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The Molecular Form of Acetylcholinesterase as Determined by Irradiation Inactivation

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The molecular size of acetylcholinesterase (EC 3.1.1.7) from the electric organ of *Electrophorus electricus* and erythrocyte 'ghosts' was estimated in both membrane-bound and purified preparations by irradiation inactivation. Results suggest that the form of the enzyme in the membrane is a monomer of molecular weight approx. 75000 and that multiple forms of the enzyme observed in solubilized preparations are aggregates of this monomer.

Nearly 20 years ago a solubilized acetylcholinesterase was shown by irradiation inactivation to be a non-spherical unit of molecular weight 105000 (Serlin & Fluke, 1956). Since then, various biochemical attempts to isolate acetylcholinesterase by solubilization and subsequent gel filtration and column chromatography have obtained results which vary with the source of enzyme and the isolation methods used (Leuzinger, 1971; Millar & Grafius, 1970; Froede & Wilson, 1970; Hollunger & Nikalsson, 1973; McIntosh & Plummer, 1973). On the basis of biochemical results, it is now generally agreed that solubilized acetylcholinesterase exists in multiple forms, ranging in molecular weight from 60000 to over 400000. The possibility that these multiple forms are due to aggregation of the solubilized enzyme makes it difficult to apply these results to the membrane-bound enzyme.

Irradiation inactivation has been successfully applied to estimate the molecular size of several other membrane-bound proteins in freeze-dried preparations (Kepner & Macey, 1968; Levinson & Ellory, 1973). The validity of this technique for biological molecules in general has been established by a number of investigators over the past 20 years (Okada, 1970; Pollard *et al.*, 1955). Briefly, these investigators applied classical target theory to inactivation data to obtain a molecular target size. This analysis gives a linear relationship between log(remaining activity) and irradiation dose, the slope of which is proportional to the size of the target molecule. Kepner & Macey (1968) simplified this analysis by combining empirically determined values for the energy disposition of sparsely ionizing radiations together with assumed values for the average density of protein. They obtained the following relation between molecular weight and D_{37} (that dose at which 37% of the original enzyme activity remains):

$$\text{Mol.wt.} = 6.4 \times 10^5 / D_{37} \text{ (in Mrd)}$$

More detailed explanations of the theory and empirical justifications of this method are given by Pollard (1959) or Okada (1970).

Previous irradiation studies on mainly solubilized preparations (Serlin & Fluke, 1956; Kepner & Macey, 1968) gave inactivation sizes consistent with a subunit structure of acetylcholinesterase. However, lack of existing biochemical data and inconsistent results prevented definite conclusions about the state of the membrane-bound enzyme.

In the present study, inactivation by 15 MeV electrons was used to estimate the molecular size of acetylcholinesterase from *Electrophorus electricus* electric organ and erythrocyte 'ghosts' in both membrane-bound and purified preparations. A particulate fraction of electric-organ homogenate was prepared as previously described (Levinson & Ellory, 1973). Erythrocyte 'ghosts' from fresh human or llama blood were prepared as described by Fortes *et al.* (1973). Although the results obtained were independent of the species of erythrocytes used, llama 'ghosts' were usually preferred, since they could be resuspended from the freeze-dried state without clumping. Commercially purified acetylcholinesterases from *Electrophorus* (types V and VI, prepared as described by Lawler, 1959) and bovine erythrocytes were obtained in freeze-dried form from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The erythrocyte and type VI eel enzymes (specific activities of 8 and 200 units/mg of protein respectively) could be weighed directly into irradiation tubes. Type V eel enzyme consisted of minute amounts of high-specific-activity material (1400 units/mg of protein) and had to be reconstituted and freeze-dried in sample tubes to obtain reproducible samples of this enzyme.

Irradiation was carried out with the 15 MeV linear electron accelerator at Addenbrooke's Hospital, Cambridge. Samples were irradiated *in vacuo* at a dose rate of 1–2 Mrd/min, the sample temperature being maintained at approx. 10°C by a jet of air cooled with solid CO_2. These conditions were chosen to eliminate non-specific inactivation by free radicals (Okada, 1970; Kepner & Macey, 1968) and heating, although the possibility of inactivation by heating can be discounted, since freeze-dried preparations were resistant to heating at 60°C for 10 min.

Acetylcholinesterase activity was measured as described by Ellman *et al.* (1961). Corrections were made for both pseudocholinesterase activity and non-enzymic hydrolysis, although the sum of all corrections was less than 5% in all cases and was usually much smaller.

Fig. 1 compares the radiation inactivation of two freeze-dried membrane-bound acetylcholinesterase preparations (from llama erythrocyte 'ghosts' and electric-organ particulate fraction) with a purified freeze-dried enzyme (type V). Clearly the radiation-sensitivity of the purified enzyme is much greater than that of the membrane-bound preparation. The lines give a D_{37} of 8.5 Mrd for the membrane-bound enzymes and 2.5 Mrd for the purified type V enzyme, corresponding to molecular weights of 75000 and 260000 respectively. Table 1 lists the estimated molecular weights for various acetylcholinesterase preparations, calculated by using the experimentally determined D_{37} values and the equation of Kepner & Macey (1968).

Several investigators have given 260000 as the molecular weight of the solubilized enzyme from several sources (Leuzinger, 1971; Millar & Grafius, 1970; McIntosh & Plummer, 1973). In addition, detergents and other disaggregating agents have been used to demonstrate the existence of a 50000–80000-mol.wt. subunit, and evidence has been presented which indicates that the 260000-mol.wt. species contains four to six subunits (Leuzinger, 1971; Froede & Wilson, 1970). As with recent work on mammalian brain (Hollunger & Nikalsson, 1973; McIntosh & Plummer, 1973), our results demonstrate that the low-molecular-weight 'monomer' is active in the two membrane-bound preparations and in two low-specific-activity purified preparations (eel type VI and bovine erythrocyte acetylcholinesterase; Table 1).

