free extracts, prepared by any of the methods outlined above, readily oxidized 4:5-dihydroxyphthalate, protocatechuate and succinate. None of these extracts, either alone or with the addition of cell debris, oxidized phthalate, 3-hydroxy- or 4-hydroxy-phthalate, or any of the substrates previously listed in the sequential-induction experiments.

4:5-Dihydroxyphthalate is metabolized with the uptake of 1 mole of O_2 and evolution of 2 moles of CO_2 per mole of substrate; with protocatechuate 1 mole of O_2 was consumed with evolution of 1 mole of CO_2 per mole of substrate (Fig. 4b). The end product in each case is 3-oxoadipate, detected by the Rothera reaction (Rothera, 1908) and

Fig. 3. Ultraviolet-absorption spectra of phthalic acid (●), 4:5-dihydroxyphthalic acid (■) and protocatechuic acid (▲). Each compound was dissolved in 0.1M-phosphate buffer, pH 7, to give a concentration of 0.03–0.04 mM. The spectra of the eluted chromatogram spots, obtained from ether extraction of cultures, are also shown; from the butanol-acetic acid-water solvent the spot of R_F 0.8 (identical with that of phthalic acid) (○); and spot of R_F 0.7 (identical with that of protocatechuic acid) (∇).

Fig. 4. Oxidation of various compounds by acetone-dried powders and cell-free extracts. Each Warburg flask contained: 0.067M-phosphate buffer, pH 7, 1.8 ml., and either (a) 1 ml. of acetone-dried cells (approx. 10 mg. dry wt. in 0.067M-phosphate buffer, pH 7) or (b) 1 ml. of cell-free extract prepared in the Mickle disintegrator (14 mg. of protein in the same buffer). Side arms contained 5 μ moles of the following substrates dissolved in 0.2 ml. of the same buffer (0.2 ml.). The centre well contained 0.2 ml. of 20% KOH. Temperature, 30°. Atmosphere, air. (1) Phthalate; (2) 4:5-dihydroxyphthalate; (3) protocatechuate; (4) no substrate.

estimation by catalytic decarboxylation. For each mole of either 4:5-dihydroxyphthalate or protocatechuic acid, 1 mole of 3-oxoadipate is formed (Table 1). This suggests that 4:5-dihydroxyphthalate is first decarboxylated to protocatechuic acid, before ring-cleavage. This decarboxylation may be accomplished under anaerobic conditions. The relative rates of decarboxylation and oxidation of 4:5-dihydroxyphthalate under aerobic conditions are shown in Fig. 5 and are consistent with the view

Fig. 5. Relative rates of decarboxylation and subsequent oxidation of 4:5-dihydroxyphthalate by cell-free extracts under aerobic and anaerobic conditions. Each Warburg flask contained 4:5-dihydroxyphthalate ($5 \mu\text{moles}$ in a volume of 0.2 ml.); 0.1 M -phosphate buffer, pH 6.0, 2.0 ml. ; cell-free extract (containing 15 mg. of protein), 1 ml. Temperature, 30° . Atmosphere was air for the direct method of estimation of O_2 uptake and CO_2 evolution, and $\text{CO}_2 + \text{N}_2$ (5:95) for the anaerobic decarboxylation of 4:5-dihydroxyphthalate. $\blacktriangle$, O_2 uptake; $\bullet$, CO_2 evolution (direct method, aerobic); $\circ$, CO_2 evolution (anaerobic, see Experimental section). Points are all corrected for oxidation and decarboxylation in the absence of substrate.

that 4:5-dihydroxyphthalate decarboxylation is an intermediate step in its overall oxidation by crude extracts.

4:5-Dihydroxyphthalate decarboxylase

Conversion of 4:5-dihydroxyphthalate into protocatechuic acid. The evolution of an equimolar amount of CO_2 on anaerobic incubation of crude extracts with 4:5-dihydroxyphthalate suggested a conversion of the substrate into protocatechuic acid. The change in a u.v.-absorption spectrum supported such an enzyme action, and protocatechuic acid was isolated from the reaction mixture as follows.

