polated to the whole body content was: serum, 3.4; skin, 0.4; muscle, 29.6; bone 1.5; marrow, 0.5.

The percentage of the radioactivity in each tissue was distributed between peaks iii (cholecalciferol), iv and v-vii respectively as follows: serum, 67, 28, 5; skin, 24, 59, 17; muscle, 76, 14, 10; bone 67, 15, 18; marrow, 12, 39, 49. Thus muscle would appear to constitute a store of vitamin D₃ largely in the unmetabolized form.

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The Metabolism of 4-Hydroxypyridine by *Agrobacterium* sp.: a New Ring Cleavage of the Pyridine Nucleus

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In a previous communication Houghton, Wright & Cain (1968) reported the identification of pyridine-3,4-diol as an intermediate in the metabolism of 4-hydroxypyridine by a bacterium, now tentatively identified as *Agrobacterium* sp. Extracts of 4-hydroxypyridine-grown cells, clarified by centrifugation (120 000g for 60 min.) and supplemented with an NADH-generating system, oxidized 4-hydroxypyridine with the consumption of 2 moles of O₂/mole of substrate. In the absence of NADH there was no oxidation of 4-hydroxypyridine, but pyridine-3,4-diol was still rapidly oxidized (1 mole of O₂/mole of substrate). The 4-hydroxypyridine hydroxylase and the 3,4-diol oxygenase could be resolved by standing at 4° for 24 hr. and by Sephadex G-200 chromatography, the very labile oxygenase being inactivated by this treatment.

The hydroxylase was extremely specific for 4-hydroxypyridine (K_m 0.24 mM) but showed product inhibition. The product of the enzymic reaction was identified as pyridine-3,4-diol by isolation from a millimolar-scale preparation and comparison with the synthetic 3,4-diol (mixed m.p. of diacetyl derivatives, u.v. and i.r. spectra, chromatography, FeCl₃ reaction and activity with the oxygenase). In crude extracts NADH was some fivefold more effective than NADPH as cofactor, but response to NADPH further diminished during hydroxylase purification. The K_m for NADH (tris-HCl, pH 8.5) was 0.04 mM. The flavin component of the hydroxylase was resolved by acid (NH₄)₂SO₄ treatment (0.6–0.8 saturation, pH 5.6,

for 3 hr.). Addition of FAD (K_m 0.23 μ M), but not FMN or riboflavin, completely restored the activity.

Pyridine-3,4-diol was metabolized by an oxygenase that lost some 10% of its original activity/hr. (pH 7.0–8.5 at 0–4°). The consumption of 1 mole of O₂/mole of 3,4-diol occurred at pH 8.5 with the release of only one titratable acid group and the formation of an uncharacterized compound (compound I) absorbing at 284 nm. at pH 7–10 and at 260 nm. at pH 3–4. Compound I was altered by such acid treatment, since on readjustment to pH 7.0 the absorption peak was displaced to 279 nm.

At pH 7.0 compound I did not accumulate and the final products of pyridine-3,4-diol metabolism by extracts were identified as pyruvate, formate and NH₃. The nature of these products, the titration value and the tautomerism of compound I strongly suggests a 'meta' cleavage of pyridine-3,4-diol. Formate was further indicated by readdition of the particle fraction to extracts, when a further uptake of 0.5 mole of O₂/mole of substrate was observed.

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The Regulation of Enzymes of Aromatic-Ring Fission in Fungi: Organisms using both Catechol and Protocatechuate Pathways

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The fungal metabolism of aromatic compounds differs from that of bacteria in several ways, among which are the multiplicity of interconversions of intact benzenoid compounds and in particular the distinct metabolic route by which they convert protocatechuate into β -oxoadipate. Although some fungi appear genetically incapable of utilizing the catechol pathway for ring cleavage, most mycelia-forming genera can synthesize the enzymes of both catechol and protocatechuate pathways.

In *Aspergillus niger*, *Penicillium spinulosum*, *Schizophyllum commune* and on unidentified *Penicillium* sp. growth on *p*-hydroxybenzoate induced all the enzymes of the protocatechuate branch (but none of the enzymes of the catechol branch) at amounts 10–80-fold the basal values obtained on glucose. Although these genera grew weakly or not at all on catechol, the enzymes of the catechol pathway, but not the protocatechuate pathway, could be elicited by growing the fungi on mixtures of glucose and catechol. In *Aspergillus niger* exposure to benzoate was accompanied by transient accumulation of *p*-hydroxybenzoate in the medium

and induction of the enzymes of the protocatechuate pathway only, a result radically different from previous bacterial experiments (Ornston & Stanier, 1966; Cánovas & Stanier, 1967). In contrast, the black yeast *Aureobasidium pullulans* produced enzymes of both pathways when induced with *p*-hydroxybenzoate.

