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Kinetic Mechanism of Cytidine Amino-hydrolase

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Previous reports from this laboratory have indicated cytidine aminohydrolase (EC 3.5.4.5) as a possible regulatory enzyme in nucleic acid metabolism (McFerran, Smyth & Orsi, 1969). Results presented in the present communication suggest cytidine aminohydrolase possesses many of the characteristics associated with other regulatory enzymes.

Gel-filtration studies on cytidine aminohydrolase with Sephadex G-200 over a range of pH values between 5.5 and 8.6 and a range of ionic strengths between 0.1 and 1.0 have shown no decrease in molecular weight at high pH and high ionic strength. However, under these conditions a marked increase in enzymic activity is observed. Temperature also plays a role in this activation: at 10°C 1.0M-NaCl has no effect on the enzyme's activity, but at 37°C a 3.6-fold activation over an NaCl-free control was observed.

A series of univalent-cation chlorides was tested for their effect on this activation. At a constant ionic strength of 1.0 the order of activation by metal ion was according to the sequence: $\text{Na}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{K}^+ > \text{Li}^+ > \text{HN}_4^+$. This sequence could correlate with their hydrated ionic radii as determined by Padova (1964) by using electrostrictive techniques; however, gross variations in values for hydrated ionic radii may be found in the literature. At high pH KCl and NH_4Cl both exhibit a saturation effect in their activation with a cytidine concentration equal to its K_m ; however, with NaCl there is a linear relationship between the NaCl concentration and the activity in the range tested (<1.5M).

Product-inhibition studies (Cleland, 1963) were used in an attempt to identify the kinetic mechanism of the enzyme. Ammonia is not inhibitory and uridine, the other product, gave competitive inhibition. These results would be compatible with an apparent Uni Bi mechanism (Crypto Ping Pong) with the first substrate cytidine and the second water (which must be saturating to prevent ammonia acting as an inhibitor); the first product would be ammonia and the second uridine. This type of mechanism is common to many hydrolytic enzymes (Bender & Kézdy, 1965). The 'modified-

enzyme' intermediate in this mechanism would be a 'uridylyl-enzyme' complex, whose hydrolysis should not be the rate-limiting step in the reaction sequence.

Further studies have indicated that the reaction is not catalysed via a simple nucleophilic displacement.

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The Alcohol Dehydrogenase of *Clostridium acetobutylicum*

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The principal neutral volatile metabolites of *Clostridium acetobutylicum* N.C.I.B. 8049 are butan-1-ol, acetone and ethanol. Formation of propan-1-ol and 2-methylpropan-1-ol has been induced by increasing the redox potential of culture media for a short period during the exponential phase of growth or by addition of certain amino acids (Ward & Fogarty, 1970). In the present study the alcohol dehydrogenase (EC 1.1.1.1) of *C. acetobutylicum* was investigated with respect to method of preparation and substrate specificity.

The bacterium was grown anaerobically in a glucose-peptone-salts broth at 37°C. The cells were harvested after 32h by centrifugation and were stored at -25°C until required. After being washed once, the cells were resuspended in 0.01M-phosphate buffer, pH 7.5, before disruption at 0°C. The following methods of cell disintegration and enzyme extraction were examined: ultrasonic sound waves in an MSE ultrasonic disintegrator, agitation with 0.1mm Ballotini glass beads in a Braun cell homogenizer and acetone-powdering followed by extraction with buffer. In addition, grinding with the following abrasives was investigated: glass powder, 1μm particles of aluminium oxide and aluminium sodium silicate. The cell homogenate was centrifuged at 20 000g for 30min at 0°C. The supernatant was retained for enzyme studies and for protein determination. Active enzyme preparations were obtained with each treatment. The alcohol dehydrogenase activity was particularly high in the cell-free extract prepared by grinding with aluminium sodium silicate.