To each of thirty-six Warburg flasks (20 ml. capacity) were added: 4:5-dihydroxyphthalate ($10 \mu\text{moles}$ in a volume of 0.2 ml.) in the side arm; 0.1 M -phosphate buffer, pH 6.5 (2 ml.), and cell-free extract (1 ml. , approx. 7 mg. of protein). The flasks were equilibrated at 30° under $\text{CO}_2 + \text{N}_2$ (5:95) and the reaction was followed as described in the Experimental section. To each flask was added 2 N-HCl (0.2 ml.) after completion of the CO_2 evolution and the flask contents were bulked. After, the contents were centrifuged and the supernatant was extracted three times with an equal volume of ether. The combined ether extracts were dried over Na_2SO_4 and distilled to dryness at 35° . An off-white crystalline material (45 mg.) remained, which melted at $185\text{--}191^\circ$. This was decolorized with activated charcoal and recrystallized twice from water. The crystals so obtained melted at $197\text{--}198^\circ$ and, upon admixture with authentic protocatechuic acid (m.p. $198\text{--}199^\circ$), melted at $197\text{--}198^\circ$. All chromatographic, chemical and absorption characteristics were consistent with the material being protocatechuic acid.

Properties of 4:5-dihydroxyphthalate decarboxylase. The enzymic decarboxylation of 4:5-dihydroxyphthalate to protocatechuic acid was assayed manometrically under anaerobic conditions by CO_2 evolution, the standard conditions described in the Experimental section being used. The effect of enzyme concentration upon rate of reaction is shown in Fig. 6.

Table 1. *Stoichiometry of 4:5-dihydroxyphthalate and protocatechuic acid oxidations by crude extracts*

Each flask contained: 0.2 M -phosphate buffer, pH 7, 1 ml. ; extract (13 mg. of protein), 1 ml. ; substrate in side arm, 0.2 ml. (neutralized to pH 7, amount as indicated) and water to make a total volume of 3 ml. The centre well contained 0.2 ml. of 20% KOH for measurements of O_2 uptake. Temperature, 30° . Incubation time, 60 min. Total CO_2 evolution was estimated by tipping in acid (0.2 ml. , 2 N-HCl) from double-side-arm flasks and the values listed are corrected appropriately for retention with similar flasks containing no substrate.

Expt. no.	Substrate	Substrate (μmoles)	Uptake of O_2 (μmoles)	Evolution of CO_2 (μmoles)	3-Oxoacidipate formed (μmoles)
1)	4:5-Dihydroxyphthalate	3	2.91	5.9	2.58
2)		6	5.9	11.7	5.6
3)	Protocatechuic acid	3	3.05	3.1	2.75
4)		6	5.96	6.1	5.65

The crude extract may be stored at -15° for up to 3 months, in either water or phosphate buffer (0.067M, pH 6.5), without appreciable loss of activity, and at 5° for 2–3 days. Protocatechuic acid and CO_2 were the only products detected, and the reaction was essentially irreversible under these conditions, the stoichiometry being shown in Table 2.

The optimum pH was between 6.0 and 6.7 in phosphate buffer and substitution by tris-HCl buffer had no effect upon activity. Dialysis against distilled water or phosphate buffer (0.067M) over a range of pH 5.9–8.5, at $0-3^{\circ}$, resulted in no appreciable loss of activity. Prolonged dialysis (3–4 days) caused a loss of about 70% of original

Fig. 6. Effect of enzyme concentration on 4:5-dihydroxyphthalate decarboxylase. Each flask contained: 4:5-dihydroxyphthalic acid (10 μ moles in a volume of 0.2 ml. in side arm); 0.1M-phosphate buffer, pH 6.5, 2 ml.; cell-free extract (15 mg./ml.) as indicated and water to give a final volume of 3 ml. Atmosphere, $\text{CO}_2 + \text{N}_2$ (5:95). Temperature, 30° .

Table 2. *Stoichiometry of 4:5-dihydroxyphthalate decarboxylase*

Each double-arm flask contained the standard reaction mixture with 1 ml. of cell-free extract containing 13 mg. of protein (prepared in the Mickle disintegrator), except that the amount of substrate was as indicated and 2N-HCl (0.2 ml.) was added to the second side arm. The acid was tipped in at the termination of CO_2 evolution to prevent any of the formed protocatechuic acid being oxidized enzymically on exposure to air. The acidified mixtures were centrifuged and samples taken to estimate the protocatechuic acid formed spectrophotometrically. A flask containing all reagents except substrate was used for a blank in the spectrophotometer.

