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STUDIES ON ($K^+ + H^+$)-ATPase

VI. DETERMINATION OF THE MOLECULAR SIZE BY RADIATION INACTIVATION ANALYSIS

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(1) A ($K^+ + H^+$)-ATPase containing membrane fraction, isolated from pig gastric mucosa, has been further purified by means of zonal electrophoresis, leading to a 20% increase in specific activity and an increase in ratio of ($K^+ + H^+$)-ATPase to basal Mg^{2+}-ATPase activity from 9 to 20. (2) The target size of ($Na^+ + K^+$)-ATPase, determined by radiation inactivation analysis, is 332 kDa, in excellent agreement with the earlier value of 327 kDa obtained from the subunit composition and subunit molecular weights. This shows that the Kepner-Macey factor of $6.4 \cdot 10^{11}$ is valid for membrane-bound ATPases. (3) The target size of ($K^+ + H^+$)-ATPase is 444 kDa, which, in connection with a subunit molecular weight of 110000, suggests a tetrameric assembly of the native enzyme. The ouabain-insensitive K^+-stimulated *p*-nitrophenylphosphatase activity has a target size of 295 kDa. (4) In the presence of added Mg^{2+} the target sizes of the ($K^+ + H^+$)-ATPase and its phosphatase activity are decreased by about 15%, while that for the ($Na^+ + K^+$)-ATPase is not significantly changed. This observation is discussed in terms of a Mg^{2+}-induced tightening of the subunits composing the ($K^+ + H^+$)-ATPase molecule.

Introduction

The ($K^+ + H^+$)-ATPase system, present in the tubulo-vesicular system of the gastric parietal cell, is able to exchange K^+ for H^+ in electroneutral fashion and is thought to play a major role in gastric acid secretion [1–4]. The enzyme resembles the ($Na^+ + K^+$)-ATPase system in several ways. Both enzymes possess a subunit (α) which has an apparent molecular weight of about 100000 on sodium dodecyl sulphate gel electrophoresis [5] and can be phosphorylated by ATP. Both enzymes are responsible for active cation transport, show an acid-stable phosphointermediate, a K^+-stimulated phosphatase activity and can undergo ion-dependent conformational changes.

Nevertheless, there appears to be a difference in subunit composition. The ($Na^+ + K^+$)-ATPase shows in addition to the α-subunit of 121 kDa an equimolar amount of a β-subunit with a molecular weight of 43000 [6]. In ($K^+ + H^+$)-ATPase there is no indication of the presence of a second subunit with a molecular weight below 100000. While Saccomani et al. [7] concluded from tryptic inactivation studies that the 100 kDa band in ($K^+ + H^+$)-ATPase consists of three different proteins present in equimolar amounts, recent studies in our laboratory [5] indicate no protein heterogeneity in this band.

In addition to this disagreement about the molecular composition of the enzyme, even the molecular weight of the whole enzyme is not yet known. The molecular weight of a native enzyme can be suitably determined by radiation inactivation analysis [8,9]. In this method the enzyme is exposed to high-energy ionizing radiation, like

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electrons and gamma rays. On the assumption that a single particle or quantum hit leads to complete inactivation of the enzyme, the target size can be calculated from the slope of the plot of log residual enzyme activity vs. radiation dose.

In the present paper we describe our determinations of the molecular weight of $(K^+ + H^+)$-ATPase from radiation inactivation experiments with gamma rays from a ^{60}Co source. In addition to a proportionality factor derived by Kepner and Macey from experiments on enzymes of known molecular weight, we have used a $(Na^+ + K^+)$-ATPase preparation as an internal standard because of its strong resemblance to the $(K^+ + H^+)$-ATPase in activity and chemical composition [5] and its known molecular weight [6]. The effects of Mg^{2+} on the target size of the $(K^+ + H^+)$-ATPase and its K^+-stimulated phosphatase activity have also been determined.

Materials and Methods

Enzyme preparations. The $(K^+ + H^+)$-ATPase preparation is isolated from pig gastric mucosa as previously described [10]. Briefly, a microsomal fraction is obtained by layering a submitochondrial membrane fraction on a 37% (w/v) sucrose layer and subsequent fractionation of the 37% (w/v) interface layer by centrifugation over a linear sucrose gradient. The fractions with a density of 1.11–1.13 are collected, diluted and centrifuged for 1 h at $100\,000 \times g_{av}$ (gradient fraction G).

