The Action of Pyruvate on Ethanol Oxidation by Intact Isolated Liver Cells

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The oxidation of ethanol to acetate by isolated liver cells from starved rats was stimulated almost fourfold to $2.96 \mu\text{mol/min per g wet wt.}$ in the presence of 10 mM -pyruvate. Both lactate and glucose formation from pyruvate were increased in the presence of ethanol, and balance studies indicated that pyruvate or its metabolites could have acted as acceptors for up to 90% of the reducing equivalents arising during ethanol oxidation to acetate. Enhancement of ethanol oxidation by pyruvate was completely suppressed by 0.25 mM -pyrazole, but in contrast with fructose-promoted ethanol oxidation the pyruvate effect was inhibited 30% by 3-hydroxybutyrate or palmitate. Rotenone prevented glucose formation from pyruvate, decreased lactate accumulation by more than 50% and inhibited stimulation of ethanol oxidation by pyruvate by almost 90%. Pyruvate-stimulated ethanol removal was also decreased by drugs metabolized within the hepatic endoplasmic reticulum. Acetanilide or aminopyrine inhibited by 40% and aniline or antipyrine inhibited by 70%. The inhibitions by 3-hydroxybutyrate and aniline or by 3-hydroxybutyrate and antipyrine were partially additive.

These results support the view that the rate-limiting step in ethanol metabolism is the reoxidation of the NADH arising from alcohol dehydrogenase-catalysed ethanol oxidation. It appears that pyruvate or its metabolites promote ethanol oxidation by acting as acceptors for the reducing equivalents generated during acetate formation. The inhibition of these interactions of pyruvate and ethanol by rotenone and by mitochondrial substrates indicates that mitochondrial electron transport may be involved in the transfer of reducing equivalents between ethanol and pyruvate or its metabolites. Moreover, the partial inhibition of the pyruvate effect by microsome-metabolized drugs raises the possibility that microsomal electron transport may also play some role in pyruvate-stimulated ethanol oxidation by the isolated liver cells.

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Determination of the Operational Molarity of Solutions of Bovine α -Chymotrypsin, Trypsin, Thrombin and Factor Xa by Spectrofluorometric Titration

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Several spectrophotometric methods for determining the operational molarity of solutions of chymotrypsin, trypsin, and thrombin have been developed here and elsewhere. No methods, however, have been described for the titration of Factor Xa. Unfortunately, spectrophotometric titrations require an inconveniently large amount of enzyme. Consequently we have synthesized spectrofluorometric titrants, since the amount of enzyme required for assay should be much less than for spectrophotometric methods. The kinetic requirements for a suitable titrant (Bender *et al.* 1966) are even more crucial for spectrofluorometric than for spectrophotometric methods, since both substrate and enzyme concentrations are very low. The rate constants for acylation and deacylation must be very high and low respectively and K_m must be very low. We have synthesized 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride, an analogue of the *p*-nitrophenyl ester used for spectrophotometric titration of trypsin and thrombin (Chase & Shaw, 1967, 1969; Baird & Elmore, 1968), and this is a good spectrofluorometric titrant for α - and β -trypsin, thrombin and Factor Xa. As little as 0.02 nmol of enzyme can be titrated, and the burst of 4-methylumbelliferone is linear with 0.02 – 3.00 nmol of enzyme and is independent of the concentration of titrant. Typically 0.1 mM -4-methylumbelliferyl *p*-guanidinobenzoate in 1 mM -hydrochloric acid containing 1% (v/v) of *N*-methylpyrrolid-2-one (10 – $250 \mu\text{l}$), 50 mM -sodium barbiturate-HCl buffer, pH 8.3 (3.0 ml), and trypsin or thrombin solution (10 – $100 \mu\text{l}$) were mixed and the increase in emission at 445 nm was measured after excitation at 365 nm . 4-Methylumbelliferyl *p*-guanidinobenzoate like *p*-nitrophenyl *p*-guanidinobenzoate (Hruska, Law & Kézdy, 1969), reacts with α -trypsin much more slowly than with β -trypsin. The rate of acylation of Factor Xa by the 4-methylumbelliferyl ester was inconveniently low and required a modified technique: enzyme and substrate were allowed to react in a small volume of solution ($50 \mu\text{l}$), which was then diluted after completion of acylation and before spectrofluorometric assay. Values for the operational molarities of trypsin, thrombin and Factor Xa solutions obtained with 4-methylumbelliferyl

p-guanidinobenzoate and with the *p*-nitrophenyl ester agreed closely.

