Biochimica et Biophysica Acta, 727 (1983) 89–100
Elsevier Biomedical Press

BBA 71489

THE K^+-INDUCED APPARENT HETEROGENEITY OF HIGH-AFFINITY NUCLEOTIDE-BINDING SITES IN $(Na^+ + K^+)$-ATPase CAN ONLY BE DUE TO THE OLIGOMERIC STRUCTURE OF THE ENZYME

PAUL OTTOLENGHI and JØRGEN JENSEN

Institute of Physiology, University of Aarhus, 8000 Aarhus C (Denmark)

(Received August 9th, 1982)

Key words: $(Na^+ + K^+)$-ATPase; Nucleotide-binding site; Heterogeneity; Ouabain; Oligomeric structure; (Pig kidney)

K^+ induces an apparant heterogeneity among an otherwise homogeneous population of nucleotide-binding sites in $(Na^+ + K^+)$-ATPase preparations from pig kidney. With the help of ouabain we show that this heterogeneity cannot be due to a mixture of different and independent sites and conclude that each enzyme molecule must contain two nucleotide site-containing units that show interaction. Na^+ induces an apparent heterogeneity among an otherwise homogeneous population of ouabain-binding sites. The argument is, therefore, extended to include one ouabain site on each of the structural units that bind nucleotide. All these structural units are shown to hydrolyse substrate at identical rates. Using the presently available molecular weight data, it is concluded that the enzyme is composed of two subunits each possessing one nucleotide-binding site, one ouabain-binding site, one α-peptide and the capacity for hydrolysing ATP and *p*-nitrophenyl phosphate.

Introduction

The binding isotherms for ADP and ATP to preparations of $(Na^+ + K^+)$-ATPase from pig kidney outer medulla microsomes maximally activated with SDS, give no indication of site heterogeneity as long as the only monovalent cations present during the binding assay are Tris, imidazolium and/or Na^+ [1–3]. The introduction of K^+ into this medium leads to the appearance of heterogeneity in the population of sites capable of high-affinity binding of nucleotides without changing the total number of sites. This apparent heterogeneity can only be explained by one of the following two general cases: (A) the enzyme preparation consists of a mixture of independent nucleotide binding site-containing units with different K^+-dependent affinities for nucleotide; (B) the enzyme consists of molecules containing at least two nucleotide binding-site units (e.g. an oligomeric enzyme) showing interaction that could be mediated via nucleotide sites and/or K^+ sites.

It is important to stress that the nucleotide-binding sites we are concerned with are those that have a high affinity for both ATP and ADP and are present in every enzyme preparation in exactly the same number as ouabain-binding sites and vanadate-binding sites [3]. Furthermore, in any enzyme preparation, ouabain binding to a fraction of the available sites leads to a very great decrease in the affinity for nucleotides of an identical fraction of the total number of nucleotide-binding sites [4].

The literature contains many references to low affinity nucleotide-binding sites on $(Na^+ + K^+)$-ATPase; such proposals have been put forth to explain the kinetic behaviour of various types of reactions involving the enzyme. These sites, if they exist, would be present in addition to those discussed above, and are, therefore, not our concern

in the context of the present study (for a review of this question, see Ref. 5).

The time has obviously come to ascertain whether all those enzyme preparations that have been in use during the past 25 years consist of a mixed population of enzyme molecules with various properties as suggested for brain [6], or whether these enzyme preparations can be assumed to consist of homogeneous populations of molecules that show apparent negative cooperativity of nucleotide binding under appropriate experimental conditions.

If the second case applies, each enzyme molecule must contain more than one high affinity nucleotide-binding site, but so far, no incontrovertible evidence has been presented that proves that a molecule of $(Na^+ + K^+)$-ATPase (whatever this term might imply) can bind more than one molecule of nucleotide with dissociation constants in the μmolar range or less. Furthermore, since models with negatively cooperating sites and models invoking the presence of independent but different sites are described by mathematical expressions of identical form [7], it is necessary to use some other method to resolve the problem imposed by the experimental observations.

In the present communication we wish to present data that provides plausible evidence that preparations of $(Na^+ + K^+)$-ATPase from SDS-activated kidney microsomes consist of enzyme molecules with at least two nucleotide-binding units, where these units are capable of interaction. We will also extend the model to the binding of ouabain by describing conditions under which previously identical ouabain sites show apparent negative cooperativity. Finally we present data that strongly suggest that all units having one nucleotide site and one ouabain site have identical molar activites towards substrate.