Further purification of gradient fraction G is achieved by means of zonal electrophoresis on a density gradient. The apparatus, developed by Walters and Bont [11], has previously been applied to the separation of basolateral membranes from smooth endoplasmic reticulum of the rat enterocyte [12]. Fig. 1 is a schematic drawing, showing the composition of the various buffers used in the procedure. The sample overlay and upper electrode buffers are carefully applied by means of a sieve to prevent disturbance of the gradient. Each electrophoretic run can accomodate 30–40 mg protein.

The gradient fraction is twice washed with the sample buffer and thoroughly homogenized (Braun teflon-glass homogenizer, 40 strokes, 500 rev./min) and is then layered on top of the stabilizing sucrose gradient. After the electrophoretic run the gradient is collected in 12-ml fractions, which are then diluted and centrifuged for 1 h at $100\,000 \times g_{av}$. The sedimented membranes, appropriately pooled (fractions E_1, E_2, E_3, E_4 in Fig. 2) are suspended in 0.25 M sucrose. The suspensions are dropwise added to liquid nitrogen, and the resulting beads are stored at −20°C.

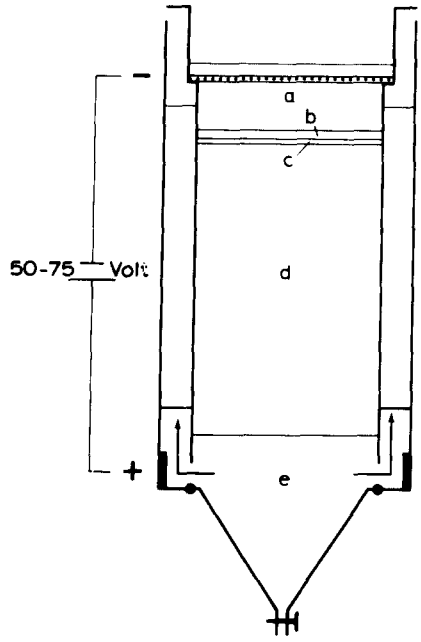

Fig. 1. Cross section of zonal electrophoresis apparatus. a, upper electrode buffer, e, lower electrode buffer; d, linear gradient; c, sample buffer; b, overlay. Buffers: a, 200 ml 100 mM Tris-acetate (pH 7.4)/0.1 mM Na_2ATP; b, 80 ml 10 mM Tris-acetate (pH 7.4)/0.1 mM Na_2ATP/0.1 mM EDTA/5.5% sucrose; c, 20 ml 10 mM Tris-acetate (pH 7.4)/0.1 mM Na_2ATP/0.1 mM EDTA/7% sucrose; d, 1100 ml 10 mM Tris-acetate (pH 7.4)/0.1 mM EDTA/10–20% sucrose; e, 550 ml 0.4 M Bicine (pH 8.0)/30% sucrose. Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

The $(Na^+ + K^+)$-ATPase preparation is isolated in highly purified form from rabbit kidney outer medulla [13].

Irradiation with gamma rays of a ^{60}Co source. As purified $(K^+ + H^+)$-ATPase preparation either gradient fraction G or electrophoretic fraction E_1 is used. The enzyme (approx. 5 mg protein) is mixed with highly purified $(Na^+ + K^+)$-ATPase preparation (approx. 0.8 mg protein). The membrane mixture is sedimented by centrifugation, and the resulting pellets are resuspended in 15 mM

Tris-HCl (pH 7.4)/0.1 M sucrose/0.2 mM ATP with or without 2 mM $MgCl_2$ to a final protein concentration of about 1.8 mg per ml. The addition of ATP and sucrose serves to stabilize the enzyme, as empirically established. Aliquots of 0.1 ml are lyophilized for at least 15 h in 1.5 ml Eppendorf plastic tubes. The tubes are kept for a week or longer in a P_2O_5-containing vacuum desiccator to achieve complete dryness, which reduces the enzyme activities of $(K^+ + H^+)$-ATPase and $(Na^+ + K^+)$-ATPase by only 6–10%. After gentle flushing with nitrogen, the tubes are immediately capped and sealed with parafilm. They are then placed in a ^{60}Co source (Gamma-Cell 200, Atomic Energy of Canada, Ottawa, Canada) for irradiation.

