RED CELL MEMBRANE ATPase: RADIATION INACTIVATION ESTIMATES OF "SIZE"*

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Sodium-Potassium stimulated, ouabain sensitive ATPase activity has been reported in a wide variety of cellular membrane preparations. The striking quantitative correlation between the kinetics of this enzyme system and the kinetics of the Na-K active transport system suggests that the enzymatic activity can serve as a marker for the transport mechanism in membrane fragments (Skou, 1964). This paper describes experiments on the radiation inactivation of the Na, K, ouabain sensitive ATPase of freeze-dried human erythrocyte ghosts. Assuming that conventional "target theory" (Lea, 1946) can be applied to this preparation, the experiments suggest an inactivation volume of $\sim 1.5 \times 10^{-18} cm^3$ with an "equivalent molecular weight" of $\sim 1 \times 10^6$ for both the ouabain sensitive and ouabain insensitive ATPase systems.

Materials and Methods

Fragmented ghost membranes were prepared from fresh, human blood (collected in ACD) by a modification of the methods of Dodge, et. al. 1963 and Post, et. al. 1960. Cells were hemolysed in (6mM Na_2HPO_4, 1mM NaH_2PO_4, 1mM EDTA, 1mM glycylglycine), washed in (10mM Tris, 1mM EDTA, 1mM glycylglycine), and finally washed twice and stored in (10mM Tris). All solutions were adjusted to pH 7.5 with HCl or NaOH. Centrifuge runs were for 20 min. at 20,000 x g in a refrigerated centrifuge (2-4°C).

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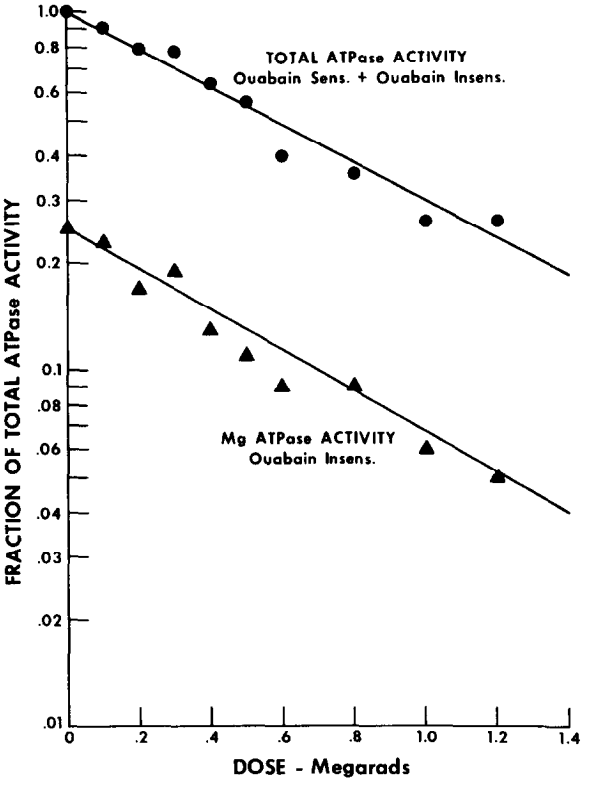
Results

FIG. 1

ATPase INACTIVATION



Each data point represents from 4 to 18 experiments. Data were fitted to a single exponential by computer using a least squares procedure. Weighting the data points equally or inversely according to their variances had no significant effect on the calculated slopes. Although not included in the plot, the data point at three megarads for the total ATPase was on the calculated line.

Two ml of suspension was pipetted into a 25ml Erlenmeyer flask quickly frozen in a dry-ice bath, and dried in a Virtis vacuum system. Once dried, preparations remained stable and active for weeks. Freeze-dried ghosts were irradiated with 7.5 Mev electrons at a dose rate of 10^5 Roentgen/minute (in air, ≈16°C) with an electron linear accelerator.