Materials

Enzyme preparation. Crude microsomes from pig kidney outer medulla [8] containing $(Na^+ + K^+)$-ATPase were washed by centrifugation with four changes of ice-cold solution of 250 mM sucrose, 12.9 mM imidazole, 0.625 mM EDTA, pH 7.15 (measured at 37°C). They were then 'activated' by mixing enzyme suspension with SDS: 3 mg protein and 0.35 mg SDS per ml, final concentrations. The enzymatic activity due to $(Na^+ + K^+)$-ATPase attained after standing overnight at room temperature is the maximal obtainable and stable. This activated enzyme preparation was washed several more times with the above solution to reduce the concentrations of contaminating cations as much as possible. Very extensive washing still left, typically, about 10–20 μM K^+ and 100 μM Na^+ at a final protein concentration of 10 mg per ml.

The activities of the enzyme used in the present study were: 9 μmol ATP split per min per mg protein and 1.6 μmol *p*-nitrophenyl phosphate split per min per mg protein. The assay conditions for ATPase included 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+} and 3 mM ATP [9]. *p*-Nitrophenylphosphatase was measured in the presence of 150 mM K^+, 20 mM Mg^{2+} and 10 mM *p*-nitrophenyl phosphate [10]. All activities mentioned in the text are those inhibitable by ouabain (approx. 97% of the total). The enzyme had a nucleotide-binding capacity and a ouabain-binding capacity of 1.17 nmol per mg protein, measured with the method of Lowry et al. [11], with bovine serum albumin as standard. The sample of highly purified enzyme (a gift of Dr. P.L. Jørgensen) used for experiments shown in Figs. 1 and 2, had an ATP-binding capacity of 3.76 nmol per mg of protein measured by the method of Lowry et al. The molar activity of *p*-nitrophenylphosphatase per nucleotide binding site of both the pure and impure preparations used in this study was $1.43 \cdot 10^3$ min^{-1}.

Reagents. The reagents used were of analytical reagent grade whenever possible. SDS was obtained from Sigma, sodium salts of ADP and ATP from Boehringer and the sodium salt of *p*-nitrophenyl phosphate from Merck. [^{14}C]ADP and [^{14}C]ATP were obtained from The Radiochemical Centre, Amersham, and [^{3}H]ouabain from NEN.

Nucleotides and *p*-nitrophenyl phosphate for use in binding experiments and for assays of activity were purified and converted to their Tris salts by chromatography on DEAE-Sephadex [12] or on Dowex 50W-X8. [^{3}H]Ouabain was purified by means of a slight modification of the method of Hansen [13]. Briefly, ouabain and kidney microsomal ATPase were allowed to react under condi-

tions giving approx. 50% binding of the added glycoside. The ouabain-enzyme complex was washed free of unbound ouabain and that which was bound was released into water by heating to 65°C for 30 min. Insoluble material (enzyme, etc.) was removed from the mixture by centrifugation.

Experimental procedure

Binding of ouabain. Determination of isotherms for the binding of ouabain was carried out by incubating enzyme with varying concentrations of ouabain + [^{3}H]ouabain in the presence of 1.5 mM Mg^{2+}, 3 mM phosphoric acid neutralized with 2-amino-2-methyl-1,3-propanediol, 10 mM imidazole, 195 mM sucrose at pH 7.25 for 180 min at 37°C. This incubation time is sufficient for binding equilibrium to be attained; enzyme incubated for the same period of time and under the same conditions (but in the absence of ouabain) shows no measurable change in enzymatic properties. The mixture was cooled to 0°C, at which temperature the enzyme-ouabain complex was found to be stable [14], and an aliquot was centrifuged at 100 000 × *g* for 60 min, at 0°C. The concentration of bound ligand was estimated as the difference between the concentrations of ouabain in the non-centrifuged aliquots and in the supernatants of the corresponding centrifuged ones. The concentration of ouabain in stock solutions was determined according to Joiner and Lauf [15].