A special holder is used for the tubes to ensure that all samples will receive an equal radiation dose. The cylindrical Perspex holder fits into the radiation chamber and contains 12 holes for sample tubes, such that these tubes are located at the same height and equidistant from the center of the radiation chamber. Control tubes are processed in exactly the same way, but are placed outside the ^{60}Co source for the duration of the irradiation experiments. After each of five irradiation periods two sample tubes are removed.

The radiation dose at the sample location is read from the isodose curves of the Gamma-Cell-200 irradiation chamber, provided by the manufacturer. The dose rate has been checked by Fricke dosimetry ($Fe^{2+} \rightarrow Fe^{3+}$; 15.5 Fe^{3+} molecules being formed per 100 eV absorbed radiation energy). It is normalized in different experiments by using a monthly decay factor of 0.98908. The dose rate decreased from 100 to 85 krad per h during the time period elapsing between the first and the last of our experiments.

Molecular weight calculations. The contents of the irradiated and control tubes are taken up in 0.5 ml of water and the $(K^+ + H^+)$-ATPase, $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities as well as the protein contents are determined. From a plot of the logarithm of the fractional residual enzyme activity versus the radiation dose we find the dose for 37% residual activity (D_{37}). This dose and the molecular weight (M) are inversely proportional according to the single target theory:

$$M = \frac{K}{D_{37}\,(\text{rads})}$$

The proportionality constant K has been empirically determined by Kepner and Macey [14] for a number of enzymes with known molecular weight to be $6.4 \cdot 10^{11}$.

In our case no corrections for the temperature are required [8], since all radiations are carried out at room temperature and the heat production at our relatively low dose rates is so small that the rise in temperature of the sample during irradiation is negligible.

Analytical methods. Membrane protein is determined by the method of Lowry et al. [15], using as standard bovine serum albumin dissolved in the same medium as the sample. $(K^+ + H^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities are determined as previously described [11]. The $(Na^+ + K^+)$-ATPase and ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase assays are performed as described by Schoot et al. [16].

SDS-polyacrylamide gel electrophoresis is carried out as earlier described [6]. Mg^{2+} contents of the $(K^+ + H^+)$-ATPase preparations are determined in an atomic absorption spectrometer (Pye Unicam sp 1950), using $MgSO_4$ standards [17].

Results

Purification of gastric vesicles by zonal electrophoresis

Gastric membranes have been isolated, as previously described [10], by centrifugation of a submitochondrial membrane suspension on a 37% (w/v) sucrose layer, followed by isopycnic centrifugation of the interface layer over a continuous sucrose gradient. The peak fractions thus obtained show a ratio of $(K^+ + H^+)$-ATPase to Mg^{2+}-ATPase activity of approx. 9.

Further fractionation of this gradient purified fraction has now been achieved by zonal electrophoresis in the apparatus depicted in Fig. 1. The distribution of $(K^+ + H^+)$-ATPase and Mg^{2+}-ATPase activities after zonal electrophoresis is shown in Fig. 2. There are a major and minor protein band. The material in the major band is collected in three fractions (E_1–E_3, in order of decreasing mobility), the minor protein band as