We have also synthesized 4-methylumbelliferyl *p*-trimethylammonium cinnamate chloride, an analogue of the *p*-nitrophenyl ester used for the spectrophotometric determination of α -chymotrypsin (Knowles & Preston, 1968). The technique for the spectrophotometric titration of α -chymotrypsin with this titrant is similar to that described above for titrations with 4-methylumbelliferyl *p*-guanidinobenzoate. Results obtained with 4-methylumbelliferyl *p*-trimethylammonium cinnamate agreed well with those obtained with *p*-nitrophenyl N^2 -acetyl- N^1 -benzylcarbazate (Elmore & Smyth, 1968).

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Observations on Rat Mast-Cell-Granule Protease

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Mammalian mast cells contain proteases that, according to their ability to hydrolyse ester substrates, appear to have active centres analogous to either trypsin or chymotrypsin (see Selye, 1965). Interrelationships between the chymotrypsin-like enzyme of rat peritoneal-mast-cell granules (Lagunoff & Benditt, 1963) and the granule heparin proteoglycan fraction (cf. Lloyd, Bloom, Balazs & Haegermark, 1967a; Lloyd, Bloom & Balazs, 1967b) have now been investigated. The studies were facilitated by the development of spectrofluorometric procedures based on the use of *N*-methoxycarbonyl-L-phenylalanine 4-methylumbelliferyl ester and 4-methylumbelliferyl *p*-trimethylammonium cinnamate chloride. These compounds respectively have been shown to be suitable as steady-state

assay substrate and titrant for α -chymotrypsin (T. Morrison, unpublished work; Roberts, Adams, Elmore, Jameson & Kyle, 1971).

Granule preparations were obtained from fractionated populations of mast cells (purity 97–99% by haemocytometer count) by using methods described by Lloyd *et al.* (1967a). The granule ultrastructure was dissociated by extraction with 2M-sodium chloride followed by sonication for 30s at 4°C before centrifuging at 35000g for 45min. At pH 7.09 and 25°C in 20mM-phosphate buffer containing 0.1M-sodium chloride the clarified extracts accomplished the turnover of *N*-methoxycarbonyl-L-phenylalanine 4-methylumbelliferyl ester and reacted with 4-methylumbelliferyl *p*-trimethylammonium cinnamate in a manner analogous to chymotrypsin.

The unique sensitivity of 4-methylumbelliferyl *p*-trimethylammonium cinnamate as a titrant for chymotrypsin-like active centres was exploited in investigations of the binding of the enzyme to the granule matrix. Granule preparations were suspended at 4°C for 30min in sodium chloride solutions in the concentration range 0.1–2.0M and residual material was then removed by centrifuging at 35000g for 45min. The operational molarity of the enzyme in each of the supernatants was titrated with the titrant and expressed as a percentage of the operational molarity in the supernatant after extraction with 2.0M-sodium chloride, which also liberated all the heparin proteoglycan into solution (cf. Lloyd *et al.* 1967a). Complete recovery of enzyme was achieved after extraction with 1.2M-sodium chloride accompanied by 22% of granule heparin. Declining amounts of enzyme were recovered on extraction with 0.9M-sodium chloride (46.4%) and 0.7M-sodium chloride (7.3%). These findings are in agreement with the view that the heparin proteoglycan is among the most stable of the components of the granule to electrolyte extraction and is likely to contribute materially to the reticular network of the granule matrix (cf. Lloyd *et al.* 1967a,b), to which other granule components are attached by weak electrostatic forces.

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