the lactating mammary gland, and few membrane-bound enzymes of this type have been so extensively purified.

Kinetic parameters of the *N*-acetyl-lactosamine synthetase reaction (2) were measured, and the results obtained were consistent with a reaction mechanism in which there was an ordered addition of UDP-galactose and *N*-acetylglucosamine and an ordered release of *N*-acetyl-lactosamine and UDP. These results are in agreement with those obtained by Morrison & Ebner (1971*a,b,c*). The strength of binding of the A protein to the affinity columns was influenced by its substrates and inhibitors in accord with this mechanism.

The binding stoichiometries between the A protein and α -lactalbumin were examined by the method of continuous variation (Job, 1928). In this the total protein concentration was held constant, but the relative concentrations of the A protein and α -lactalbumin were varied. The ability to synthesize lactose was then used as a measure of complex-formation, and the positions of maximum activity indicated the molar ratios of the complexes formed. Two major complexes were formed, corresponding to A protein/ α -lactalbumin molar ratios 1:3 and 1:1. The existence of these complexes in solutions containing the A protein and α -lactalbumin in the presence of glucose was confirmed by the use of a bifunctional cross-linking reagent, dimethyl suberimidate (Davies & Stark, 1970). After reaction with this reagent the incubation mixtures were submitted to analysis by electrophoresis in gels containing sodium dodecyl sulphate. Species were apparent with molecular weights corresponding to those of the A protein, α -lactalbumin and complexes corresponding to 1:1, 1:2 and 1:3 molar ratios respectively of these proteins. An additional complex containing the A protein and α -lactalbumin in 1:2-molar ratio was found by this method. The formation and stability of these complexes, however, will be a function of their equilibrium constants, and it may be that the lactose synthetase assay is not sensitive enough to pick up more than the most active and stable species in the method of continuous variation.

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The Separation of Functional and Non-functional Xanthine Oxidase by Affinity Chromatography

By VINCENT MASSEY, DALE EDMONDSON, GRAHAM PALMER, LOWRIE M. BEACHAM, III, and GERTRUDE B. ELION (*Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich. 48104, U.S.A., and Burroughs Wellcome Co. Research, Triangle Park, N.C. 27709, U.S.A.*)

Since 1949 evidence has accumulated that even the most active preparations of milk xanthine oxidase contain non-functional active sites, even though they possess their full complement of FAD, molybdenum and iron-sulphur chromophores (Lowry *et al.*, 1949; Morell, 1952; Bray *et al.*, 1961; Massey *et al.*, 1970*a*). Recent work on the powerful inhibitory effects of pyrazolo[3,4-*d*]pyrimidines on xanthine oxidase has revealed that these compounds form inactive complexes with the enzyme when the molybdenum component of the latter is in a reduced state, e.g. Mo^{IV} (Massey *et al.*, 1970*a,b*; Spector & Johns, 1970). It was found that only functional enzyme formed such tight complexes; non-functional enzyme has been found to complex only weakly with pyrazolo[3,4-*d*]pyrimidines. We have made use of this property to devise an affinity-chromatography method for the separation of functional and non-functional xanthine oxidase.

6-Amino-1-propyl-3-(1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamino)hexanoate was synthesized and attached to Sepharose 6B by reaction with CNBr (in the manner described by Cuatrecasas *et al.*, 1968). This was converted into the 6-hydroxy derivative by prolonged aerobic incubation with xanthine oxidase. The structure of the derivative used in affinity chromatography was therefore as shown in formula (I).

(I)

The activated Sepharose was packed in a column and equilibrated with 0.1M-pyrophosphate buffer, pH 8.5, containing 10mM- $\text{Na}_2\text{S}_2\text{O}_4$, and kept under an atmosphere of N_2 . Milk xanthine oxidase of approx. 70% functionality, as normally isolated (Massey *et al.*, 1969), was applied to the column and allowed to equilibrate overnight at room temperature to ensure full complex-formation with functional enzyme. A fraction that had very little activity (being mainly non-functional enzyme) was then eluted with $\text{Na}_2\text{S}_2\text{O}_4$ -containing buffer. The remaining enzyme was very tightly bound to the activated Sepharose, and was not eluted with $\text{Na}_2\text{S}_2\text{O}_4$ -containing buffer. The column was then equilibrated with aerobic buffer, and equilibrated for several days at 4°C to permit the slow oxidation of enzyme-bound molybdenum to the Mo^{VI} state, with the accompanying dissociation of enzyme from its complex with the pyrazolo[3,4-*d*]pyrimidine (Massey *et al.*, 1970*a,b*). The enzyme was then eluted with aerobic buffer, and found to be mainly fully functional. From previous studies (Massey *et al.*, 1970*a,b*; McGartoll *et al.*, 1970) the AFR^{25°C} value for fully functional enzyme had been predicted to be approx. 210. [The AFR^{25°C} value is the activity/ E_{450} as defined by Avis *et al.* (1955) but determined at 25°C instead of 23.5°C.] The first fraction obtained by affinity chromatography had AFR values ranging from 0 to 30 across the peak; the second fraction eluted mainly had an AFR value of 200–210.

Both functional and non-functional enzyme have been found to have very similar spectral properties and cofactor composition; they both contain FAD, Mo, Fe and acid-labile S in the proportions 1:1:4:4. The essential difference between the two forms has been found to be in the content of active-centre persulphide, recently discovered in this enzyme, which on reaction with cyanide is split to yield a protein thiol group, with the release of CNS^- (Massey & Edmondson, 1970). This reaction can also be monitored by a distinctive decrease in the absorption of the enzyme, maximal at 320nm (Coughlan *et al.*, 1969). Non-functional enzyme was found to release no CNS^- on reaction with CN^- ; fully functional enzyme was found to release 0.95mol of CNS^- /mol of enzyme-bound FAD. In analogy with the results with cyanide-inactivated enzyme (Massey & Edmondson, 1970), it was found that non-functional enzyme could be activated by incubation with Na_2S . Thus it is clear that the reason for non-functionality is the lack of the essential persulphide group. It is proposed that the role of this group in catalysis is to promote H^- -transfer from the substrate to the enzyme by nucleophilic attack, with a resulting covalent link between the electron-deficient carbon atom and the persulphide as an intermediate. Hydroxylation is then proposed to occur by attack of OH^- , to regenerate

the enzyme persulphide and liberate the hydroxylated product. The role of O_2 , or other electron acceptors, in the catalytic reaction, is proposed to be simply the reoxidation of the reduced chromophores of the enzyme.

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Some Studies on Insolubilized Nicotinamide Nucleotides

By P. D. G. DEAN and C. R. LOWE (*Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.*)

Affinity chromatography on insolubilized cofactors is an attractive proposition for the purification of groups of enzymes with similar catalytic functions. One such group which interests us particularly is the NAD(P)-dependent dehydrogenases.

The chemistry of the nicotinamide nucleotides is not well documented, and the search for methods for the covalent attachment of these coenzymes to insoluble matrices is hindered by their relative instability and their lack of reactivity. Of the functional groups of the adenine moiety of NAD(P) the amine at position 6 is potentially available for linkage by CNBr-activated polysaccharide polymers (Axén *et al.*, 1967) or the epichlorohydrin method (Porath & Fornstedt, 1970). [This relatively unreactive amine (like 2-aminopyridine) probably does not have a completely free lone pair of electrons on the amine nitrogen atom (R. M. Acheson, personal communication).] Methods based on attacking the purine ring system with electrophiles such as diazonium