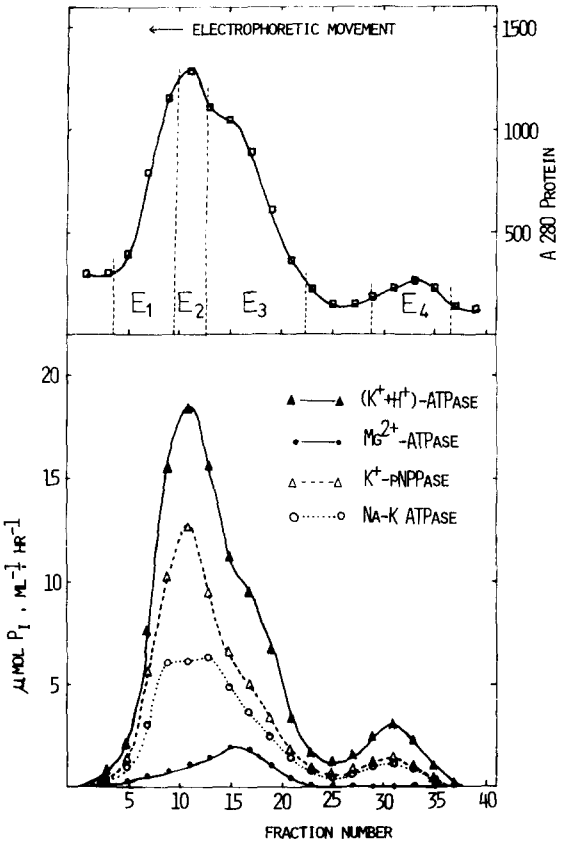

Fig. 2. Protein and enzyme activity distribution after zonal electrophoresis of the gradient purified $(K^+ + H^+)$-ATPase containing membrane preparation. E_1–E_4 indicate the pooled fractions.

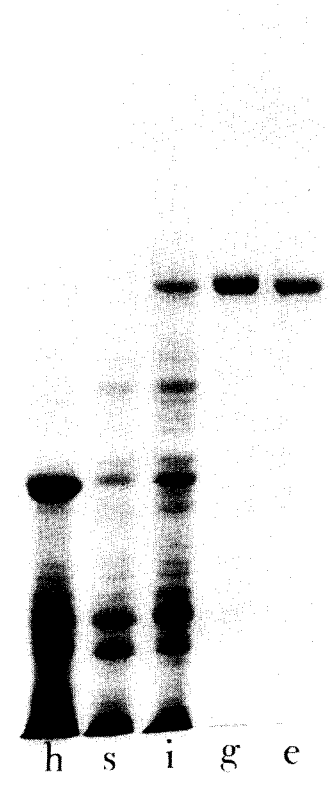

Fig. 3. SDS-gel electrophoretic patterns of fractions obtained during the purification of $(K^+ + H^+)$-ATPase fraction from pig gastric mucosa. h, crude homogenate; s, 20000 × *g* supernatant; i, interface fraction; g, gradient purified fraction and e, zonal electrophoretic fraction (E_1 from Fig. 2).

fraction E_4. The ratio of $(K^+ + H^+)$-ATPase to Mg^{2+}-ATPase activity in fractions E_1 and E_2 is about 20, indicating a further purification over that achieved by the gradient purified step.

The shoulder in the major protein peak, also reflected in the $(K^+ + H^+)$-ATPase curve, suggests the existence of an additional peak. However, we have been unable to resolve the major peak into two clearly separated membrane fractions, either by zonal electrophoresis or by free flow electrophoresis according to Saccomani et al. [18]. This is true even if we include ATP, which appears to be essential for good separation.

Fig. 3 shows the protein composition of the main fractions obtained at the various steps of our purification procedure, as analyzed by SDS gel electrophoresis. The 100 kDa protein is present in the interface fraction (I) and represents approx. 70% of total protein after gradient centrifugation (G). At first sight, further fractionation by means of zonal electrophoresis (E) does not seem to result in a much purer 100 kDa protein. However, scanning of the gels reveals some decreases in the relative amounts of the other proteins in the respective fractions (not shown). As mentioned above, the $(K^+ + H^+)$-ATPase to Mg^{2+}-ATPase activity ratio is increased to 20. The specific $(K^+ + H^+)$-ATPase activity of the electrophoretically purified enzyme preparations ranges from 90 to 140 μmol P_i per mg protein per h, about 20% higher than that of the gradient purified fraction. The endogenous and tightly bound Mg^{2+} contents amount to 18 (S.E. 0.8, $n = 4$) μmol Mg^{2+}/g protein, considerably lower than for the gradient purified fractions (47, S.E. 8, $n = 8$).

Inactivation by gamma rays

In order to determine the molecular weight of $(K^+ + H^+)$-ATPase, a mixture of this enzyme with purified $(Na^+ + K^+)$-ATPase is lyophilized and irradiated by γ-rays from a ^{60}Co source. Figs. 4 and 5 show the residual activities of the two enzymes as a function of the irradiation time in a typical experiment. The semilogarithmic plots are all linear for the 21 h experimental period, indicating the applicability of the single-hit-target theory. The two *p*-nitrophenylphosphatase activities (ouabain-sensitive and ouabain-insensitive) are less sensitive to the ionizing radiation than the two ATPase activities, indicating that they involve smaller functional units.

