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Preparation and properties of cholinesterases covalently bound to Sepharose

Enzymologists gradually become aware that the study of *in vivo* membrane-bound enzymes in solution may not give all the information wanted. This procedure is not suited for investigating factors of importance in the organized cell such as flow of substrates and inhibitors to and of products from the membrane-bound enzyme, pH conditions in the microenvironment of the enzyme and the influence of the structure of the surrounding membrane on the enzymes and its reactions. Model systems obtained by enzymes bound to a synthetic matrix may provide some information as to factors influencing the activity of membrane-bound enzymes. Other reasons for the study of matrix-supported enzymes are their suitability for analytical purposes.

Cholinesterase (acetylcholine hydrolase, EC 3.1.1.7) has been immobilized by entrapment of the enzyme in starch gel[1], in starch gel fixed on polyurethane resin[2], in a Silastic resin[3] and by covalent fixation to poly-L-alanine–glutamic acid, CM-cellulose, poly-L-aspartic acid and polygalacturonic acid[4]. In order to obtain products with great stability, a covalent bond seems to be expedient. Such methods[5–10] have been described; among them are chemical fixation of proteins to polysaccharides[11–13]. The present paper describes the preparation and some properties of acetylcholinesterase (Winthrop Labs., New York) and butyrylcholinesterase (horse serum, Organon, The Netherlands) bound to Sepharose 2B (Pharmacia, Sweden). Despite their impurity, these enzymes have been chosen for two reasons: firstly, membrane-bound acetylcholinesterase most probably plays an important role in cholinergic nerve transmission and, secondly, matrix-supported cholinesterases may be used as analytical tools in the determination of insecticide and nerve-gas-type organophosphorus compounds.

Sepharose 2B in water was filtered. 4 g of the gel (approx. 100 mg dried polysaccharide) were transferred to a double-jacketed reaction vessel thermostated at 25° and stirred. 3.5 ml of water and 4 ml of a BrCN solution containing 25 mg BrCN per ml water were added. The pH of the suspension was rapidly raised to 11.0 by adding 2 M NaOH and was kept there for 6 min. The activated gel was washed on a glass filter with 1 l of cold water and 50 ml of cold 0.1 M $NaHCO_3$. 100 mg of the enzyme preparation dissolved in 10 ml of cold 0.1 M $NaHCO_3$ were added to the activated gel in a test tube. The test tube was rotated end-over-end at 3° for 20 h. The gel was packed on a column attached to a peristaltic pump and washed with the following solutions in the order given: 0.1 M $NaHCO_3$ (24 h); 0.5 M NaCl (24 h); 0.1 M sodium acetate, pH 5.5 (24 h); aq. dest. (12 h). For batch-wise operations, the gel was transferred to 125 ml of aq. dest. and stored in this form. Before withdrawing aliquots, the suspension was stirred. The concentration of the enzyme conjugate in the suspension was determined by transferring a series of suspension volumes to weighed glass filters. Acetone was used to replace water. Finally, the gel was dried for 24 h over P_2O_5 in vacuum, and the filter with the conjugate was weighed. The amount of fixed protein was calculated from amino acid analysis of the starting enzyme preparation and the conjugate. This value had to be corrected for gelatine present

TABLE I

SPECIFIC ACTIVITIES OF FREE AND CONJUGATED ENZYMES

Specific activities of free and conjugated enzymes (pH 7.4, 25°, acetylcholine iodide for acetylcholinesterase 3.1 and for butyrylcholinesterase 19 mM), coupling yields and retained activities as percent of those of the free enzymes.

Enzyme	*Specific activity (μmoles acetic acid · min^{-1} · mg^{-1})*	*Coupling yield (mg protein per g dry conjugate)*	*Specific activity of the conjugate (% of that of the free enzyme)*
Free enzyme			
Butyrylcholinesterase	4.25		
Acetylcholinesterase, Prep. I	5.45		
Prep. II	6.18		
Conjugated enzyme			
Butyrylcholinesterase,	1.76	87.5	41
Acetylcholinesterase, Prep. I	0.68	52.8	12
Prep. II	0.70	53.6	11

TABLE II

THE EFFECT OF COUPLING ON SUBSTRATE SPECIFICITY (pH 7.4, 25°)

Substrate	*Concn. (mM)*	*Activity (μmoles acetic acid · min^{-1} · mg^{-1})*	
		Free acetylcholinesterase	*Conjugated acetylcholinesterase*
Acetylcholine iodide	3.1	6.18	0.70
Propionylcholine iodide	2.5	4.63	0.82
Butyrylcholine iodide	2.5	—	—
Acetyl-β-methylcholine iodide	10.0	2.18	0.39
Benzoylcholine iodide	2.5	—	—

TABLE III

THE EFFECT OF COUPLING ON K_m, V AND k_2 (pH 7.4, 25°)

The rates of inhibition (k_2) were measured with acetylcholinesterase protected by 3.1 mM acetylcholine iodide.