Expt. no.	Substrate (μ moles)	CO_2 evolved (μ moles)	Protocatechuic acid formed (μ moles)
1	5	4.82	4.75
2	10	9.67	9.6

activity; this was not restored by addition of boiled enzyme or of thiol compounds or both. Supplements of cocarboxylase, Mg^{2+} , Mn^{2+} , reduced glutathione (GSH), coenzyme A (CoA) and adenosine triphosphate (ATP) at concentrations up to 1mM to the dialysed residues or cell-free extracts did not increase the activity in any combination tried; in fact CoA, ATP and cocarboxylase were slightly inhibitory. No intermediates of the reaction were detected with hydroxylamine as a trapping agent.

4:5-Dihydroxyphthalate decarboxylase was partially purified from crude extracts by $(\text{NH}_4)_2\text{SO}_4$ fractionation. All operations were conducted between 2 and 5° . To the crude extract in 0.067M-phosphate buffer, pH 7.0 (100 ml.), prepared in the Mickle disintegrator, was added saturated $(\text{NH}_4)_2\text{SO}_4$ (adjusted to pH 7.0 with NH_3 soln.) up to 33% saturation, and, after standing for 30 min., the precipitate was centrifuged. The supernatant was brought to 66% saturation by further addition of saturated $(\text{NH}_4)_2\text{SO}_4$ solution and the flocculent precipitate collected and redissolved in water (100 ml.). Refractionation of this with saturated $(\text{NH}_4)_2\text{SO}_4$ up to 40, 50 and 66% showed that most of the activity was present in the 40–50% fraction (Table 3). This fraction was dissolved in water (50 ml.) and used for inhibition experiments (Table 4).

Oxidation of protocatechuic acid by cell-free extracts

Oxygen uptake and evolution of CO_2 are consistent with the oxidation of protocatechuic acid to 3-oxoadipate, by extracts incubated with protocatechuic acid as the reaction product. This was confirmed by the isolation of 3-oxoadipic acid as the reaction product. The twice-recrystallized material had m.p. $118-119^{\circ}$ and the mixed m.p. with an authentic sample of 3-oxoadipic acid (m.p. $120-121^{\circ}$) was $118.5-120^{\circ}$.

Dialysis of the crude extracts against tap water at $0-3^{\circ}$ for 60 hr. did not suppress protocatechuic acid oxidation, but stopped the sequence of reactions at some point before 3-oxoadipate. The product formed has the u.v. spectrum of *cis-cis*- β -carboxymuconic acid (MacDonald, Stanier & Ingraham, 1954). This was confirmed by the isolation of the trisodium salt, as described previously by MacDonald *et al.* (1954). Incubation of the isolated *cis-cis*- β -carboxymuconate with undialysed extracts yielded 3-oxoadipate, as detected by the Rothera reaction and identification as the 2:4-dinitrophenylhydrazone of laevulinic acid (Kilby, 1951).

DISCUSSION

The reaction sequence as illustrated in Fig. 7 would account for the observed results.

Whole cells grown on phthalic acid as sole carbon source readily oxidized this substrate, but

Table 3. *Summary of 4:5-dihydroxyphthalate-decarboxylase purification by precipitation with ammonium sulphate*

4:5-Dihydroxyphthalate decarboxylase was assayed under standard conditions as described in the Experimental section.

Fraction	Concn. of protein (mg./ml.)	Activity (μ moles of CO_2 /mg. of protein/hr.)
Crude Mickle cell-free extract	16.4	2.46
(1) $(\text{NH}_4)_2\text{SO}_4$ ppt. (33–66%, w/v, saturated)	8.45	4.42
(2) $(\text{NH}_4)_2\text{SO}_4$ ppt. prepared from (1) (35–50%, w/v, saturated)	3.8	11.5

Fig. 7. Proposed scheme of dissimilation of phthalate. (a) Phthalate; (b) 4:5-dihydro-4:5-dihydroxyphthalate; (c) 4:5-dihydroxyphthalate; (d) protocatechuate; (e) *cis-cis*- β -carboxymuconate; (f) 3-oxoadipate.

Table 4. *Effect of various inhibitors on 4:5-dihydroxyphthalic acid decarboxylase*

Each Warburg cup contained the standard reaction mixture (see Experimental section) with inhibitors as indicated, the partially purified enzyme being used (950 μ g. of protein per flask). EDTA, Ethylenediamine-tetra-acetic acid.