From the time needed to obtain 37% residual activity the D_{37} dose is calculated, which yields the molecular weight by means of the formula given under Materials and Methods. The values obtained for the various enzyme activities are summarized in Table I. The average value of 332 kDa (S.E. 27, $n = 6$) obtained for the $(Na^+ + K^+)$-ATPase is in good agreement with the value of 327 kDa (S.E. = 9.4, $n = 6$) calculated for the protein part of this enzyme by Peters et al. [6]. Target analysis of the $(Na^+ + K^+)$-ATPase by Ellory et al. [19] has recently yielded a molecular weight of 330 000 for this enzyme from human erythrocytes, close to our value. However, for the ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase activity we find a radiation sensitive weight of 262 kDa (S.E. 21, $n = 4$), which is considerably higher than the values of 164 and 180 kDa reported by Ellory and co-workers [19,20].

The close agreement between the molecular weight calculated from the subunit protein weights and that obtained from radiation inactivation analysis by means of the Kepner-Macey factor has permitted the use of the latter method for the calculation of the molecular weight values for $(K^+ + H^+)$-ATPase. The value thus obtained for the whole enzyme in the absence of Mg^{2+} is 444 kDa and for the ouabain-insensitive *p*-nitrophenylphosphatase 295 kDa (Table I).

When gastric $(K^+ + H^+)$-ATPase and renal $(Na^+ + K^+)$-ATPase preparations are suspended

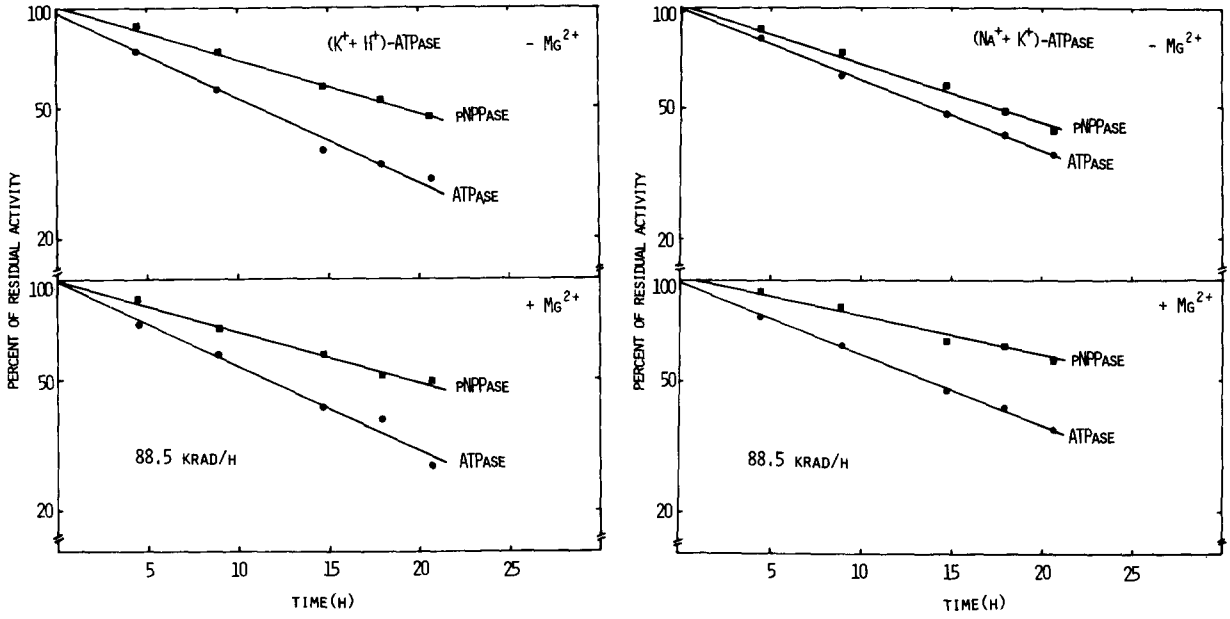

Fig. 4. Inactivation of $(K^+ + H^+)$-ATPase and ouabain-insensitive K^+-stimulated *p*-nitrophenylphosphatase by gamma rays in the presence or absence of 2 mM $MgCl_2$. Irradiation in a ^{60}Co Gamma-Cell-200 is carried out at room temperature as described under Materials and Methods. Semilogarithmic plot of log residual activity vs. irradiation time. Typical experiment out of four.