Experiments with both ouabain and ADP binding. Where nothing else is mentioned, all experiments were carried out in media of the same composition, including concentration of enzyme. The experiments consisted of determinations of the binding isotherms for ADP to $(Na^+ + K^+)$-ATPase that had been preincubated without ouabain and with two different concentrations of ouabain. The procedure employed was as follows.

Preincubation conditions: 7.75 ml of enzyme suspension containing 10 mg protein per ml, 0.5 ml 30 mM Mg^{2+}, 1 ml 30 mM phosphoric acid (buffered with aminomethylpropanediol), 10 mM imidazole, 195 mM sucrose and 0.75 ml redistilled water containing 0, 10^{-4} M or 10^{-2} M ouabain. This mixture had a pH of 7.25 and was incubated at 37°C for 1 h. The result of these preincubations was enzyme free of ouabain (= experiment A), enzyme with all its ouabain-binding sites occupied (= experiment B), and enzyme with 81% of its ouabain-binding sites occupied (= experiment C).

The above mixtures were cooled on ice for 15 min and to each 10 ml of mixture was added 0.8 ml ice-cold 150 mM EDTA (imidazole salt, pH 7.1 measured at 37°C) and 1.2 ml of an ice-cold mixture containing 690 mM Tris-HCl and 10.7 mM KCl (pH 6.3 measured at 37°C). The EDTA was added to complex the Mg^{2+} in the medium so as to minimize nucleotide hydrolysis and/or dismutation by adenylate kinase contaminating the enzyme preparation.

ADP binding assays. A rate dialysis procedure [16] as described by Nørby and Jensen [12] was used. To 1.2 ml of the final ice-cold mixture obtained above were added 80 µl of 150 mM Tris-HCl (pH 6.3 measured at 37°C) containing labelled and unlabelled ADP in varying concentrations. The final concentrations of reagents in the binding assay were: 78.5 mM imidazole; 151 mM sucrose; 9.75 mM EDTA; 2.34 mM inorganic phosphate; 1.17 mM Mg^{2+} (total); 74 mM Tris. The final concentrations of substances that are of principal importance for the experiment were: 1 mM K^+; 6 mg protein per ml; an ADP-binding capacity of approx. 7.0 µM; final pH of 7.6 measured at 0°C. The binding assays were all carried out at 0–2°C. The determination of equilibrium concentrations of free and bound ADP at each concentration of free ADP used took 10 min.

Analysis of binding data

The results of binding data are presented in the form of Scatchard plots with concentration of bound ligand ([B]) plotted against [B]/[F], where [F] represents the concentration of free ligand. Unless otherwise stated, the data were fitted to one of the two following mathematical models:

$$[B] = [B]_t \cdot \frac{\alpha \cdot [F]}{1 + \alpha \cdot [F]} \quad (1)$$

for straight-lined isotherms in Scatchard plots, and

$$[B] = \frac{[B]_t}{2} \cdot \frac{\beta \cdot [F] + 2\gamma \cdot [F]^2}{1 + \beta \cdot [F] + \gamma \cdot [F]^2} \quad (2)$$

for the data shown in figures with upward curved

isotherms in Scatchard plots. $[B]_t$ is the total concentration of sites, and α, β and γ are constants the exact meaning of which will depend upon the model obeyed. Eqn. 1 describes systems consisting of sites with identical binding properties while Eqn. 2 is a mathematical description of either one of two system: (i) two equal sized populations of different and independent sites, and (ii) one population of enzyme molecules with two sites that show apparent negative cooperativity of ligand binding [7]. In the context of the following discussion, 'ligand' will represent nucleotide or ouabain.

A non-linear least-squares fit of these equations was applied to the experimental data, weighted so as to take into account the experimental errors.

Results

The apparent heterogeneity of nucleotide-binding sites elicited by K^+.

As can be seen from Fig. 1, Scatchard plots of data representing ADP and ATP-binding to ($Na^+ + K^+$)-ATPase in the absence of K^+ are straight. Fig. 2 shows that addition of K^+ to the binding medium reduces the apparent affinity of the enzyme for nucleotides and it also induces a curvature in the binding isotherms. In the present work, the ($Na^+ + K^+$)-ATPase consisted of a pig kidney, crude microsomal preparation, maximally activated with SDS; the purity of the enzyme, however, is immaterial as far as the results are concerned: the purest preparations obtainable from the same tissue behave in identical fashion. It was therefore easier to use the crude preparations to fulfill the experimental requirements for rather large amounts of enzyme.

