Preliminary Notes

PN 10066

Epoxidation of α-olefins by heptane-grown Pseudomonas cells

Our investigation on the dissimilation of heptene-1 (ref. 1) by a Pseudomonas showed that this hydrocarbon, when incubated with heptane-grown cells, gave rise to ω-unsaturated fatty acid intermediates which could be accumulated in yields of 30–40%, calculated on a molar basis. It was concluded that these cells oxidize heptene-1 preponderantly at C-7 whilst primary attack was thought to be effected by an oxygenating rather than a dehydrogenating enzyme (see also refs. 2–5). An alternative pathway of minor importance via oxidation of the double bond was, however, not excluded. No conclusions were drawn with respect to heptene-1 oxidation by heptene-1-adapted cells.

Epoxidation of the α-olefinic double bond by heptane-grown cells has now been demonstrated. Heptane-grown cells (52 mg dry wt.) in 40 ml phosphate buffer (pH 7.0) were shaken vigorously with 0.5 ml octene-1 in a stoppered 250-ml flask (30 min; 30°). The reaction was terminated with phosphoric acid (0.25 ml) and one drop of a non-ionic detergent* was added. After transfer into a 50-ml volumetric flask (in the cold), pentane (10 ml) was added, and the flask was shaken for 3 min.

Gas–liquid chromatography of the hydrocarbon layer showed the presence of octene 1,2-epoxide, further identified as follows:

(a) Boron-trifluoride-catalysed methanolysis of the biochemical epoxide and of a synthetic octene 1,2-epoxide sample resulted in the formation of two isomeric hydroxymethoxyoctanes[6] in each case. The two pairs of isomers showed the same ratio of peak areas and the same retention volumes. The procedure was repeated for ethanolysis catalysed by sulfuric acid.

(b) Treatment of the biochemical epoxide solution in pentane with HCl (ref. 6) led to 1-chloro-2-hydroxyoctane, identical (gas–liquid chromatography) with the chlorohydrin prepared from the model octene 1,2-epoxide. The isomeric chlorohydrin is formed in small amount only.

(c) The biochemical epoxides of heptene-1 and nonene-1 were prepared in an analogous way by use of heptane-grown cells. The logarithms of the retention times of these epoxides and of the octene epoxide, plotted against the number of C atoms, constitute a straight line. This line perfectly matches the line obtained by plotting the corresponding data of synthetic hexene and octene 1, 2-epoxides.

There is a strong indication that the biochemical epoxidations, with which this note is concerned, are enzymatic, since epoxidation is not brought about by *e.g.* peptone-grown cells and because epoxidation by heptane-grown cells is specific for terminal double bonds. Octene-2, which is smoothly epoxidized by the usual chemical

* Triton X 45. Alkylphenyl polyglycol ether (Rohm and Haas).

agents, remains unchanged under the conditions of the biochemical reaction using alkane-grown cells.

The octene epoxide yield was about 1.0 mg, which is extremely high for a system employing 52 mg dry weight bacteria during 30 min and in a reaction which is in competition with the fast oxidation at the saturated end of the molecule. Chloramphenicol—an inhibitor of protein synthesis and, hence, of adaptation phenomena—did not affect the epoxide yield, showing that the formation of epoxidizing enzymes has not been induced by the α-olefin during the experiment (secondary adaptation).

Epoxides were not detected in the oxidation of *n*-alkanes. In addition, they are poorly utilized by alkane-grown cells. Moreover, the adaptation of the Pseudomonas cells to epoxide substrates is slow or absent. Hence, there are no arguments to assume that epoxides are intermediates in alkane oxidation. Nevertheless, the epoxides are shown to be formed when alkane-grown cells are confronted with an α-olefinic double bond. Therefore, we must conclude that epoxides are formed by an enzyme system already present in the alkane-grown cell and closely related to—if not identical with—the alkane oxidizing system.

α-Olefin epoxidation by alkane-grown cells bears a close relation to the epoxidation of unsaturated steroids by various fungi[7] and by a Nocardia[8]. Unsaturated steroids are epoxidized only when they are incubated with microorganisms capable of introducing a hydroxyl group into the same position of the corresponding saturated steroid. Secondary adaptations were not prevented in the studies cited.

Another oxygenase which seems to have the peculiar property of catalysing at least two different reactions is the Pseudomonas benzoate oxidase[9]. The enzyme preparation of benzoate-grown cells causes 1,2-dihydroxylation of the aromatic ring. This preparation oxygenates *o*-toluate as well, but instead of attacking the ring it causes a hydroxyl group to be introduced into the methyl substituent.

MASON suggested earlier[10] that reduced coenzyme-dependent oxygenases, which hydroxylate aromatic rings, may give rise to epoxides, dihydrodiols and phenols through a common intermediate and without one type of compound being the precursor of the others. He suggests this common intermediate to be a ferrous-enzyme oxygen substrate complex. The nature of the interactions within this complex (depending on enzyme and substrate) is thought to determine which compound(s) is (are) formed and in what relative amounts. Following MASONS suggestion one can imagine that the action of an alkane-oxygenating enzyme, when confronted with an α-olefinic double bond, will lead to an epoxide, a diol, an aldehyde or a ketone (but not to an alcohol), again without one compound being the precursor of the other.

This hypothesis, combined with the fact that alkane-grown cells do not oxidize the epoxide, explains the accumulation of the epoxide. The hypothesis also implies that a pathway via oxidation of the double bond, comprising, for example, the aldehyde and the acid, may exist as an alternative to the oxidation via the saturated end of the molecule. This might be the case in particular in the α-olefin-grown cell, which, by adaptation, might even show a preference for the unsaturated end of the molecule.

For the moment it can be concluded only that alkane-grown Pseudomonas cells do epoxidize the α-olefinic double bond at a high rate and without adaptation. Hence, an oxygenating system is definitely present in these cells. This system is presumed

to be closely related to—if not identical with—the system effecting aerobic hydroxylation of alkanes to give the primary alcohol.

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Induced, soluble phenylalanine hydroxylase from Pseudomonas sp. grown on phenylalanine or tyrosine

The formation of tyrosine by the phenylalanine hydroxylase (EC 1.99.1.2) of mammalian liver has been well studied[1–4]. The biosynthesis of tyrosine in microorganisms is generally via an entirely different pathway[5,6]. Although it has been reported that intact cells of certain species of microorganisms can form tyrosine from phenylalanine under the proper conditions[7–9], the enzyme or enzymes involved have not been obtained in cell-free extracts. This communication describes a soluble, cell-free preparation from Pseudomonas sp. which hydroxylates phenylalanine to form tyrosine.

Pseudomonas sp. (ATCC 11299a) was grown on minimal media containing 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4$, 0.2% NH_4Cl, and either 0.2% L-phenylalanine, 0.1% L-tyrosine, or 0.2% L-asparagine (pH 6.8). The cells were harvested during log phase and washed five times with 0.2% phosphate buffer containing 0.02% $MgSO_4$ (pH 6.8). The concentrated cell suspension was lysed in an osmotic pressure cell and the lysate centrifuged in a Spinco centrifuge, Model L, at 40 000 rev./min for 1 h. The clear supernatant fraction from this preparation was used for these experiments.

The supernatant fraction was incubated in air at 30° with DPNH and L-phenylalanine. At the end of the incubation period trichloroacetic acid was added to a final concentration of 6%. Tyrosine[10] and phenylalanine[11] were determined fluorometrically in aliquots of the protein-free filtrate.

High-speed supernatant preparations from cells grown on phenylalanine con-