Fig. 5. Inactivation of $(Na^+ + K^+)$-ATPase and ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase by gamma rays in the presence or absence of 2 mM $MgCl_2$. Experimental conditions and presentation of the results as in Fig. 4.

TABLE I

MOLECULAR WEIGHTS OF GASTRIC $(K^+ + H^+)$-ATPase AND RENAL $(Na^+ + K^+)$-ATPase AND THEIR K^+-STIMULATED *p*-NITROPHENYLPHOSPHATASE ACTIVITIES DETERMINED BY RADIATION INACTIVATION

Preparations of the two enzymes are mixed and lyophilized in the absence or the presence of 2 mM $MgCl_2$ as described under Materials and Methods. Molecular weights are calculated with the formula $M = K/D_{37}$, where $K = 6.4 \cdot 10^{11}$ and D_{37} is the radiation dose leaving 37% residual activity, which is deduced from semi-logarithmic plots as shown in Figs. 4 and 5. Data are presented with standard error of the mean and in parentheses the number of determinations. The S.E. from two experiments is calculated as 0.63-times the range, according to the approximation method of Davies and Pearson [34]. K^+-*p*NPPase, K^+-stimulated *p*-nitrophenylphosphatase.

Enzyme activity	Molecular weight ($\times 10^{-3}$)			
	$-Mg^{2+}$	$+2$ mM Mg^{2+}	Average	Ratio $+Mg^{2+}/-Mg^{2+}$
$(K^+ + H^+)$-ATPase	$444 \pm 10(4)$	$371 \pm 39(3)$	$413 \pm 22(7)$	0.84
K^+-*p*NPPase (ouabain-insensitive)	$295 \pm 16(4)$	$250 \pm 12(3)$	$276 \pm 13(7)$	0.85
$(Na^+ + K^+)$-ATPase	$336 \pm 14(3)$	$328 \pm 23(3)$	$332 \pm 12(6)$	0.98
K^+-*p*NPPase (ouabain-sensitive)	$283 \pm 6(2)$	$242 \pm 27(2)$	$262 \pm 21(4)$	0.86

in a medium containing 2 mM $MgCl_2$ before lyophilization, the radiation-sensitive masses of the four enzyme activities seem to be decreased by some 15%, much less for the $(Na^+ + K^+)$-ATPase activity. Application of the Wilcoxon nonparametric test to each of the four enzyme activities with and without added Mg^{2+} shows no significant difference, neither does combined testing of the $(Na^+ + K^+)$-ATPase and phosphatase activities. However, combined testing of the $(K^+ + H^+)$-ATPase and its phosphatase activities gives a $P = 0.0086$. This would indicate that Mg^{2+} tightens the subunits of $(K^+ + H^+)$-ATPase, thus leading to a decreased radiation sensitive cross-section of the enzyme molecule.

Discussion

Purification of gastric vesicles by zonal electrophoresis

Zonal electrophoresis reveals an enzyme distribution pattern, which shows a close resemblance to the pattern obtained by Saccomani et al. [18] with free flow electrophoresis. The complete absence of Mg^{2+}-ATPase activity in fraction E_4 suggests that this fraction may be identical to their GII-FIII fraction. In contrast to their results, we are unable to resolve the major peak into two clearly separated membrane fractions, even when ATP is included in the medium.

Nevertheless, from the final specific activities (90–140 μmol $P_i \cdot (mg\ protein)^{-1} \cdot h^{-1}$) and the high ratio [20] of $(K^+ + H^+)$-ATPase to Mg^{2+}-ATPase activity it appears that the relatively simple and inexpensive zonal electrophoresis method has the same fractionating capability as the free flow electrophoresis method, as previously claimed by Van Os et al. [12].

