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MEMBRANE ENZYME SYSTEMS
MOLECULAR SIZE DETERMINATIONS BY RADIATION INACTIVATION

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SUMMARY

Classical radiation target theory is applied to radiation inactivation data on lyophilized membranes to obtain values for the molecular weights of membrane-bound enzymes. A molecular weight of 250000 is estimated for the $(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) of human erythrocyte ghosts, guinea pig kidney cortex microsomes or plasma membrane preparations, and crayfish nerve cord. Other results suggest that the Mg^{2+}-dependent ATPase and the $(Na^+ + K^+)$-ATPase have the same molecular weight. Studies with a K^+-stimulated microsomal alkaline phosphatase (orthophosphoric acid monoester phosphohydrolase, EC 3.1.3.1) indicate a molecular weight of 140000 for this enzyme which is thought to be responsible for the dephosphorylation step in the hydrolysis of ATP *via* the ATPase system. The volume fraction of the red cell membrane devoted to active cation transport is estimated at 0.002–0.04%. Microsomal and plasma membrane preparations apparently contain the same $(Na^+ + K^+)$-ATPase judged by their similar responses to ouabain inhibition, cation stimulation, and radiation inactivation. Radiation inactivation estimates of the molecular weight of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) and of mitochondrial ATPase are also presented. Studies with intact red cells and ghosts, irradiated while frozen, suggest that it might be possible to estimate the molecular weight of enzymes in intact cells.

INTRODUCTION

This work attempts to exploit classical radiation target theory[1] to obtain estimates of the molecular size of membrane enzyme systems. Various cell membrane preparations containing as yet unisolated enzymes have been irradiated *in vitro*. Target theory is applied to data from radiation inactivation curves to obtain estimates for the molecular weights of enzyme systems. Especially interesting are those membrane enzyme systems thought to be associated with the active transport of salt across membranes, *i.e.*, the $(Na^+ + K^+)$-activated ATPase. The basis for this association arises from the striking correlation of the kinetics of the enzyme system with the kinetics of active transport of Na^+ and K^+. It is commonly assumed that the

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$(Na^+ + K^+)$-ATPase can be identified with, or serve as a marker for the ion pump. Comparative radiation studies on the $(Na^+ + K^+)$-ATPase from different membranes as well as the actual values for molecular weights are of consequence for theories of active transport and for the more general problem of the structural organization of enzyme systems in membranes.

The Mg^{2+} and $(Na^+ + K^-)$-ATPases from red cell ghosts, kidney microsomes and plasma membrane, and nerve cord were selected for a comparative study. Alkaline phosphatase activity in microsomes was studied to assess the possibility that the ATPase system consists of two enzymes, a phosphorylase and a phosphatase, operating in sequence and having molecular weights differing from that of the ATPase system.

Divalent cation-stimulated ATPase in mitochondria was also investigated since this ATPase has not been associated with active transport of Na^+ and K^+. Additionally the radiation estimate for the molecular weight of this membrane enzyme can be compared to a value obtained for the purified soluble enzyme[2].

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) in red cell ghosts and commercial preparations of electric eel electroplaque was also studied. The enzyme in ghosts is another example of a membrane-bound enzyme that has resisted efforts at isolation. The enzyme from eel provides an opportunity to compare the radiation estimate for the molecular weight with that obtained on a highly purified, crystalline preparation[3].

Because of uncertainties associated with the application of target theory in general, and in particular to a heterogeneous preparation of enzymes, investigations of the suitability of this approach were undertaken. A comprehensive analysis is presented elsewhere[4]. The conceptual and experimental development of target theory is presented in various review articles[5–7].

METHODS

Radiation procedure

An electron linear accelerator at the Lawrence Radiation Laboratory delivering $7.5 \cdot 10^6$ eV electrons was used. Dosimetry was conducted with cobalt glass chips (Bausch and Lomb Optical Co.) calibrated against a Fricke dosimeter[8]. The beam was continuously monitored by a transmission detector of the secondary emission foil type[9]. Doses measured in $FeSO_4$ are considered to be equivalent to the dose delivered to the biological material.

Samples, lyophilized in 25-ml erlenmeyer flasks, were positioned so that the beam struck the base of the flask which was perpendicular to the beam direction. Dose rates were typically Mrad/min and an air jet was routinely directed at the base of the flask to facilitate heat removal. This was needed mainly at doses above 5 Mrad. A rubber valve was attached at the neck of the flask and opened to a vacuum pump and thermocouple gauge. The vacuum was applied for 2 min before irradiation began and throughout the exposure.

Enzyme preparations

Red cell ghosts were prepared from fresh or expired normal human blood[10,11]. Aliquots of ghosts (5 mg protein/ml)[12] were lyophilized for 2–4 h and stored in the cold until used.

A procedure suggested by Dr. K. UTSUMI was used to isolate mitochondria[13]. Rat liver was minced in 0.25 M sucrose, 0.2 mM EDTA, 5 mM Tris (pH 7.4) and homogenized, in the cold, by one stroke of the teflon pestle while operating the stirring motor at 600 rev./min. The suspension was centrifuged for 7 min at 100 $\times$ g and 20 ml of the supernatant were layered over 20 ml of 0.34 M sucrose, 0.2 mM EDTA, 5 mM Tris (pH 7.4). After centrifuging 10 min at 5000 $\times$ g the mitochondrial precipitate was washed twice in 5 mM Tris and resuspended to 10 mg protein per ml. Aliquots of 1.5 ml, diluted to 0.1 mg/ml, were used for lyophilization.

