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Benzoate Oxidation by Bacterium N.C.I.B. 8250

By C. A. FEWSON, A. LIVINGSTONE and P. J. ROACH.
(Department of Biochemistry, University of Glasgow,
Glasgow W.2, U.K.)

Although Ichihara, Adachi, Hosokawa & Takeda (1962) described a cell-free system for benzoate oxidation in *Pseudomonas aeruginosa* and *Micrococcus ureae*, several workers have experienced difficulties in measuring benzoate oxidase activity in bacteria and have had to resort to experiments with intact organisms (e.g. Mandelstam & Jacoby, 1965; Kemp & Hegeman, 1968; Kennedy & Fewson, 1968).

We have developed a specific, rapid and sensitive method for measuring benzoate consumption by whole cells or by extracts. This is based on u.v.-absorption measurements of residual benzoic acid after acidification of reaction mixtures and extraction through chloroform. It has several advantages over previous methods, which have generally depended on measurement of oxygen consumption with consequent uncertain corrections for oxygen uptake caused by endogenous metabolism, NADH oxidation or metabolism of the products of benzoate oxidation.

Whole cells of bacterium N.C.I.B. 8250 grown in a benzoate-salts medium oxidise benzoate at a rate of about 70nmol/min per mg of protein. After ultrasonic disruption and centrifugation at 10000g for 20min the supernatants show a low but reproducible benzoate oxidase activity (about 2nmol/min per mg of protein). Optimum activity is obtained by extraction and assay in tris buffer, pH 7.5. The enzymic activity has a specific requirement for Fe²⁺ ions. Preincubation with cupferron (30μM) or 2,2'-bipyridyl (100μM), but not sodium diethyldithiocarbamate (30μM), prevents benzoate oxidation. There is a requirement for FAD (5μM). Enzymic activity also depends on the continuous regeneration of NADH (ethanol and alcohol-NAD oxidoreductase, EC 1.1.1.1). NADPH is not effective. Benzoate does not disappear under anaerobic conditions. Various combinations of ATP, CoA, thiamin pyrophosphate, lipoic acid, Mg²⁺, mercaptoethanol and GSH have no beneficial effect on the rate of benzoate oxidation. A compound giving a positive Rothera reaction is formed as benzoate disappears from the reaction mixture. Presumably the active catechol oxygenase (EC 1.13.1.1) (Kennedy & Fewson, 1968) accounts for the absence of catechol accumulation.

The addition of extracts of bacterium N.C.I.B. 8250 inhibits benzoate oxidation by intact cells. This is superficially reminiscent of the situation in *Rhodopseudomonas palustris* (Dutton & Evans, 1968), but it is unlikely that the mechanism of inhibition is the same since the pathways of benzoate metabolism are quite different in the two organisms.

A.L. and P.J.R. are in receipt of Science Research Council Studentships.

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Metabolism of Myricetin, Myricitrin and 3,4,5-Trihydroxyphenylacetic Acid

By G. E. SMITH and L. A. GRIFFITHS. (Department of Biochemistry, University of Birmingham, Birmingham 15, U.K.)

Previous studies on the metabolism of flavonoid compounds in mammals have largely been restricted to flavonoids showing hydroxylation in the 4'- or 3', 4'-positions only in the B ring, although flavonoids possessing a 3',4',5'-trihydroxyphenyl grouping are known to occur in plants of dietary importance.

Administration of myricetin (3,5,7,3',4',5'-hexahydroxyflavone) (200mg) to rats maintained on our standard diet gave rise to a major metabolite in the urine that has been identified as 3,5-dihydroxyphenylacetic acid by chromatographic and spectral methods. No glucuronide conjugates of myricetin were detected.

Since earlier studies have shown that the degradation of flavonoids is in part mediated by intestinal micro-organisms (Griffiths, 1964; Nakagawa, Shetlar & Wender, 1965; Das & Griffiths, 1968), the ability of intestinal micro-organisms to degrade myricetin *in vitro* was studied by incubating myricetin in a thioglycollate broth with mixed inocula of micro-organisms obtained by sterile section of the rat caecum. The incubation was carried out under anaerobic conditions at 37°C. The metabolites were recovered by a modification of a previously described ether extraction procedure (Griffiths, 1964) and shown to be 3,5-dihydroxyphenylacetic acid, 3,4,5-trihydroxyphenylacetic acid and unchanged myricetin.

