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Stereospecificity of Hydride Transfer in the Flavin Mono-oxygenase Orcinol Hydroxylase

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Orcinol hydroxylase, a flavoprotein mono-oxygenase, catalyses the oxidation of orcinol to 2,3,5-trihydroxytoluene. It has been purified to homogeneity and crystallized from a strain of *Pseudomonas putida* grown on orcinol (Ohta & Ribbons, 1970). The enzyme catalyses a typical mixed-function oxidation, using NADH, NADPH or reduced acetylpyridine as electron donor, and is active with several analogues of orcinol, including resorcinol and *m*-cresol. *m*-Cresol, however, is not hydroxylated, equimolar amounts of reduced nucleotide and O₂ being consumed while equimolar quantities of H₂O₂ are formed. Resorcinol is partly hydroxylated to hydroxyquinol, but there is some concomitant uncoupling of electron transfer from the hydroxylation reaction, and H₂O₂ is also formed (Ribbons & Ohta, 1970; Ribbons *et al.*, 1971).

4S-[4-³H]NADH was prepared from [4-³H]NAD⁺ by reduction with ethanol and yeast alcohol dehydrogenase. The opposite isomer (4R-[4-³H]₁NADH) was prepared in analogous experiments with NAD⁺ and [1-³H₂]ethanol, and their authenticity was confirmed by using pyruvate and rabbit muscle lactate dehydrogenase.

The specifically labelled nucleotides were incubated with orcinol hydroxylase in duplicate reaction mixtures for each isomer and each aromatic substrate. When orcinol was substrate, 3.1% of the tritium was recovered in water, from the 4S species of NADH. Similar results were obtained when the 4S-[4-³H]NADH was oxidized in the presence of resorcinol or *m*-cresol as effector; only slightly more radioactivity appeared in the water. The complementary experiments with 4R-[4-³H]NADH confirmed the stereospecificity of the transfer of hydride when orcinol was the substrate, 83% of the tritium being found in the water. However, when resorcinol was the aromatic substrate, there was a large isotope effect with 4R-[4-³H]NADH, and this was more pronounced when *m*-cresol was substrate; only 42.3 and 27.1% of the radioactivity supplied was

recovered in the water respectively. At higher temperature (55°C) with orcinol as substrate there was also an isotope effect with the 4R species of NADH; uncoupling of hydroxylation from electron flow also occurred at 55°C. The recovery of radioactivity in the water and that retained in the NAD⁺ formed was at least 90% in all experiments.

These findings will be discussed in relation to the mechanism of the enzymic reaction.

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The Modification of Bongkreic Acid Inhibition of Mitochondrial Adenine Nucleotide Translocation by Coenzyme A

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The tricarboxylic bongkreic acid is a potent inhibitor of the translocation of adenine nucleotides across mitochondrial membranes, but a time-lag occurs between the addition of bongkreic acid and the appearance of inhibition (Henderson & Lardy, 1970; Klingenberg *et al.*, 1970; Henderson *et al.*, 1970). The lag period can be shortened by prior incubation with adenine nucleotides (Kemp *et al.*, 1970) and is much longer at lower temperatures (Henderson *et al.*, 1970). Shug *et al.* (1971) demonstrated that an inhibition of adenine nucleotide transport by fatty acids is in fact dependent on their conversion into the CoA derivative, and this suggested to us that CoA may also be involved in the inhibition by bongkreic acid.

A direct assay of the adenine nucleotide exchange activity has been utilized (Henderson *et al.*, 1970) under conditions where inhibition by bongkreic acid appeared after 7–10 min and was complete in a further 2 min. The presence of 200 μM-CoA shortened the lag period to ½–1½ min. Carnitine did not substitute for CoA, nor did it prevent the CoA effect, and neither carnitine nor CoA inhibited translocation in the absence of bongkreic acid. Uncoupling agents did not prevent the CoA 'activation', and CoA did not appear to affect inhibition of the adenine nucleotide translocation by atractyloside.