Guinea pig kidney cortex microsomes[14] were suspended in 1 mM EDTA, 1 mM NaCl (pH 7.4) at a protein concentration of 10 mg/ml. Aliquots at 0.1 mg/ml were lyophilized.

Plasma membrane preparations were obtained using the procedure developed for liver cell membranes[15] and suspended in 1 mM $NaHCO_3$.

Ventral nerve cords and claw nerve fibers were dissected from 15–20 crayfish, *Procambarus clarkii*, and a fraction isolated containing $(Na^+ + K^+)$-ATPase[16].

Electric eel acetylcholinesterase was obtained commercially (Sigma, St. Louis, Type III). The stock solution (250 σ units) was diluted 50 times and 1.5-ml aliquots lyophilized.

Frozen cells

Red cells were glycerolized for studies in the frozen state[17]. After irradiation at dry-ice temperatures the cells were thawed and hemolysed to obtain ghosts. Protein analysis of each sample was employed to normalize measured values of enzyme activity. Suspensions of ghosts were also treated with glycerol, frozen, irradiated and deglycerolized before assaying enzyme activity.

Enzyme assays

ATPase activity was evaluated using the DREISBACH method[18] to measure P_i liberation. Alkaline phosphatase activity in microsomal preparations was assayed following a procedure suggested by Dr. J. G. FORTE. After preincubation of the enzyme at 25° for 10 min, the substrate, *p*-nitrophenyl phosphate was added and incubation continued for 15–60 min. Non-specific activity was assayed without K^+ present. K^+-stimulated activity was measured in the presence of 10 mM K^+. The 4 ml reaction volume contained 20 mM Tris (pH 9.0), 250 mM sucrose, 5 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate. The reaction was stopped by addition of 2 ml of cold 0.05 M NaOH. Samples were centrifuged to clarify the suspension and their absorbance determined at 400 mμ on a Beckman DB spectrophotometer.

Acetylcholinesterase activity in ghosts or eel preparations was assayed following a procedure of D. KOBLICK (personal communication). The reaction mixture, 5 ml volume, consisted of 3.7 ml of salt solution (170 mM NaCl or 20 mM $MgCl_2$) *plus* 0.5 ml of substrate (30 mM acetylcholine chloride) *plus* 0.8 ml of enzyme solution (dried ghosts or eel enzyme resuspended in water). The temperature was kept constant at 26° and production of H^+ by enzyme hydrolysis of the substrate was compensated, at pH 8.0, with NaOH (usually 0.015 M) added from a microliter syringe. The NaOH added per unit time was directly proportional to the activity of the enzyme. Since the enzyme exhibits a peak in the substrate dependence curve at 2–4 mM, initial rates were obtained by limiting the incubation times to periods such that less than 20 % of the substrate was utilized.

CALCULATIONS

According to classical target theory, molecular weights and volumes of enzymes can be calculated from radiation inactivation data on the basis of the following assumptions:

(1) The primary energy releases generated by ionizing radiation in matter are discrete, highly localized clusters of ionizations.

(2) One such event is assumed to inactivate the function of the target molecule if it occurs in the "effective volume" for that function.

(3) The primary events are randomly distributed with respect to volume, *i.e.* sparsely ionizing radiations must be employed (*e.g.* ^{60}Co γ-rays or electrons from a linear accelerator).

(4) There are no indirect effects due to free radical diffusion. Therefore, the irradiations must be conducted on dried preparations.

Target theory predicts that the fraction, A/A_0, of enzyme activity units which survives the radiation dose D (inactivating events per cm^3) will be given by

$$A/A_0 = e^{-VD} \tag{1}$$

where V is the target volume in cm^3. Hence V can be obtained from the slope of a semi-log plot of A/A_0 *versus* D. The basic problem with this approach is to specify the absorbed dose in the microscopic region of a target molecule (in units of inactivating events per cm^3) on the basis of a measured macroscopic dose (units of 100 ergs absorbed per g = rad). It therefore becomes necessary to establish independently the energy deposited per inactivating event. This involves a factor Q which specifies the electron volts per inactivating event.

An alternative approach, taken in this work, is to use the data available in the literature on radiation inactivation of enzymes of known molecular weight (Table I). A calibration curve is constructed (Fig. 1) relating the known molecular weight to the dose (rads) required to achieve 37 % survival, *i.e.* the D_{37}. The data are fitted to the equation

$$\log D_{37} \text{ (rads)} + \log \text{ mol. wt.} = \text{constant} \tag{2}$$

which is a consequence of Eqn. 1 together with the fact that molecular weight is proportional to molecular volume. The constant involves the factor required for converting the dosage units from inactivating events per cm^3 to rads. In particular, it contains Q (see Eqn. 5), and thus this procedure allows a comparison to be made with Q values determined by other means. The value found here was $Q = 66$ eV per inactivating event as contrasted with the values 75 (ref. 6) or 100 (ref. 1) which are often cited[1].

Letting ρ denote the density of the target molecule and N Avogadro's number, the molecular weight is then calculated from the target volume V as follows:

$$\text{mol. wt.} = \rho N V \tag{3}$$

If the dose is measured in inactivating events per cm^3, then from Eqn. 1 and the definition of D_{37} we have

$$V = 1/D_{37} \text{ (inactivating events per } cm^3) \tag{4}$$

TABLE I

RADIATION INACTIVATION DATA FOR BIOMOLECULES

D_{37} values are taken from graphs of the original article or by calculation from data presented therein. These data were selected because the radiation used conformed to the requirement of being sparsely ionizing. All data found to meet these requirements were included. Irradiations were carried out at room temperature. Consult cited references for molecular weight values, some of which have been updated for inclusion in this table (changes not greater than 10%).