The specificity of the alcohol dehydrogenase of

cells harvested from cultures containing butan-1-ol and ethanol was examined by following the reduction of NAD^+ by the method of Vallee & Hoch (1955). The activity of the enzyme preparation was examined with respect to the following primary and secondary alcohols: ethanol, propan-1-ol, propan-2-ol, butan-1-ol, 2-methylpropan-1-ol, butan-2-ol, 2-methylbutan-1-ol and 3-methylbutan-1-ol. Results obtained indicate that the alcohol dehydrogenase of *C. acetobutylicum* is non-specific with regard to substrate. The synthesis of 2-methylpropan-1-ol, propan-1-ol and any of the above-mentioned alcohols is therefore possible on provision of a suitable precursor. Further studies on the properties of this enzyme preparation are in progress.

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The Alkylation of Yeast Hexokinase A

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Hexokinase A is one of the two isoenzymes isolated from baker's yeast by a method that avoids proteolysis (Lazarus, Ramel, Rustum & Barnard, 1966). Evidence suggests that 4 out of the 8 thiol groups/molecule of mol.wt. 100 000 are not essential for catalytic activity (Lazarus, Derechin & Barnard, 1968). However, inactivation of hexokinase A by iodoacetate at low ionic strength is accompanied by carboxymethylation of all 8 thiol groups (Lazarus *et al.* 1968).

Hexokinase A was treated with iodoacetate (10–700 mM) at pH 8.4 in tris-HCl buffer, *I* 0.1, when the enzyme is presumed to exist as a dimer of mol.wt. 50 000 (see Lazarus *et al.* 1968). At 25°C inactivation of the enzyme is first-order with respect to the concentration of each reactant with a rate constant of $0.9 \times 10^{-3} \text{ l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. At 30°C inactivation of hexokinase A is first-order with respect to the concentration of the enzyme, but the relationship between reaction rate and the concentration of iodoacetate is hyperbolic. The data are consistent with a reversible equilibrium between protein and inhibitor (K_i 0.03 M) before alkylation of the enzyme. At low concentrations of iodoacetate and at pH 8.4 the apparent second-order rate constant for inactivation at 30°C is $28 \times 10^{-3} \text{ l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and it is very sensitive to changes in pH or temperature. These changes may reflect the conformational state of the protein (see also Jones, 1970).

Inactivation (86%) of hexokinase A at 30°C by 16 mM-iodo[^{14}C]acetate leads to the incorporation of 3.2 g-atoms of ^{14}C /mol of enzyme. Glucose (20 mM) prevents this inactivation but 0.8 g-atom of ^{14}C is still incorporated/mol of enzyme. When this latter experiment was repeated with 0.2 M-iodoacetate the enzyme still retained over 80% of its activity. After being desalted this preparation could be inactivated by fresh iodoacetate at 25°C, the kinetics of inactivation being identical with that described earlier for native hexokinase A. In one experiment the partially alkylated active enzyme described above was treated with iodo[^{14}C]acetate, when complete inactivation was accompanied by the incorporation of 1.9 g-atoms of ^{14}C /mol of enzyme.

The collective results confirm that hexokinase A possesses both essential and non-essential thiol groups. Hence glucose prevents inactivation but only decreases the degree of alkylation of enzyme by iodoacetate.

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The Hydrolysis of Glycine Oligopeptides by Guinea-Pig Intestinal Brush Borders

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Previous studies have shown that, whereas diglycine peptide hydrolase (EC 3.4.3.1) occurs predominantly in the cytosol from intestinal mucosa, significant amounts of triglycine peptide hydrolase (EC 3.4.1.3) are localized in the brush-border fraction (Peters, 1968). It was therefore decided to study the specificity of oligopeptide hydrolysis in further detail.

Brush borders were prepared from guinea-pig intestinal mucosa (Eichholz & Crane, 1965) and were demonstrated to be substantially free of other subcellular organelles. The brush borders were shown to hydrolyse glycine oligopeptides up to and including hexaglycine, but did not hydrolyse polyglycine (mol.wt. approx. 10 000). The oligopeptides studied were not hydrolysed by pepsin, trypsin, α -chymotrypsin, elastase, carboxypeptidase