Compound (addition)	Concn. (mm)	Inhibition (%)
KF	1	24.5
8-Hydroxyquinoline	1	0
EDTA	1	87.9
EDTA	0.6	54
EDTA	0.3	35
EDTA + $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3 mm)	0.3	27
EDTA + $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (3 mm)	0.3	13
Iodoacetate	2	84
Cystine	1	20
<i>p</i> -Chloromercuribenzoate	1	100
HgCl_2	1	100
Phenyl mercuric nitrate	1	100
Oxythiamine diphosphate	1	0

crude cell-free extracts, prepared by the methods described, were unable to do so. A factor involved might have been removed or diluted out during their preparation, since relatively dilute suspensions of the cells were disintegrated (see Dagley & Patel, 1957).

Neither the 3-hydroxyphthalate nor the 4-hydroxyphthalate was metabolized by whole cells grown on phthalate; this might mean that either a

permeability barrier exists which prevents these substances from reaching the appropriate intracellular-enzyme sites, or that there is an absence of such enzymes. Cell extracts, prepared by a variety of different methods, were also inactive on the monohydroxyphthalates. Acetone-dried cell powders could still oxidize phthalate (although at a much decreased rate compared with whole cells) but were without any action on the monohydroxyphthalates. These observations exclude both the monohydroxyphthalates as intermediates in phthalate metabolism. We therefore suggest that 4:5-dihydro-4:5-dihydroxyphthalate may well be formed initially, by perhydroxylation of phthalate, followed by dehydrogenation to 4:5-dihydroxyphthalate. A test of this hypothesis awaits the preparation of the relevant diol. A similar reaction is believed to be of widespread occurrence in the bacterial and mammalian metabolism of many aromatic compounds (Williams, 1950; Parke & Williams, 1958; Evans, 1956, 1957; Mason, 1957).

The decarboxylation of 4:5-dihydroxyphthalate to protocatechuate was the earliest reaction in the sequence we could demonstrate by cell-free extracts. This simple decarboxylation, directly from the benzene nucleus, is a new enzymic reaction in aromatic-ring biochemistry. Other decarboxylations from the ring previously reported have been oxidative in nature, e.g. salicylate (Walker & Evans, 1952; Murphy & Stone, 1954), benzoate

(Sleeper, 1951) and 6-hydroxynicotinate (Stanier & Behrmann, 1957). The decarboxylation of anthranilic acid to aniline (McCullough, Piligian & Daniel, 1957) is pyridoxal phosphate-linked.

Attempts to determine the cofactor requirements for this new enzyme did not meet with much success. The inactivity of CoA, ATP, cocarboxylase and pyridoxal phosphate with crude and dialysed extracts could have been due to the presence of phosphatases, which were detected in the extracts. However, the inhibition by ethylenediaminetetraacetic acid and subsequent partial reversal by Mn^{2+} and Mg^{2+} ions suggest that a bivalent ion may be required for optimum activity, in addition to essential thiol groups of the enzyme. Attempts to trap intermediates in the decarboxylation, with hydroxylamine, were negative, suggesting that phosphate or thio-esters are not involved.

The isolation of 3-oxoadipate and *cis-cis*- β -carboxymuconate from protocatechuate oxidation by the use of cell-free extracts and diffusates respectively are in accordance with the work of MacDonald *et al.* (1954).

Dialysis of the extracts did not suppress protocatechuate oxidation, indicating that there is no easily dissociable cofactor involved in this case. This was also found by Stanier & Ingraham (1954). Other systems which perform similar oxygenations, e.g. pyrocatechase (Suda, Hashimoto, Matsuoka & Kanahe, 1951), homogentisic acid oxidase (Ravdin & Crandall, 1951), gentisic acid oxidase (Lack, 1959), 3-hydroxyanthranilic acid oxidase (Wiss, 1957), *Neurospora* protocatechic acid oxidase (Ottey & Tatum, 1956) and the *Pseudomonas* protocatechic acid oxidase of Dagley & Patel (1957), have all been shown to require Fe^{2+} ions for optimum activity. The enzyme in Stanier's and our systems may well be Fe^{2+} ion-dependent, but we could not demonstrate stimulation by Fe^{2+} ions in extracts or dialysed extracts. We presume that Fe^{2+} is very tightly bound to the apo-enzyme.