Biomolecule	*Mol. wt.*	D_{37} (*Mrads*)	*Atmosphere*	*Ref. No.*
Penicillin	356	1200	Vacuo	19
Coenzyme A	767	800	Vacuo	20
α-Melanophore Stimulating hormone	1 670	282	Ar	21
α-Melanophore Stimulating hormone	1 670	660	Vacuo	22
Adrenocorticotropic Hormone	4 540	91/180	Air/Ar	21
Ribonuclease	13 700	22/38	Air/vacuo	23
Ribonuclease	13 700	63	Vacuo	24
Ribonuclease	13 700	43	Vacuo	25
Lysozyme	14 400	26/49	Air/N_2	26
Lysozyme	14 400	45	Vacuo	24
Trypsin	23 800	30	Vacuo	27
Trypsin	23 800	10	Air	28
Trypsin	23 800	12/18	Air/vacuo	29
Trypsin	23 800	36	Air	30
Trypsin	23 800	27	Vacuo	25
Trypsin	23 800	35	Vacuo	24
Chymotrypsin	24 300	26	Vacuo	25
Human chorionic gonadotropic hormone	30 000	24	Air	31
Lactate dehydrogenase	35 000	21.5	Vacuo	32
Thrombin	35 000	21.7	Vacuo	33
Alcohol dehydrogenase	36 000	25	Air	20
Deoxyribonuclease	63 000	6.5	Vacuo	34
Deoxyribonuclease	63 000	11.5/6.3	Vacuo/air	35
Deoxyribonuclease	63 000	8.5/5.8	Vacuo/air	36
Prothrombin	63 000	9	Vacuo	33
Hyaluronidase	65 000	12	Vacuo	37
Creatine phosphotransferase	81 000	10	Vacuo	38
RNA ϕR17	$1.1 \cdot 10^6$	0.78	Air dry/frozen	39
DNA ϕX174	$1.75 \cdot 10^6$	0.34	Air dry/frozen	39
RNA TMV	$2.1 \cdot 10^6$	0.28	Air dry/frozen	39
E. coli ribosomes	$2.6 \cdot 10^6$	0.27	Air	40

To convert the measured dose (in rads) to dose in inactivating events per cm³ we use

$$D_{37}\left[\frac{\text{inactivating eV}}{\text{cm}^3}\right] = \left[\frac{\rho\ (\text{g/cm}^3)}{Q\ (\text{eV per inactivating event})} \times \frac{10^2\ (\text{erg/g}\cdot\text{rad})}{1.6\cdot 10^{-12}\ (\text{erg per eV})}\right] D_{37}\ (\text{rads}) \qquad (5)$$

Finally, combining Eqns. 3, 4 and 5 with the empirically determined value $Q = 66$ leaves

$$\text{mol. wt.} = 6.4\cdot 10^{11}/D_{37}\ (\text{rads}) \qquad (6)$$

Note that the final Eqn. 6 is independent of ρ. Although Q is simply a fitting parameter to which no physical significance need be ascribed, a value of 66 eV per inactivating event is in excellent agreement with recent experimental studies of energy deposition by ionizing radiation[41,42].

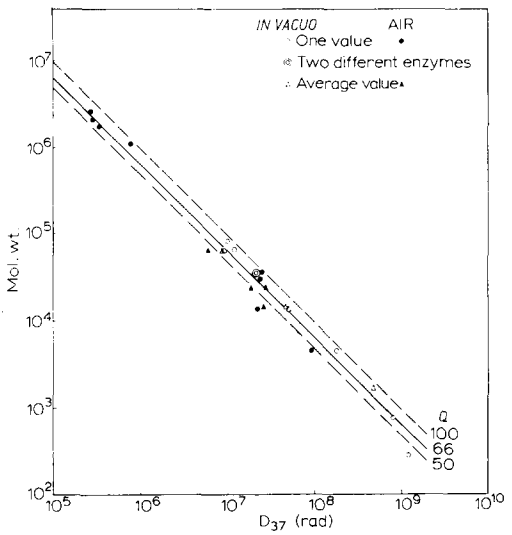

Fig. 1. Log-log plot relating D_{37} to molecular weight based on the literature survey listed in Table I. Differences between measured and known molecular weights were converted to percentages of the known values. The data were then fitted to Eqn. 2 by minimizing the sum of the squares of the percentage deviations from the known values. A value of $Q = 66$ eV per inactivating event is obtained for the *in vacuo* data.

Experiments run *in vacuo* yield more consistent responses (Fig. 1) although in principle the same approach is valid for irradiations in air or even in frozen systems (indirect effects of diffusion are also minimized in this case).

RESULTS

The basic experiment included the following conditions: samples dried from water or minimal buffer (approx. 1–10 mM salt or buffering compound), irradiations conducted *in vacuo* at room temperature, samples stored cold after irradiation until assayed, dose-rates equal to 1–3 Mrad/min.