There is a dispute whether the basal Mg^{2+}-ATPase activity is an integral part of the gastric $(K^+ + H^+)$-ATPase [21,22] or is simply due to contamination [18]. During purification this activity is considerably lowered, although even in the purest preparations a minor amount of this enzyme activity is still present. This favors the assumption that the Mg^{2+}-ATPase activity represents a contamination, or else there would have to exist a complex regulation of the ratio of the two activities. The dephosphorylation of the phosphorylated enzyme preparation in the absence of K^+ [21] and the relative increase of the Mg^{2+}-ATPase activity after treatment with phospholipase A_2 [22] would lend some support to the latter explanation. Hence, the matter remains undecided.

Validity of the radiation inactivation method

Radiation inactivation analysis has been shown to be a simple but powerful technique for obtain-

ing information about the molecular size of enzymes, particularly for membrane-bound enzymes and for the detection of regulatory interactions, such as association between discrete but functionally connected membrane components and subunits [8,9,14,23,24]. The method is based on the assumption that an enzyme molecule is inactivated by a single hit from a quantum of ionizing radiation. The molecular weight determined with this method represents the functional unit of the enzyme, since the enzyme activity is the measured parameter. The linearity of semi-logarithmic plots obtained in all of our experiments indicates that the inactivation process obeys first-order rate kinetics, and thus that the single hit target theory holds for these enzymes at least under the conditions of our experiments.

A prerequisite for valid application of the method is that inactivation is limited to direct action of the radiation. This is achieved by the use of freeze-dried samples under nitrogen, thus avoiding the formation of free radicals from water and oxygen leading to secondary inactivation phenomena and falsely high molecular weight values [8,25]. It is assumed, in the absence of evidence to the contrary, that lyophilization will not change the subunit configuration of the enzyme.

In our experiments 1.3 MeV γ-rays from a ^{60}Co source at the relatively low dose rate of 85–100 krad · h^{-1} are used. Thus, our irradiation times (up to 21 h) are longer than in studies with high energy electrons at high dose rates and the maximal inactivation is only 70%. Nevertheless, our results for $(Na^+ + K^+)$-ATPase closely agree with those of Ellory et al. [19], which were obtained with high fluxes (150 Mrad · h^{-1}) of 20 MeV electrons produced by a linear accelerator. Furthermore, in preliminary studies in collaboration with Dr. J.C. Ellory (Department of Physiology, University of Cambridge, U.K.) using the same electron source, the apparent molecular weight ratio for $(K^+ + H^+)$-ATPase and $(Na^+ + K^+)$-ATPase is 1.28 in the absence of added Mg^{2+} and 1.15 in the presence of 2 mM Mg^{2+}, which values are in good agreement with the corresponding ratio's of 1.32 ± 0.06 and 1.13 ± 0.14 found in our experiments with gamma rays (Table I). These findings indicate that type, energy and dose rate of the radiation do not significantly affect the results.

Since the proportionality factor K in the formula $M = K/D_{37}$ has been empirically determined by Kepner and Macey [14] for a number of soluble enzymes, we wondered whether this factor would also apply to insoluble, membrane-bound enzymes. Hence, we have used as an internal standard $(Na^+ + K^+)$-ATPase, for which we have determined the molecular weight from the subunit molecular weights [6]. The excellent agreement between the two molecular weight values (332, S.E. 12, $n = 6$ vs. 327, S.E. 9.4, $n = 6$) indicates that the Kepner-Macey factor of $6.4 \cdot 10^{11}$ is valid for the membrane-bound ATPase. Thus, this factor has been used to calculate the molecular weights for the $(K^+ + H^+)$-ATPase and its phosphatase activities.

It should be added that the value of 327 000 is the protein molecular weight of $(Na^+ + K^+)$-ATPase, whereas the value including carbohydrates is 386 000. Hence the agreement of the protein molecular weight with the radiation inactivation value would imply that hits on the carbohydrate moiety do not abolish the activity of the enzyme molecule. This conclusion is supported by a recent observation of Lowe and Kempner [26] that the radiation target size for the invertase activity from *Saccharomyces cerevisiae* is not diminished after enzymatic removal of nearly all of the carbohydrates, which comprise 50% of the molecular weight of the enzyme.