Since it appeared probable that 3,4,5-trihydroxyphenylacetic acid was an intermediate in the

formation of 3,5-dihydroxyphenylacetic acid, the former was incubated under similar conditions with intestinal micro-organisms, when the formation of 3,5-dihydroxyphenylacetic acid was shown. Administration of 3,4,5-trihydroxyphenylacetic acid to rats resulted in the excretion of 3,5-dihydroxyphenylacetic acid.

To determine whether myricetin breakdown is dependent on the activity of the intestinal microflora *in vivo*, the antibiotic neomycin (100mg) was fed daily to rats before and during the period of myricetin administration, when the formation of 3,5-dihydroxyphenylacetic acid was suppressed.

Incubation of the glycoside myricitrin (myricetin 3-rhamnoside) with intestinal micro-organisms gave rise to free myricetin, 3,4,5-trihydroxyphenylacetic acid and 3,5-dihydroxyphenylacetic acid, indicating that rat intestinal micro-organisms are capable of effecting the cleavage of the glycosidic linkage as well as the heterocyclic ring system of myricitrin.

This work was supported by a grant from the Agricultural Research Council.

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The Metabolic Fate of 4-Chloro-2-methylphenoxyacetic Acid in Peas

By D. J. COLLINS and J. K. GAUNT. (*Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Caerns., U.K.*)

This report describes preliminary results from an investigation into the metabolism of the selective herbicide, MCPA,* in plants.

Radioactive MCPA, ³⁶Cl- or carboxy-¹⁴C-labelled, was applied to the leaves of 21-day-old pea plants (*Pisum sativum* var. Progress no. 9). After 24h the plants were extracted with boiling ethanol. Extracts were concentrated and fractionated by partition into ether. Fractions were examined by t.l.c. for labelled metabolites. Two of the breakdown products (MX₁ and MX₂) were found to be ether-soluble, whereas a third was not. In each case both the chlorine atom and the carboxyl carbon remained on the molecule.

This application technique did not permit the use of enough herbicide to isolate sufficient of any metabolite for its chemical identification. Thus incubation experiments with excised plant tissue were set up. Light-grown pea shoot sections were

* Abbreviation: MCPA, 4-chloro-2-methylphenoxyacetic acid.

incubated for 48h in a medium containing 0.5mM-MCPA. Examination of tissue extracts showed the presence of all three previously observed metabolites. Repeated t.l.c. of ether-soluble fractions led to the isolation of products MX₁ and MX₂ as crystalline compounds.

Both substances are non-phenolic acids with u.v. spectra identical with that of MCPA. Compound MX₁ has an i.r. spectrum characteristic of a hydroxylated compound. It was shown to be 4-chloro-2-hydroxymethylphenoxyacetic acid, after synthesis of this compound. Compound MX₂ has an i.r. spectrum characteristic of an amido acid. Acid and alkaline hydrolysis yielded MCPA and a ninhydrin-positive ether-insoluble component. Though still impure, the chemical, chromatographic and spectroscopic properties of compound MX₂ are similar to those of synthetic *N*-(4-chloro-2-methylphenoxyacetyl)-L-aspartic acid.

The ether-insoluble metabolite is also acidic and unstable to hydrolysis. Its purification and chemical characterization are currently under investigation.

D.J.C. is indebted to Ranks Hovis McDougall for financial support. This work was in part supported by grants from the Agricultural Research Council and Imperial Chemical Industries Ltd.

The Microbial Metabolism of Biphenyl

By D. LUNT and W. C. EVANS. (*Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Caerns., U.K.*)

Pure cultures of Gram-negative bacteria were isolated from tropical and temperate soils capable of utilizing biphenyl as sole carbon source in an otherwise mineral salts medium. 2,3-Dihydroxybiphenyl was detected and isolated from the culture; this was cleaved by a particulate fraction from biphenyl-grown cells to give α -hydroxy- β -phenylmuconic semialdehyde [(m.p. 122°C, $\lambda_{\max}$ 350nm (acid) and 430nm (alkali)]. A soluble cell-free extract converted this initial ring-fission product into phenylpyruvate through some, as yet, unidentified intermediates. The subsequent metabolic pathways of phenylpyruvate are known. Unsubstituted biphenyl is therefore hydroxylated in the 2,3-positions, to be followed by a 'meta'-type cleavage between C-3 and C-4 of the aromatic ring; this is to be contrasted with the fate of naphthalene, where a 'meta'-type fission occurs between the angular carbon atom and C-1 of 1,2-dihydroxynaphthalene (Davies & Evans, 1964). Several plant aromatic polymers contain the biphenyl type bond (e.g. the biflavonoids).

Multicomponent mixtures of chlorinated bi-