Experience showed that temperature treatment of the samples during irradiation (0–20°) or storage time after irradiation (stored in the cold for 24 h or kept at room temperature up to 5 h after irradiation before assay) was not critical. Variations in dose-rate from 0.5 to 5 Mrad/min did not alter the amount of enzyme inactivation at fixed dose (ghost ATPase and acetylcholinesterase, eel acetylcholinesterase). Maintaining samples *in vacuo* for as long as 20 min before irradiation gave the same response as the adopted procedure of maintaining samples *in vacuo* for 2 min prior to irradiation. To study the possible influence of the buffer and salt concentrations during drying and subsequent irradiation, various dilutions of the microsomal preparation were prepared using distilled water (microsomes initially suspended in 25 mM NaCl, 13 mM histidine–HCl, 0.1 mM H_4 EDTA). The D_{37} values

for ATPase inactivation were substantially the same (± 30 %) for dilutions of 0, 1:3, 1:8, 1:20, and 1:80. Similar results were obtained with microsomes suspended in NaCl–EDTA at concentrations of 0.1, 0.5, 1 and 2 mM. In addition, microsomal preparations in sucrose solutions at 1.3, 13, 130 and 200 mM were freeze-dried and irradiated. The one experiment conducted showed no apparent variation in the D_{37}.

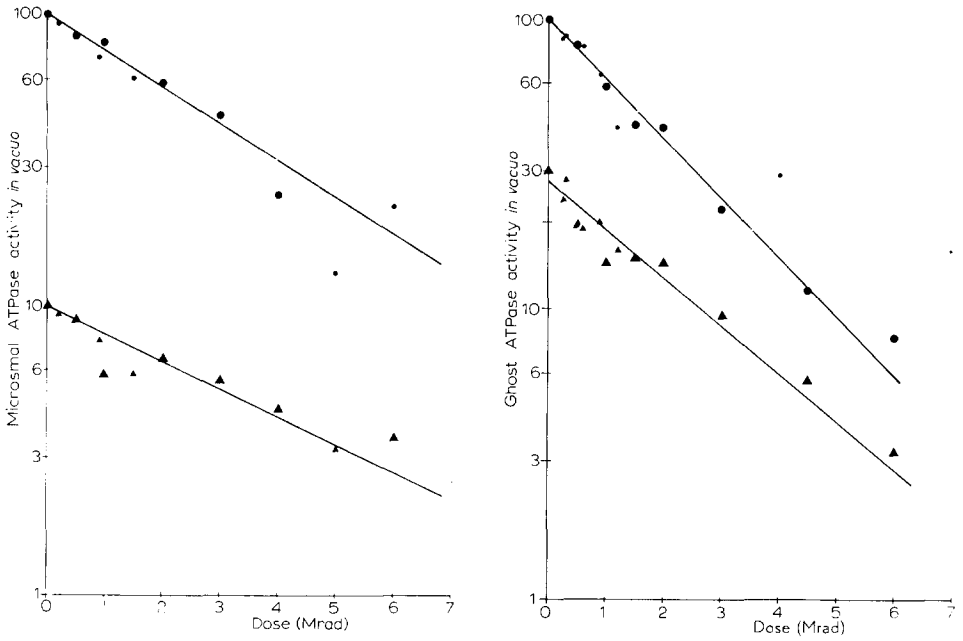

Fig. 2. Radiation inactivation of microsomal ATPase. For the upper curve (●) the assay medium contained in a volume of 2 ml: ATP, 3 mM; $MgCl_2$, 4 mM; imidazole, 20 mM; glycylglycine–HCl, 20 mM (pH 7.5); NaCl, 150 mM; KCl, 30 mM. Assays for the lower curve (▲) employed the same medium with the addition of ouabain, 0.1 mM. (In a few instances the KCl was replaced by NaCl.) Incubation time: 15 min at 37°. Each assay contained 0.3 mg protein and 100 % activity corresponds to 36 μmoles P_i/mg protein per h. The larger symbols indicate that more experiments were run at those points.

Fig. 3. Radiation inactivation of red cell ghost ATPase *in vacuo*. For the upper curve (●) the assay medium contained in a volume of 2 ml: ATP, 1 mM; $MgCl_2$, 1 mM; EDTA, 1 mM; Tris, 15 mM (pH 7.4); NaCl, 115 mM; KCl, 20 mM. Assays for the lower curve (▲) employed the same medium with the addition of ouabain, 0.1 mM. (In a few instances KCl was replaced with NaCl.) Incubation time: 1 h (without ouabain) or 2 h (with ouabain) at 37°. Each assay contained 9 mg protein and 100 % activity corresponds to 0.13 μmole P_i/mg protein per h. The larger symbols indicate that more experiments were run at those points.

However, mitochondrial preparations at 30 or 50 mM sucrose exhibited a D_{37} greater by a factor of two when compared to the D_{37} without sucrose in the medium during freeze-drying and irradiation.

The radiation inactivation curves for ATPase activity in ghosts and microsomes are presented in Figs. 2–4. D_{37} values from the *in vacuo* curves are used for calculating the molecular weight of the enzyme system (Table II). Data from several experiments were pooled and individual dose points were weighted according to the number of experiments run at that dose. The larger data point symbols in the figures are intended to indicate that more experiments were run at those points (*i.e.* these points have

larger weights). The lines drawn in the figures were determined by a least-squares fit of the weighted data to the theoretical exponential decay of activity with dose (Eqn. 1). The estimated standard deviation of the decay constants was generally within 10% of the estimated value of the decay constant. As illustrated in the inactivation curves the experiments assayed total ATPase activity ($Na^+ + K^+ + Mg^{2+}$)