The enzymes of the protocatechuate branch in these fungi exhibited a low degree of co-ordinacy, only β -carboxymuconate-lactonizing enzyme, β -carboxymuconate delactonase and β -oxoadipate transferase being co-ordinately induced. Earlier stages appeared to be controlled by sequential inductions, thus permitting protein conservation in organisms utilizing many different aromatic compounds entering the pathway at various levels. *p*-Hydroxybenzoate hydroxylase was substrate-induced and independently regulated in these fungi.

The convergence of the two pathways in fungi is at β -oxoadipate instead of β -oxoadipate enol lactone. The transferase converting this product into β -oxoadipyl-CoA was strongly induced in mycelia of *Aspergillus niger* grown with *p*-hydroxybenzoate or catechol; both compounds induced the same transferase. In *Aureobasidium pullulans*, however, distinct transferases were induced by these substrates and, furthermore, growth of permeability mutants at the expense of β -oxoadipate resulted in the induction of the β -carboxymuconate-lactonizing enzyme and β -carboxymuconate delactonase.

Although not supporting growth of wild-type *Aspergillus niger*, probably owing to permeation difficulties, exposure to 5 mM- β -oxoadipate resulted in an eightfold increase of only two enzymes of the catechol branch but not of the transferase in this fungus.

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The Regulation of Enzymes of Aromatic-Ring Fission in Fungi: Organisms using only the Protocatechuate Pathway

By R. B. CAIN. (*Department of Botany, University of Newcastle upon Tyne*)

Certain fungal genera can neither grow upon nor metabolize catechol or its precursors or metabolic products, whereas their ability to utilize protocatechuate or its precursors is unimpaired. In some respects, therefore, their regulatory pattern is

easier to investigate, since growth on protocatechuate precursors is uncomplicated by the appearance of enzymes of the catechol pathway. Two such organisms are represented by *Neurospora crassa* and the yeast *Rhodotorula mucilaginosa*.

N. crassa grew sparingly on shikimate, protocatechuate and, to a smaller extent, on *p*-hydroxybenzoate, with which substrates all the enzymes of the protocatechuate pathway are induced to high activities. Vanillate acted as a gratuitous inducer of the same group of enzymes. An 'aromaticless' mutant of *N. crassa* blocked before shikimate and requiring all four aromatic amino acids for growth in a minimal medium also showed high activities of the enzymes of the protocatechuate pathway. Basal activities of these enzymes in *Moraxella* have been attributed to shikimate, formed in biosynthesis of aromatic amino acids, leaking into the protocatechuate pathway (Cánovas, Wheelis & Stanier, 1968). Although shikimate dehydrogenase was virtually absent from *N. crassa*, this situation still appeared to be true because the presence in the medium of excess of aromatic amino acids [which should stop shikimate formation by inhibiting the first enzyme (deoxyheptulosonate phosphate synthetase) in the biosynthetic sequence] brought about a marked decrease in the basal activities of the enzymes of the protocatechuate pathway. β -Carboxymuconate-lactonizing enzyme, β -carboxymuconate delactonase and β -oxoadipate transferase were co-ordinately induced and repressed in *N. crassa*. There was evidence that regulatory control of the protocatechuate pathway in *N. crassa* was effected from shikimate. Exposure of mycelia to β -oxoadipate, for instance, elicited the synthesis of only a transferase.

In *R. mucilaginosa* co-ordinacy was equally extensive, all four enzymes of the protocatechuate pathway being co-ordinately controlled. Although *p*-hydroxybenzoate hydroxylase remained independently regulated, a mutant of this organism devoid of the hydroxylase (and thus a means of generating protocatechuate) still had full activity of all four enzymes when grown on *p*-hydroxybenzoate plus glucose, suggesting that this compound may be a substrate inducer for the whole protocatechuate pathway. Revertants from this mutant ($1/10^5$ cells) had fully restored hydroxylase function. Spontaneous mutants selected for growth on β -oxoadipate also had high activities of all enzymes of the protocatechuate pathway even under constitutive conditions.

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