Although K^+ is the factor leading to the curvature of Scatchard plots of the data of ADP and ATP binding to ($Na^+ + K^+$)-ATPase of Fig. 2, it is still an essential requirement for the existence of such curvature that the preparation contain two or more independent nucleotide-binding site popula-

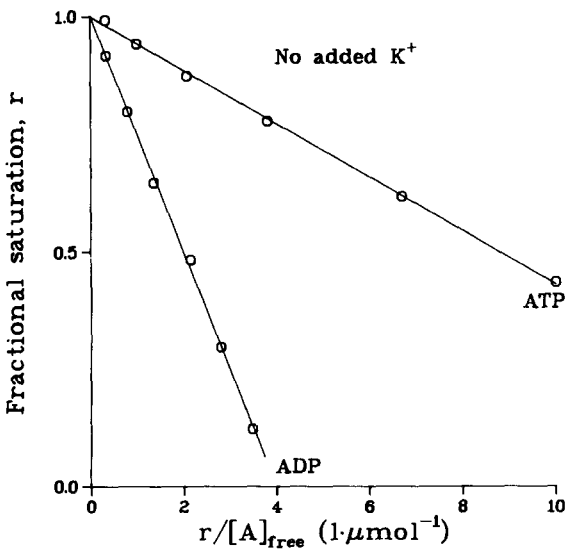


Fig. 1. Normalized Scatchard plots (ordinate: Bound/$[B]_t$) of ADP binding data to SDS-treated microsomes similar to those used in the present study and of ATP binding data to pure kidney ($Na^+ + K^+$)-ATPase, kindly supplied by Dr. P.L. Jørgensen. The procedure for ATP binding was similar to that described under Methods for ADP binding. Binding was measured in the absence of K^+. The data fit a straight line which corresponds to the model represented by Eqn. 1 (see Methods); there is no evidence of site heterogeneity.

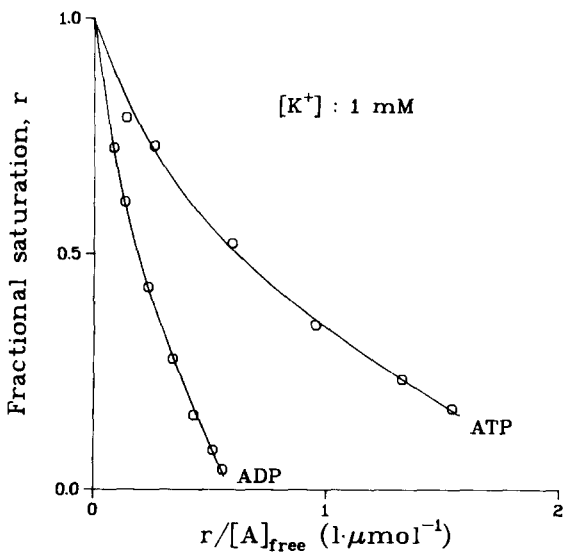
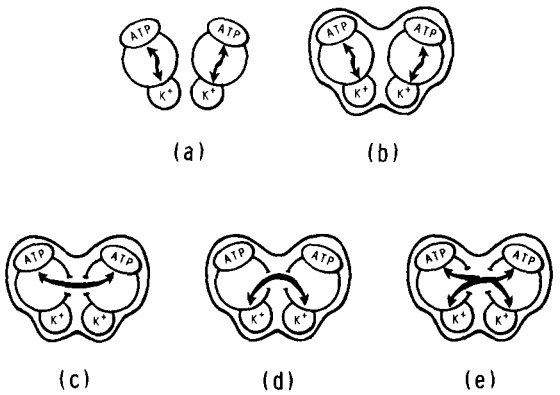


Fig. 2. Normalized Scatchard plots of ADP and ATP binding to ($Na^+ + K^+$)-ATPase in the presence of 1 mM K^+. For other relevant information, see Fig. 1. The ADP binding isotherm was obtained with enzyme that had undergone the preincubation procedure described under Methods, without the addition of ouabain. That the lines in this figure are curved indicates that K^+ has apparently induced a heterogeneity in the binding-site population of both impure (ADP curve) and pure enzyme (ATP curve). The curves have been fitted to the data by using Eqn. 2.

tions with different K^+-induced nucleotide-binding properties or that the preparation contains one nucleotide-binding enzyme with at least two nucleotide-binding subunits per molecule and where there is apparent negative cooperativity in the binding of nucleotide between at least two of these subunits. The exact mathematical description of these two cases is identical [7]. Scheme I illustrates these two general cases.