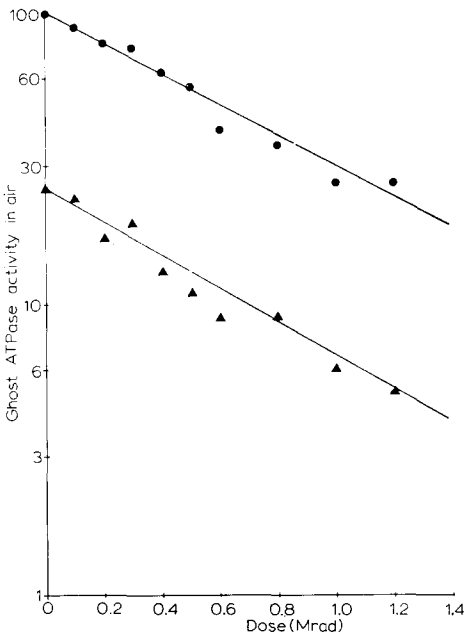

Fig. 4. Radiation inactivation of red cell ghost ATPase in air. Assay conditions were as described for Fig. 3 except that the 4 ml assay medium contained 10 mg protein and incubation was for 1 h. The 100% activity point corresponds to 0.12 μmole P_i/mg protein per h.

and Mg^{2+} activity ($Na^+ + Mg^{2+}$ or $Na^+ + K^+ + Mg^{2+}$ + ouabain). Therefore, the ($Na^+ + K^+$)-stimulated activity is the difference of the two measured activities. In most cases the least-squares fits were on the total and Mg^{2+} activities. The ($Na^+ + K^+$)-stimulated activity usually had the same ($\pm$ 5%) D_{37} as the D_{37} for the total activity. This is partly a consequence of the fact that the total activity is 3–10 times the activity without the particular cation(s) whose stimulatory effect is being studied. Thus the slope of the inactivation curve for total activity is hardly changed by subtracting the activity observed with no stimulating cation present. The largest number of experiments and the most exhaustive analysis was performed on ghosts irradiated in air. Results are summarized in Fig. 4. Weighting the points equally or inversely according to their variances had no significant effect on the computed slopes. The slopes are identical to within 6% and the calculated value for $\pm 2\sigma$ is 8% of the average value of the three slopes (total, Mg^{2+}, ($Na^+ + K^+$)-stimulated activities).

Inactivation of the nerve and plasma membrane ATPase activities gave plots similar to those of Figs. 2 and 3. The D_{37} values (Table II) are similar to the corresponding values for ghosts and microsomes.

Because of the similarities in the radiation estimates of the molecular weight

TABLE II

ESTIMATES OF MOLECULAR WEIGHTS FROM RADIATION INACTIVATION *in vacuo*

Values of D_{37} and molecular weight are taken from a least squares fit of pooled and weighted data. Estimated standard deviations for molecular weight determinations fell within 10% of the listed molecular weight. In most cases the least squares fits were on the total and Mg^{2+}-ATPase activities. Within experimental error the D_{37} values for total and $(Na^+ + K^+)$-ATPase are equivalent (see text). The number in parentheses is the number of experimental runs. A "run" consisted of 1–4 samples at each of several doses.

Preparation	D_{37} (*Mrads, in vacuo*)	*Mol. wt.*
$(Na^+ + K^+)$-ATPases:		
ghosts	2.1 (6)	300 000
nerve cord	2.4 (2)	270 000
microsomes	3.4 (8)	190 000
plasma membrane	3.2 (2)	200 000
Mitochondrial ATPase:		
Mg^{2+}-stimulated	2.2 (3)	290 000
Ca^{2+}-stimulated	1.7 (3)	360 000
No divalent cation	1.7 (3)	360 000
Alkaline phosphatase:		
K^+-stimulated	4.6 (3)	140 000
No K^+	9.1 (4)	70 000
Acetylcholinesterase:		
eel	3.7 (3)	170 000
ghosts	11.4 (4)	56 000

for the $(Na^+ + K^+)$-ATPase in microsomes and plasma membrane, studies were undertaken to further characterize these ATPase activities. The response of the enzyme from the two preparations to varying ouabain concentration as well as the stimulation by various concentrations of Na^+ and K^+ indicated they were the same.

It is not yet established that the standard procedure for obtaining cell membrane in liver cells can be used for kidney cells. Evidence bearing on this point was sought by assaying non-specific alkaline phosphatase activity since it has been histochemically identified in plasma membranes, but not microsomal membranes, of kidney cortex cells[43]. When compared on the basis of activity per mg protein, the non-specific alkaline phosphatase activity (*i.e.* K^+ absent) of plasma membranes was found to be about 4 times higher than the microsomal activity. Both preparations exhibited K^+ stimulation.

Table II summarizes some results of the radiation inactivation studies on the various enzymes. D_{37} values for the non-specific and the K^+-stimulated alkaline phosphatase from microsomes differed by a factor of 2. Mitochondrial ATPase curves (Fig. 5) are presented for purposes of comparing divalent cation-stimulated ATPases with the monovalent cation-stimulated ATPases involved in active transport. Acetylcholinesterase from eel or in ghosts presents differing responses to radiation as indicated by Fig. 6.