Substrate	k_2 ($l \cdot min^{-1} \cdot mole^{-1}$) × 10^{-5}		$K_m \times 10^4$ (*M*)	*V (μmoles acetic acid · min^{-1} · mg^{-1})*
	Sarin	*Methylfluorophosphoryl β-methylcholine*		
Free acetylcholinesterase				
Acetylcholine iodide	7.7	10	1.66	7.95
Propionylcholine iodide			1.96	5.84
Conjugated acetylcholinesterase				
Acetylcholine iodide	4.3	6.0	1.40	1.29
Propionylcholine iodide			1.62	0.90

in the enzyme preparation used. The correction was made by evaluating the amount of oxyproline of the conjugate, and assuming that the enzyme should not contain this amino acid (Table I). Both acetylcholinesterase and butyrylcholinesterase were active after fixation (Table I), the retained specific activity being higher for butyrylcholinesterase than for acetylcholinesterase.

The kinetic measurements (only acetylcholinesterase so far) were performed with the aid of an automatic recording titrator.

Substrate specificity was tested with acetylcholine, propionylcholine, butyrylcholine and acetyl-β-methylcholine (mecholyl), all iodides. No changes in substrate specificity due to fixation of the enzyme was observed (Table II).

Table III shows K_m and v for acetylcholinesterase and the substrates acetylcholine and propionylcholine. The observed decrease in K_m may be due to enrichment of substrate due to attraction between the positively charged substrate and some negative charges present in the matrix[9]. A configurational change in the enzyme seems less likely. As seen, there is proportionality in the K_m values obtained with both substrates.

V (expressed as activity per mg protein) decreased about 6 times as the result of the fixation. This could be due to a decrease in the number of active sites present per mg bound enzyme protein. Further experiments will elucidate this. A decrease was also observed in the rate of inhibition (k_2) by uncharged methylisopropoxyphosphoryl fluoride (Sarin) as well as by the positively charged methylfluorophosphoryl β-methylcholine iodide. The values again indicate an enrichment of positively charged compounds at the enzyme surface.

The pH–activity curve obtained is displaced approx. 1 pH unit towards alkaline pH. Such effects have been suggested[10] to be due to lowering of the pH in the microenvironment of the enzyme caused by the generation of hydrogen ions after ester hydrolysis. The thermostability of the matrix supported enzyme preparation was tested at 25°, 37° and 60° and found to be equal to that of the free enzyme. At 4° the matrix supported preparation was stored as a water suspension for 4 months with a 30% loss of enzyme activity.

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Lysosomal localization of sphingomyelinase in rat liver

Several recent studies have shown that rat-liver lysosomes contain an acid hydrolase that splits sphingomyelin to ceramide and phosphorylcholine[1–4]. However, it is not clear from the data given whether the enzyme is restricted solely to lysosomes or is present also in other hepatic cell organelles. The results of HELLER AND SHAPIRO[1] suggest a predominant lysosomal localization of sphingomyelinase, but these authors have found no detectable activity in the post-mitochondrial supernatant fraction, even though this fraction contained 30% of the total acid phosphatase activity. The data of WEINREB *et al.*[3] are even more puzzling. The distribution pattern reported by these authors for the enzyme in normal rat liver allows for an association with

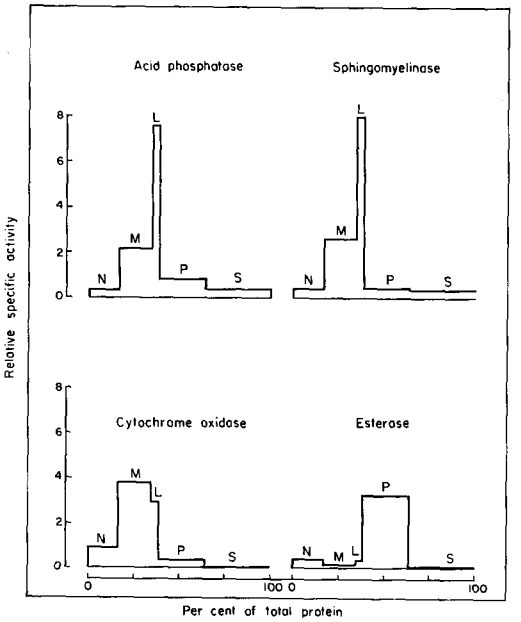

Fig. 1. Intracellular distribution of enzymes in livers of normal rats. Fractions are represented separately in the ordinate scale by their relative specific activity (percentage of total recovered activity/percentage of total recovered protein). In the abscissa scale, each fraction is represented (cumulatively from left to right) by its protein content, expressed as percentage of total recovered protein.