Molecular size of $(K^+ + H^+)$-ATPase

The calculated target size of $(K^+ + H^+)$-ATPase in the absence of added Mg^{2+} is 444 kDa. This is in good agreement with the results obtained by analytical ultracentrifugation [5]. There a gross molecular weight of 498 000 was obtained, which after correction for bound lipid and carbohydrate leads to a protein molecular weight of 440 000. The molecular weight of 440 000–444 000 indicates a tetrameric structure for the enzyme, since the resulting subunit weight of 110 kDa gives best agreement with the value of 100 kDa from gel electrophoresis and the value of 121 kDa obtained for the structurally and functionally very similar α-subunit of $(Na^+ + K^+)$-ATPase [5,6]. Saccomani et al. [27] have reported a value of only 270 kDa from radiation inactivation by high-energy electrons. However, their experiments were carried out on

frozen membrane suspensions at a temperature of −45°C to −60°C. At such low temperatures a correction is necessary [8], which according to the results of Fluke [28] amounts to a factor of 1.6 in the temperature range from −50°C to +20°C. Application of this correction factor to the value of Saccomani et al. [27] yields a molecular weight of 432 000, close to our value of 444 000. Although they include a value of only 320 kDa obtained with a frozen-dried preparation at 23°C, we are inclined to discount this value. The factor of 1.6 for a temperature increase of 70 K has namely been reconfirmed by Kempner and Haigler [29] for 25 enzymes. They ascribe deviating findings for four enzymes [27,30–32] to either inadequate temperature measurements or use of temperature-sensitive dosimeters.

Molecular size of the phosphatase unit

For both ATPases we find that their phosphatase activities offer a smaller target size than those of the overall ATPase activities. This could mean that a particular subunit, which is essential for the ATPase activity, is not required for the *p*-nitrophenylphosphatase activity. The difference of 50–80 kDa for the $(Na^+ + K^+)$-ATPase complex, approximates the molecular weight of two β-subunits, suggesting that β-subunit integrity would not be essential for the phosphatase activity. However, the difference of 120–150 kDa for the $(K^+ + H^+)$-ATPase, which we find to have only a single type of subunit, lies between one and two subunit weights. Therefore, it is more likely that the phosphatase target in each case involves a functionally distinct part of the oligomeric enzyme molecule. The same conclusion was reached by Richards et al. [20] who find a very small target size (40–60 kDa) for the K^+-occluding mechanism of the $(Na^+ + K^+)$-ATPase.

Effects of Mg^{2+} on the target size

Pretreatment of the enzyme with 2 mM Mg^{2+} appears to decrease the target sizes of the enzyme activities by some 15%, much less for the $(Na^+ + K^+)$-ATPase activity. Statistical evaluation shows that this effect is significant for $(K^+ + H^+)$-ATPase, but not for $(Na^+ + K^+)$-ATPase. This suggests that Mg^{2+} increases the interaction between the subunits. A similar conclusion has been drawn from our studies of the effect of Mg^{2+} on binding of Ado*PP*[NH]*P* to the enzyme [17], whereas the present findings appear to rule out the alternative explanation by dimerization offered there. To our knowledge this is the first report of an ion-induced decrease in the radiation target size of an enzyme. So far, only detergent-induced structural changes have been observed with the radiation-inactivation technique [33].

The apparent Mg^{2+}-induced decrease in target size may offer an alternative explanation of the earlier mentioned difference in the molecular weights of gastric $(K^+ + H^+)$-ATPase reported by Saccomani et al. [27] and by us, namely if their preparation had higher endogenous Mg^{2+} content than ours.

We have thus shown that radiation inactivation analysis can under proper safeguards provide the approximate size of a membrane bound enzyme and of the functional unit of one of its partial activities as well as information about ion-induced conformational changes of the enzyme. For renal $(Na^+ + K^+)$-ATPase we obtain a target size agreeing with the protein molecular weights of the enzyme. For gastric $(K^+ + H^+)$-ATPase we find a target size of 444 kDa, suggesting a tetrameric assembly of the enzyme molecule, and one of 295 kDa for its K^+-stimulated phosphatase activity, as well as Mg^{2+}-induced tightening of the enzyme conformation.

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