Table III contains data on studies in air and data in Table IV are from an experiment designed to test some variations in the treatment of a frozen system for

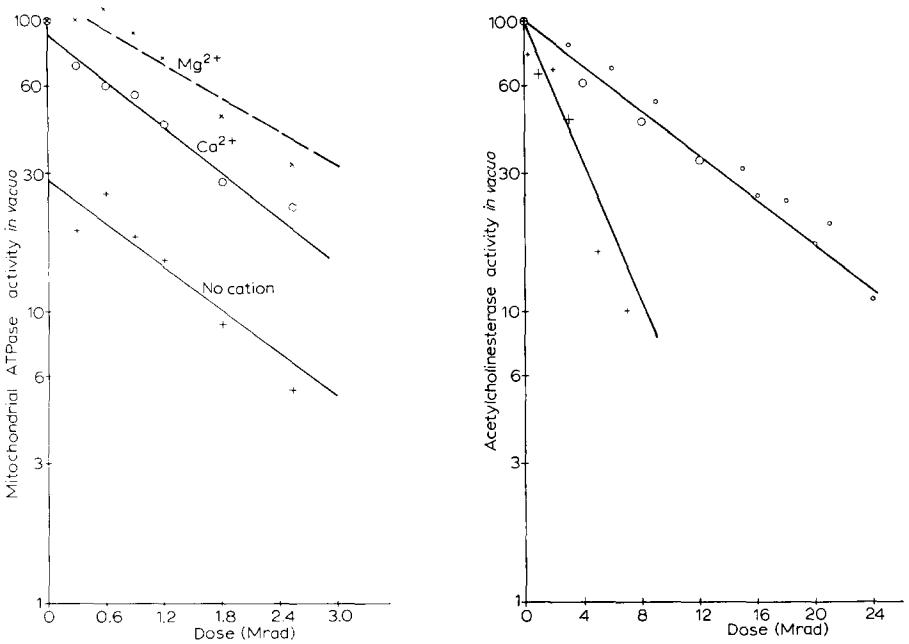

Fig. 5. Radiation inactivation of mitochondrial ATPases. The 3 ml incubation volume contained: ATP, 3 mM; Tris–acetate, 40 mM (pH 8); and either $MgCl_2$, 3 mM (×); or $CaCl_2$, 3 mM (○); or no additions (+). Incubation time: 30 min. The 100% activity point corresponded to 29 μmoles P_i/mg per h. Each assay contained 0.22 mg protein.

Fig. 6. Radiation inactivation of acetylcholinesterase in red cell ghosts and eel preparations. Typically the 5 ml reaction volume contained 2 mg of protein for ghost assays and 65 μg for the eel enzyme assays. 100% activity corresponded to 0.75 μmole acetylcholine chloride hydrolyzed per mg protein per min for ghosts. For eel 100% activity was 23 μmoles per mg per min. ○, ghost data; +, eel data.

TABLE III

RADIATION INACTIVATION IN AIR AND SUMMARY OF D_{37} VALUES

Individual experiments were analyzed by fitting (by eye) straight lines to log activity *versus* dose plots. The D_{37} values listed below represent the number of individual experiments all of which fell within the indicated range. Each experiment consisted of 1–4 samples at each of several doses. The ratio D_{37} (air)/D_{37} (vacuo) was calculated using data of Table II.

Preparation	D_{37} (*Mrad, in air*)	*Range* (*Mrad*)	$\frac{D_{37}\ (air)}{D_{37}\ (vacuo)}$
$(Na^+ + K^+)$-ATPase:			
ghosts	0.95 (24)	0.5–1.4	0.45
microsomes	2.8 (20)	1.7–4.2	0.80
Mitochondrial ATPase:			
Mg^{2+}-stimulated	0.9 (8)	0.7–1.2	0.41
Ca^{2+}-stimulated	0.7 (8)	0.5–1.2	0.41
no divalent cation	0.6 (8)	0.4–1.0	0.35
Acetylcholinesterase:			
eel	2.9 (9)	1.6–3.7	0.78
ghost	11.4 (30)	7.7–17.8	1.0

TABLE IV

RADIATION INACTIVATION IN FROZEN SOLUTIONS OF RED CELL GHOSTS

Individual experiments were analyzed by fitting (by eye) straight lines to log activity *versus* dose plots. The D_{37} values represent average values. Numbers in parentheses represent the number of individual experiments all of which fell within the indicated range. Each experiment consisted of 1–4 samples at each of several doses. The ratio D_{37} (frozen)/D_{37} (vacuo) was calculated using data of Table II. Data for acetylcholinesterase obtained from a single experiment is also included.

Preparation	$(Na^+ + K^+)$-*ATPase*			*Acetylcholinesterase*	
	D_{37} (*Mrads, frozen*)	*Range* (*Mrads*)	$\frac{D_{37}\ (frozen)}{D_{37}\ (air)}$	D_{37} (*Mrads, frozen*)	$\frac{D_{37}\ (frozen)}{D_{37}\ (air)}$
Red blood cells	4.0 (2)	1.9–6.2	1.9	19.0	1.7
Glycerolated red blood cells	4.6 (4)	2.9–8.1	2.2	15.4	1.4
Ghosts	3.2 (4)	1.9–5.6	1.5	18.5	1.6
Glycerolated ghosts	4.2 (5)	1.3–5.4	2.0	16.0	1.4

effects on D_{37}. A few experiments were performed on wet suspensions of ghosts or eel preparations. The inactivation curve for the eel acetylcholinesterase was exponential and the slope increased as the concentration of enzyme material decreased. That is, the enzyme was less sensitive to radiation at higher concentrations. For ghosts a similar pattern was observed. Radiation in the kilorad range was capable of inactivating the enzyme in the wet state. With the ATPase of ghosts the exponential inactivation curve exhibited two components. Inactivation began at about 50 krad and by about 1 Mrad the second component (noticeable by the large change in slope) became apparent.

DISCUSSION

The view that target theory is useful for estimating enzyme size is supported by the evidence of Table I and Fig. 1. Further support is provided by the results with eel acetylcholinesterase preparations (Table II). The interpretation of the radiation inactivation volume of membrane-bound enzymes refers to the enzyme *in situ*. Isolation of the enzyme(s) in homogeneous soluble forms might involve different entities; preparative procedures may produce dissociation or aggregation of the original enzyme unit.