Scheme I. An illustration of the two general cases that can give rise to upward curved isotherms in Scatchard plots. The arrows show the pathway of various types of interaction between nucleotide sites and K^+ sites on enzyme molecules or their subunits. (a) Apparently identical nucleotide-binding sites on different populations of enzyme molecules having different K^+-induced nucleotide-binding properties. (b) This example shows apparently identical nucleotide-binding sites on two subunits of an enzyme molecule; the sites are affected in different manner by K^+ bound on the same subunit as the nucleotide: i.e. the subunits are independent of each other. (c), (d) and (e) are three examples of subunit-subunit interaction that can give rise to apparent negative cooperativity. The interaction can be between nucleotide sites; K^+ sites on different subunits; between a nucleotide site on one subunit and K^+ site(s) on the other subunit. Both examples (a) and (b) belong to the general case described as preparations consisting of mixtures of different and independent sites. Cases (c), (d) and (e) show examples of enzyme having two non-independent (i.e interacting) subunits, each having one nucleotide-binding site. Since K^+ decreases the apparent affinity of $(Na^+ + K^+)$-ATPase for nucleotide (and vice versa) [12,17–19], interactions between nucleotide site and K^+ site(s) within a subunit are also likely to exist, in addition to those shown for cases (c), (d) and (e).

In order to identify the cause of the curvature we (and others) have observed in Scatchard plots such as those shown in Fig. 2, we have chosen to test whether preparations of $(Na^+ + K^+)$-ATPase consist of mixtures of independent nucleotide-binding site populations differing in K^+-dependent nucleotide-binding properties (i.e. cases (a) or (b) of Scheme I).

Preliminaries to testing the hypothesis

There is very good evidence in the literature that the number of nucleotide-binding sites in ATPase preparations is identical to the number of ouabain-binding sites, and that binding of ouabain to sites on the enzyme greatly reduces the affinity for nucleotide of an identical number of nucleotide-binding sites (in the paper where this observation was originally reported [4], the decrease in affinity was seen as an apparent disappearance of sites). It is, therefore, safe to assume that ouabain and nucleotide-binding sites are structurally linked pairwise.

We will first choose experimental conditions such that all ouabain-binding sites in the preparation have identical affinity for ouabain, proceed to occupy a fraction F of these sites with ouabain and then block reversibility of this occupation. A consequence of identical affinities is the random occupation of available sites. If the preparation originally contained n nucleotide-binding sites of type N and m nucleotide-binding sites of type M, the treatment with ouabain will have left an identical fraction $(1 - F)$ of each enzyme type unlabeled. $(1 - F) \cdot n$ of sites of type N and $(1 - F) \cdot m$ of type M will not have reacted with ouabain.

We then choose experimental conditions under which nucleotide binding gives rise to curved Scatchard plots, and compare the isotherms for nucleotide binding obtained before and after reaction with ouabain as described above.

The results of this procedure will be diagnostic: any qualitative change in nucleotide-binding properties of that fraction of the population that has not reacted with ouabain would be unambiguous proof that the postulate being tested was wrong and that the curvature of Scatchard plots obtained with nucleotide-binding data was not due to a mixture of diverse and independent nucleotide-binding sites.

Before proceeding further it is necessary to demonstrate that the experimental conditions required by the test can be fulfilled in practice.