TABLE 1

*Calculations from the data of figure 1

ATPase Activity	$V\ \theta \times 10^5$	$^{**}V \times 10^{18} cm^3$	"Equiv. M. Wt." $\times 10^{-6}$
TOTAL	.12 ± .01***	1.50	1
OUABAIN INSENS.	.13 ± .01	1.65	1.1
OUABAIN SENS.	.12 ± .01	1.50	1

*Equations used:

1) activity = $e^{-V\ \theta\ D}$

V = inactivation volume cm^3

θ = conversion factor = $\dfrac{\rho\ gm/cm^3 \times 100 ergs/gm\text{-}rad}{1.6 \times 10^{-12} ergs/ev \times \frac{85\ ev}{1\ inactivating\ event}}$

D = Dose rads

2) $\rho\ V\ N_{avog.}$ = "equivalent molecular weight"

** ρ = density of dried preparation: assumed = 1.1

*** $\pm 2\sigma$ = .01

Cobalt glass chips (Bausch and Lomb) were used for the dosimetry.

Ghosts were assayed for ATPase activity a few hours after irradiation. Three ml of incubation medium (5mM Tris, 115mM NaCl, 20mM KCl, pH 7.4) was added to each flask and incubated at 37°C for 20 minutes in a Dubnoff shaker. One ml of substrate suspension (4mM each of Na_2-ATP, EDTA, $MgCl_2$) was added and the reaction stopped after one hour by addition of 2ml of 18% TCA. P_i was measured following the method of Dreisbach, 1965. Ouabain insensitive ATPase was determined by duplicating the above assay in the presence of 10^{-5} M ouabain. The addition of ouabain at this concentration

gave ATPase values very similar to those obtained when K was omitted from the incubation and reaction media. Accordingly, we identify the difference between the total and the ouabain insensitive ATPase as the Na-K stimulated ATPase. Typical control values for ATPase activities were: 25μg P_i/hr./ml freeze-dried ghosts for Na-K activated ATPase and 8μg P_i/hr./ml for ouabain insensitive ATPase. Ghost suspension hemoglobin (determined by cyanmethemeoglobin) was less than .1% that of whole blood and the Biuret reagent (calibrated with BSA) gave "protein" values ≈5mg/ml ghosts.

Discussion

Radiation inactivation has frequently been used to estimate the molecular weight of dried enzymes (Hutchinson and Pollard, 1961) and of macromolecules in biological preparations (Alper, et. al. 1966; Haanen, et. al. 1965). The application of target theory to biological preparations rests on a number of assumptions (Augenstein, 1963; Brustad, 1962). In particular these include:

a) Inactivating events are randomly distributed in the material and the target volume is small compared to the separation between initial ionizing events. The effect of indirect inactivations is minimized if the specimen is in a dehydrated state during irradiation.

b) Inactivation of the highly-specific biological function assayed is due only to those ionizations occurring within the target volume.

c) The energy released in the initial ionizing event and the ensuing secondary ionizations is highly localized and sufficient to inactivate the biological function if it occurs in the target volume. The energy released per inactivating event is assumed to be ≈70-100 ev (Hutchinson, 1965).

The presence of impurities, the possibility of radiation-induced excitations leading to thermal inactivations, and the errors in estimating the energy release in the target volume result in a significant degree

of uncertainty as to the results obtained by this technique. Other work has suggested that the method estimates the molecular weight to within a factor of 2-3 of the "true value"; but larger errors are sometimes encountered (Hutchinson and Pollard, 1961). Work is in progress to improve the basis for interpreting the results on this preparation and to investigate radio-biological mechanisms in membranes.

The inactivation curves of figure 1 correspond with the single exponential predicted from one-hit target theory. This does not necessarily imply that each ATPase corresponds to a single molecular species. The data could also result from ATPase systems consisting of a tightly coupled sequence of biochemical steps arranged in series. Inactivation of any one of these steps would inactivate the entire sequence. Our calculated volumes correspond to the inactivation volume of the entire sequence which may have one or many steps. The appearance of parallel slopes suggests two interpretations of the molecular configuration of the ATPase inactivation volumes.

I) The two ATPase sites occur in a single inactivation volume. An inactivation event in the volume inactivates both enzyme functions.

II) The two ATPases have separate but equal inactivation volumes.

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