In each case the inactivation curves corresponded reasonably well with the exponential decay predicted by target theory. This does not necessarily imply that each enzyme unit corresponds to a single molecule. The result is equally consistent with an enzyme system composed of a tightly coupled sequence of steps arranged in series. Inactivating any of these steps disrupts the entire sequence. The calculated molecular weights would then correspond to the sum of the individual weights of each member of the sequence. This qualification may be particularly pertinent in the case of ATPase.

The calculated values for $(Na^+ + K^+)$-ATPase compiled in Table II range from 190000 to 300000. If the $(Na^+ + K^+)$-ATPase activity in each preparation was a manifestation of the same enzyme or enzyme system with molecular weight equal to 250000, then departures from this figure would all be less than 25 %. Considering

the accuracy of the technique together with variations in enzyme contamination in the different preparations, this variability seems reasonable. If our plasma membrane preparation was truly representative of cell surface membrane, then the radiation data and the kinetic response to variations in salt and ouabain concentrations both suggest that the $(Na^+ + K^+)$-ATPase systems in the microsomal portion and in the cell surface membranes are the same. In short our data support contemporary ideas of a universal membrane $(Na^+ + K^+)$-ATPase, to which we assign an approximate molecular weight of 250000.

Data for Mg^{2+}-ATPase (ouabain insensitive) can be compared with $(Na^+ + K^+)$-ATPase in the same preparations. The data are limited by the difficulties in assaying the low activities of the irradiated Mg^{2+}-ATPase. However, on the basis of numbers of experiments performed, the most accurate data arise from studies on microsomes *in vacuo* (Fig. 2), ghosts *in vacuo* (Fig. 3), and ghosts in air (Fig. 4). In these experiments, the D_{37} values obtained from the illustrated "best fit" straight lines of the Mg^{2+}-ATPase do not differ from the D_{37} values of the corresponding $(Na^+ + K^+)$-ATPase by more than 15 %. This close agreement could arise if the two ATPase sites occur within the same inactivation volume or, alternatively, if the two ATPases have separate but equal inactivation volumes.

NAKAO *et al.*[44] have recently reported a radiation study which estimates the molecular weight of $(Na^+ + K^+)$-ATPase in NaI-treated brain microsomes at 500000. They do not specify whether the preparation was irradiated *in vacuo* or in air. Target theory calculations are most applicable to irradiation of dry samples *in vacuo*. Our results (Table III) suggest a marked variation in the ratio D_{37} (air)/D_{37} (vacuo) among the enzymes studied. Data in the literature place the ratio cited at about ½ although considerable variation is reported (see Table I, Fig. 1). If, in fact, the experiments of NAKAO *et al.*[44] were performed in air, then their data would be consistent with ours; *i.e.* differences between in air, and *in vacuo* radiation may account for the major discrepancy of the two estimates. Our earlier estimate[11] is subject to the same remarks[45].

Other molecular weight estimates have been based on gel chromatography of extracted preparations. Using guinea pig brain microsomal extracts MEDZIHRADSKY, KLINE AND HOKIN[46] found a $(Na^+ + K^+)$-ATPase fraction with an estimated molecular weight of 670000. Two Mg^{2+}-ATPase fractions were also found with molecular weight values of 265000 (a value very close to our estimate) and 775000. The authors point out the possibility that these molecular weights may be overestimated due to aggregation with other proteins. Another report for a Mg^{2+}-ATPase fraction from human erythrocyte ghosts suggested a molecular weight of 150000 (ref. 47).

Knowledge of the molecular weight of the $(Na^+ + K^+)$-ATPase can be used in a number of interesting calculations and comparisons which bear on the organization of enzyme systems in membranes. By assuming a protein density of 1.3, the calculated enzyme volume equals $3.2 \cdot 10^{-19}$ cm^3. If the enzyme were spherical, its diameter would be 85 Å, a linear dimension which is similar to current estimates of the membrane thickness. The actual shape and orientation of the enzyme remain unknown. The molecular weight together with the enzyme turnover number can be used to calculate the specific activity to be expected in a pure preparation. Although the magnitude of these turnover numbers is difficult to obtain, some estimates are available from the work of BADER, POST AND BOND[48] who compared the uptake of

^{32}P from γ-labeled ATP into a proposed intermediate, with the $(Na^+ + K^+)$-ATPase activity. Despite the large range of enzyme activities (400-fold) that was encountered in the various tissues examined, the estimated turnover numbers fell within the narrow range of 8000–16000 per min. If the reaction stoichiometry requires one enzyme molecule per ATP split, then since each mole of enzyme weighs 250000 g, these figures imply a specific activity in a pure preparation of the order of 1900–3800 μmoles P_i/mg per h. This can be compared with recent attempts to purify the enzyme which result in specific activities of the order of 300 μmoles/mg protein per h (ref. 49). Either the turnover number is much different in these preparations, or there is one order of magnitude remaining in the purification process.