A time-lag was also apparent when the inhibition

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of mitochondrial oxidative phosphorylation by bongkreikic acid was measured. Again, the presence of CoA considerably shortened the time required for bongkreikic acid to act.

A possible explanation for these observations is that bongkreikic acid must first be converted into a CoA derivative before it can inhibit adenine nucleotide translocation. Further work is aimed towards the isolation of such a derivative and identification of an enzyme (probably an acyl-CoA synthetase) responsible for its formation,

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Ureolysis in the Ovine Rumen

By A. R. COOK (Department of Microbiology, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K.)

The rumen contents of sheep and cattle on all diets possess ureolytic activity. Although this activity was shown to be of bacterial intracellular origin, so confirming earlier work (Gibbons & McCarthy, 1957), only a few ureolytic bacteria have so far been isolated and positively identified (Gibbons & Doetsch, 1959). In the present work only ten ureolytic strains out of over 1000 bacteria were isolated from a sheep fed on hay and dried grass.

The bacteria were isolated by two methods. In the first, 15 different media varying from the non-selective counting media to several selective media were used in the standard roll-tube method. The media should provide growth conditions for all types of rumen bacteria, but the only ureolytic bacteria isolated were five strains of *Staphylococcus* (group II), two strains of group VI and a strain of *Lactobacillus casei* var. *casei*. These were isolated from the 10^6 -dilution cultures.

In the second method, media that enriched the ureolytic bacteria of undiluted rumen fluid were used to enrich serial dilutions of rumen fluid, and *Klebsiella aerogenes* and a *Streptococcus* sp. (intermediate in properties between *Streptococcus faecium* and *Streptococcus faecalis*) were isolated from dilutions 10^5 and 10^7 respectively.

No ureolytic strains of obligate anaerobes were isolated from the rumen, and the results of this survey suggest that ureolytic activity is associated with bacteria present in comparatively small numbers and not with those bacteria present in high numbers and considered important in rumen function.

The *Streptococcus* sp. was ureolytic only under anaerobic conditions, and investigations of factors controlling the level of the activity per unit growth show that the activity would account for most of the ureolytic activity of the sheep rumen contents.

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Factors Affecting the Inhibition of Mitochondrial Adenosine Triphosphatase by Trialkyltin Compounds

By M. J. SELWYN, S. J. DUNNETT, R. D. PHILO and A. P. DAWSON (School of Biological Sciences, University of East Anglia, Norwich NOR 88C, U.K.)

The 2,4-dinitrophenol-stimulated ATPase* of rat liver mitochondria is potently inhibited by trialkyltin compounds (Aldridge, 1958; Aldridge & Street, 1971). However, unless Mg^{2+} ions are present, at concentrations greater than or equal to the concentration of ATP, the apparent K_i for inhibition by trimethyltin is very much increased.

At pH 6.8 the effectiveness of trimethyltin as an inhibitor of the 2,4-dinitrophenol-stimulated ATPase of whole mitochondria is diminished by the presence of 10 mM-P_i . In whole mitochondria the situation is complicated by intramitochondrial pH changes resulting from the Cl^- - OH^- exchange mediated by trialkyltins (Selwyn *et al.*, 1970). At pH 6.8 the effectiveness of trimethyltin as an inhibitor of the Mg^{2+} -stimulated ATPase of fragmented mitochondria is also diminished by the presence of P_i , but at pH 7.5 the effect of P_i is much less. Rose (1969) has reported that trialkyltins are complexed by P_i and that the affinity decreases above pH 7. In the absence of P_i the K_i for inhibition of the ATPase of broken mitochondria by trimethyltin is about one-quarter of the dissociation constant measured directly by Aldridge & Street (1970). However, Aldridge & Street (1970) measured the binding at pH 6.8 in the presence of 15 mM-P_i . When allowance is made for the effects of P_i there is little discrepancy between the two estimates of the dissociation constant.

The failure of atractyloside to inhibit the ATPase of broken mitochondria supports the view (Stockdale

* Abbreviation: ATPase, adenosine triphosphatase.