The conversion of *cis-cis*- β -carboxymuconic acid into 3-oxoadipate by *Pseudomonas* still requires further elucidation. Ribbons & Evans (1959*b*) have shown that 2:6-dioxa-3:7-dioxo-bicyclo[3:3:0]-octane (dilactone) is converted into 3-oxoadipate by extracts of cells grown on phthalate. Such a symmetrical intermediate or its open-chain counterpart $\beta\beta'$ -dihydroxyadipic acid is required from the isotopic evidence of Gross, Gafford & Tatum (1956). Whether the dilactone is a true intermediate has yet to be determined.

SUMMARY

1. Protocatechic acid was detected and identified early in the logarithmic phase of growth in phthalate cultures of *Pseudomonas* sp.

2. Washed whole cells were sequentially induced to phthalate, 4:5-dihydroxyphthalate and protocatechuate.

3. Cell-free extracts catalysed the conversion of 4:5-dihydroxyphthalate, protocatechuate and *cis-cis*- β -carboxymuconate into 3-oxoadipate. The amounts of gaseous exchange supported data from experiments with whole cells, that the compounds participated in a reaction sequence in that order.

4. Extracts incubated with 4:5-dihydroxyphthalate under anaerobic conditions produced equimolar amounts of carbon dioxide and protocatechuate. After a fivefold purification of 4:5-dihydroxyphthalic acid decarboxylase, the enzyme showed inhibition by sulphhydryl-group reagents and ethylenediaminetetraacetic acid; the latter effect was partially reversed by manganese and magnesium ions.

5. The oxidation of protocatechuate by these extracts proceeded through *cis-cis*- β -carboxymuconate and 3-oxoadipate, which were isolated. No requirement for ferrous ions could be detected with this protocatechic acid oxidase.

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Synthesis of Optical Isomers of Cystinylvaline

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It has been shown (Arnstein & Grant, 1954*a, b*; Arnstein & Crawhall, 1957) that L-cystine, presumably after reduction to cysteine, is incorporated intact into the β -lactam-thiazolidine nucleus of penicillin; L-valine can also be utilized without deamination or transamination in the biosynthesis of the nucleus (Stevens & De Long, 1958).

The elucidation of the mode of formation of the β -lactam-thiazolidine ring system from cystine and valine is of considerable interest and it has been suggested (Arnstein, 1957) that cystinylvaline or its disulphide form may function as a precursor. The present paper deals with the synthesis of several optical isomers of cystinylvaline, including two labelled with ^{14}C in the valine carboxyl group.

The main purpose of the synthesis with unlabelled cystine and valine was to find methods which would yield L-cystinyl-L-valine and L-cystinyl-D-valine in an optically pure state. A number of diastereoisomeric amino acids and peptides have been separated by ion-exchange chromatography on Dowex 50 (Blackburn & Lee, 1955; Blackburn & Tetley, 1956; Blackburn & Schöberl, 1956), and it was thought possible that L-cystinyl-DL-valine could be separated into its three isomers in this way. Accordingly, this peptide was synthesized by the route shown in Fig. 1; benzyloxycarbonyl-S-benzyl-L-cysteine was coupled with DL-valine ethyl ester by the mixed-anhydride method of Vaughan (1951) and, after saponification of the resulting protected dipeptide ester, both the

benzyloxycarbonyl and S-benzyl groups were removed simultaneously with sodium in liquid ammonia (pathway B, Wood & Du Vigneaud, 1939). Crystallization of the crude product, as described in the Experimental section, gave, surprisingly, optically pure L-cystinyl-D-valine. The mother liquors were evaporated to dryness and the residue was placed on a column of Zeo-Karb 225 (The Permutit Co. Ltd., London) and subjected to gradient elution with hydrochloric acid (0.5–3.0*N*); at an acid concentration of 2.6*N* a component, running as one spot in solvent A, was obtained which, after hydrolysis and separation of the cystine and valine, was shown to be L-cystinyl-DL-valine. No evidence for the separation of L-cystinyl-L-valine from the mixture by this method was found. This approach to the synthesis of the two peptides was therefore abandoned. L-Cystine was found to be racemized to a considerable extent under conditions of acid hydrolysis; the optical configurations of the cystine and valine, isolated after hydrolysis of the peptides, were determined by comparison of their specific rotations with those of cystine and valine obtained from synthetic mixtures of optically pure amino acids, which had undergone identical treatment with acid. Cystinylvaline is fairly resistant to hydrolysis, complete reaction requiring 64 hr. with 6*N*-hydrochloric acid at 110°.

Synthesis of the two required peptides was commenced with L-cystine and either L- or D-valine. The coupling method with dicyclohexylcarbodi-