To calculate the relative volume of the cell membrane occupied by $(Na^+ + K^+)$-ATPase requires the molecular weight, and in addition some notion of the number of ATPase sites per cell. This latter figure can be estimated by comparing the above cited turnover numbers with measured specific activities of cells. The turnover number estimated for red cell ghosts is of the order of 12000 per min while the $(Na^+ + K^+)$-ATPase activity is of the order of 1 mmole P_i/h per l cells[50]. Assuming $1.1 \cdot 10^{13}$ cells/l, these figures imply approx. 80 sites/cell. The total volume occupied by the transport ATPase would then be $80 \times 3.2 \cdot 10^{-19}$ cm^3 $= 2.6 \cdot 10^{-17}$ cm^3/cell. Now the average surface of a human red cell is about $1.55 \cdot 10^{-6}$ cm^2, and assuming a thickness of 100 Å the volume occupied by the membrane is approx. $1.55 \cdot 10^{-12}$ cm^3. Comparing this volume with that occupied by the ATPase we arrive at the estimate that only 0.002 % of the membrane is occupied by the enzyme system. This figure is based on the site estimate obtained from turnover numbers. An alternative approach would be to identify each ATPase site with transport sites for K^+. In a study of glycoside inhibition of K^+ transport GLYNN[51] estimates an upper bound of 1000–3000 sites/cell. Choosing 2000 sites, for example, would raise the above estimate from 0.002 % to an upper bound of 0.04 % for the relative volume occupied by the enzyme system.

It has been frequently suggested that the hydrolysis of ATP by the $(Na^+ + K^+)$-ATPase takes place in at least two steps, a Na^+-dependent phosphorylase reaction followed by a K^+-stimulated phosphatase step. Experiments on K^+-stimulated phosphatase in microsomes were motivated by this suggestion. The estimated molecular weight for this enzyme (140000) was 25 % lower than the estimated molecular weight of microsomal $(Na^+ + K^+)$-ATPase (190000). This apparent difference was obtained in the same preparation (microsomes) so that many errors inherent in the radiation inactivation technique would tend to cancel when the two enzymes are compared. Nevertheless the small difference between the two estimates may still reside within the errors of the technique. Although it is not clear whether the molecular weight of K^+-phosphatase is smaller than or equal to the molecular weight of $(Na^+ + K^+)$-ATPase, the K^+-phosphatase seems definitely different from, and larger than, the non-specific phosphatase. With regard to the phosphorylase step, it is of interest that from recent radiation experiments, NAKAO *et al.*[44] suggest a molecular weight of a phosphorylated intermediate at about 59 % of their estimated molecular weight of $(Na^+ + K^+)$-ATPase.

Since the characterization of mitochondrial membrane enzymes is advanced, this system appears promising for providing a test of the radiation technique for estimating the molecular weight of enzymes in a membrane. The value of 360000 for the radiation molecular weight of cation-stimulated ATPases in mitochondria

(Fig. 5, Table II) can be compared to the value of 284000 for the ATPase from beef-heart mitochondria (ultracentrifuge analysis of a relatively pure enzyme preparation)[2]. The good agreement (27 %) is further support for the reliability of the radiation technique. A recent radiation study presented a value of 290000 for mitochondrial ATPase from rat liver[52]. Mitochondria were irradiated in air after being dried in sucrose (250 mM). Questions are raised in interpreting these data because sucrose may afford protection, and because the study was not conducted *in vacuo* as required by the target theory calculation that was applied to the data. Interestingly this paper reports the same D_{37} for the ATPase whether the samples were lyophilized, frozen, or in a sucrose solution when irradiated.

Acetylcholinesterase is another membrane-bound enzyme of interest. The size of the acetylcholinesterase system in eel electroplaque has recently been reported to be 240000 (ref. 3). The radiation study suggested a value of 170000 (Table II). Although the acetylcholinesterase activity from eel and from red cell ghosts exhibited similar responses to varying the substrate concentration, pH and salt concentration, the radiation inactivation curves were quite different (Fig. 6). Perhaps the enzymes are different or the enzyme molecule in the red cell membrane is different from the isolated form, as obtained from eel. The eel value seems correct so that it is also possible that our estimate of the red cell acetylcholinesterase molecular weight is in error.

Frozen systems are potentially quite valuable for radiation inactivation studies. Some evidence supports the view that the D_{37} of frozen samples may be similar to that obtained *in vacuo*[39]. Investigations carried out in the course of these studies attempted to test this assumption (Table IV). Data on frozen red cells were obtained in experiments designed to use the ATPase enzyme of red cell membranes as a test system for evaluating different treatment procedures that might be used. Table IV presents the results of these experiments and it would appear that although the ratio is not one, it is sufficiently close to indicate that it is within reason to employ modified target theory ideas. More importantly, the ratio is quite constant, so that once it has been established for a specific cell membrane system, it may not be necessary to measure every enzyme in the lyophilized, *in vacuo* state. It is understood that this approach represents even more of a compromise with classical target theory. However, it has a great practical advantage in terms of the ease of performing the experiments and the fact that many more cells are potential candidates for investigation of their functioning systems *in vivo*.

Finally, we turn to the experiments on the irradiation of wet ghost suspensions. BRESCIANI, AURICCHIO AND FIORE[53] have reported (in human erythrocyte ghosts) radiation inactivation of the $(Na^+ + K^+)$-ATPase closely paralleling radiation-induced reduction of the active ion movement. This occurred at doses of 0.5–10 krads. Further the ATPase activity decreased as a two-component exponential which they identified as the Mg^{2+} and $(Na^+ + K^+)$-ATPases. On the other hand, MYERS AND LEVY[54] were unable to detect any effect of X-irradiation on ghosts from rat or human erythrocytes at doses up to 60 krads. We have confirmed their results by finding that it required doses greater than 50 krads to show appreciable inactivation of the ATPase activity. It is essential to recognize that in wet systems dose–effect curves are strongly dependent on the concentration of the material (enzyme or cells) irradiated. At increased concentrations the enzyme is less sensitive to radiation. It is difficult to establish

any one set of data as being representative since the correct procedure requires presentation of results as a function of concentration. In the work reported here the ghosts were irradiated at protein concentrations of 5 mg/ml.

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