Although our results with freeze-dried preparations correlate well with existing biochemical studies, it was considered that the freeze-drying process in itself might effect aggregation of the enzyme. Therefore the feasibility of irradiation in aqueous solution was considered. In general, samples are irradiated in the freeze-dried state to minimize non-specific effects of free-radical formation. Thus in the present study, when acetylcholinesterase was irradiated in aqueous solution at 10°C, inactivation was virtually complete with doses in the range 100–200krd. Further, the radiation-sensitivity of these dilute aqueous samples

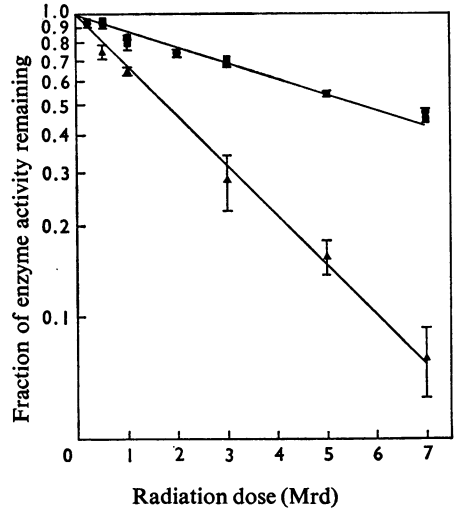

Fig. 1. *Semi-log inactivation plot of three freeze-dried acetylcholinesterase preparations*

●, Llama erythrocyte 'ghosts'; ■, electric-organ particulate fraction; ▲, type V acetylcholinesterase (Sigma). For further details see the text.

Table 1. *Molecular weights of acetylcholinesterases as determined by irradiation inactivation*

Statistical data (when given) are the means±S.E.M. for four or more determinations.

Preparation	Freeze-dried		Frozen	
	D_{37} (Mrd)	Mol.wt.	D_{37} (Mrd)	Mol.wt.
Membrane-bound				
Electric-organ particulate fraction	8.5±0.4	75000±3000	8.3	77000
Erythrocyte 'ghosts'	8.5±0.5	75000±4000	9.0	71000
Purified				
Electric-organ type V (Sigma)	2.5±0.2	255000±15000	2.7	240000
Electric-organ type VI (Sigma)	9.0±0.7	71000±5000	2.65	240000
Bovine erythrocyte (Sigma)	8.9	72000	—	—
Type V in 0.2% Triton X-100	—	—	4.2	150000

(less than 1%, w/v) was dependent on enzyme concentration, indicating that inactivation by free radicals was the predominant mechanism. In contrast, irradiation in frozen aqueous solution, as previously suggested by Kepner & Macey (1968), gave consistent results independent of enzyme concentration, and corresponding to known molecular sizes for the enzyme (see Table 1). Interestingly, however, although the membrane-bound preparations were still inactivated as the low-molecular-weight 'monomer', purified preparations, including type VI eel enzyme, which previously behaved as a monomer, gave D_{37} values corresponding to a tetrameric form. We consider this to be further evidence for the aggregation of purified acetylcholinesterase in aqueous solution. In addition, irradiation of type V enzyme frozen in 0.2% Triton X-100 gave inactivation results characteristic of a 150000-mol.wt. form (i.e. approximately dimeric). This is in accord with results of Millar *et al.* (1973) and McIntosh & Plummer (1973), who found significant quantities of a component of this size in Triton extracts of electric organ and pig brain respectively.

In the above studies we failed to obtain conclusive results indicating the coexistence of several multimeric forms in the same preparation. Such a situation has been observed under certain conditions in mammalian brain preparations (Jackson & Aprison, 1966; McIntosh & Plummer, 1973; Hollunger & Nikalsson, 1973), and if it did exist in an irradiated preparation it would give an inactivation curve consisting of several linear components when plotted as in Fig. 1. The only occasions on which such curves were observed were when the purified enzymes were quick-frozen in liquid N_2. This would suggest that aqueous solutions of purified enzymes consist of heterogeneous forms of acetylcholinesterase which gradually aggregate to become homogeneous on freeze-drying or slow freezing. Before freezing, our enzymes were made up in low-ionic-strength buffers (10mM-Tris–HCl, pH7.4), and considerable aggregation of enzyme has been reported under these conditions (Grafius & Millar, 1965). However, these results are only preliminary, since precise information has not yet been obtained on the number and size of the linear components in the inactivation data or on the dependence of these components on the rate of freezing.

To summarize, our results show that membrane-bound acetylcholinesterase is inactivated as a species of mol.wt. 75000. In addition the inactivation volume of the purified enzyme depends on conditions of preparation of the enzyme, but the forms found by irradiation-inactivation analysis are in the range 160000–280000 and are similar to results obtained for other preparations by biochemical techniques. The precise mechanism of inactivation of enzymes by ionizing radiation remains to be fully elucidated, but all available evidence indicates that polymers or aggregates of molecules are inactivated according to the molecular size of the total complex (Okada, 1970). Our results therefore suggest that the membrane-bound form of acetylcholinesterase is a monomer of mol.wt. 75000 and that the multiple forms of the enzyme found in purified preparations are aggregates caused by the solubilization of this enzyme. Such a conclusion is not unique in irradiation studies. Several other examples exist where irradiation-inactivation analysis indicates that the form of the enzyme *in situ* is either a subunit or polymer of the form of the enzyme found in biochemically purified preparations (Pollard *et al.*, 1955; Okada, 1970). Such examples further demonstrate the utility of irradiation inactivation as a technique for the study of membrane-bound proteins.

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