Fig. 3 shows that, under the experimental conditions used, Scatchard plots of ouabain-binding

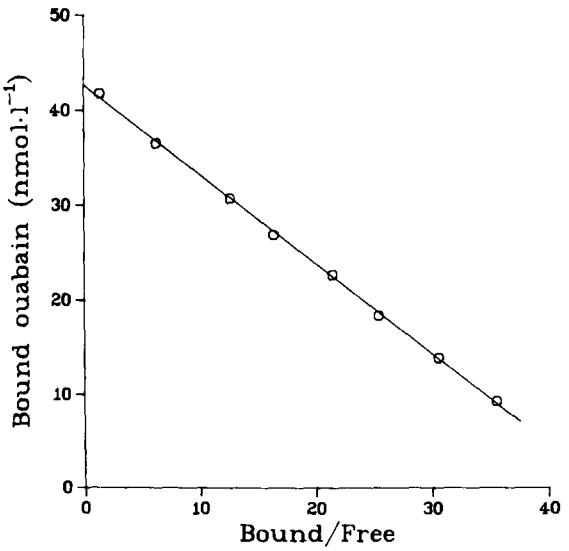


Fig. 3. Scatchard plot of ouabain-binding data obtained under conditions given in Methods. Due to its high affinity for ouabain (1 nM^{-1}), the experiment had to be performed with a lower enzyme concentration. According to the model represented in Eqn. 1, the straight line in this plot indicates that the ouabain site population is homogeneous and, therefore, that ouabain will bind randomly to the available sites. One might note than the enzyme is insoluble and membrane bound. Therefore, although the concentration of enzyme in the reaction mixture of this experiment has been reduced, its concentration in the membrane fragments of which it is a part remains unaltered.

data are straight lines and, therefore, that all available sites in the preparation have identical affinity for ouabain.

It is also important for the whole procedure that the ouabain-binding equilibrium reached in the first part of the experiments be maintained unchanged while ADP binding in the second part of the experiment is taking place. The change in experimental conditions between the first and second parts of the procedure include lowering the temperature to near 0°C, which is known to stabilize enzyme-ouabain complexes [14], and the removal of Mg^{2+} with EDTA, which precludes any further binding of ouabain. At this point in the procedure K^+ also is added to induce the required curvature of nucleotide-binding isotherms in Scatchard plots. Two hours of incubation of enzyme-ouabain complex under the conditions preparatory for ADP binding assays (i.e. 0°C, 1 mM K^+ and 9.75 mM EDTA) produced no change whatsoever in the amount of ouabain bound. The subsequent addition of ADP in concentrations higher than those used in the ADP-binding experiments did not lead to any dissociation of ouabain from its complex with enzyme during a period of time 5-times longer than that taken for an ADP-binding experiment.

Finally, since the concentration of K^+ used is very much larger than the possible concentration of K^+-binding sites on the enzyme, the concentration of free K^+ will remain virtually unchanged regardless of any effects that ouabain or nucleotide might have upon K^+ binding to ATPase.

Results of the experiments that test the hypothesis

To test our hypothesis we start with the assumption that preparations of $(Na^+ + K^+)$-ATPase consist of mixture of independent nucleotide-binding site populations that, in the presence

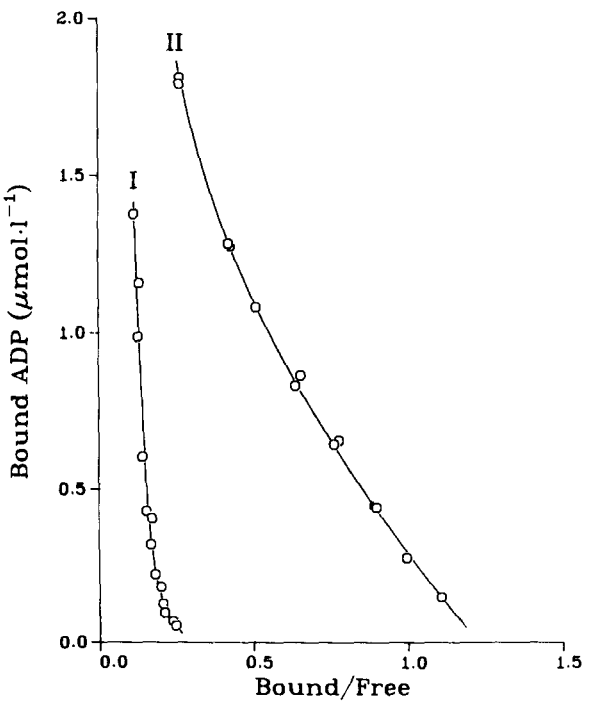


Fig. 4. Scatchard plots of ADP binding to an enzyme preparation that had all its ouabain-binding sites occupied (curve I = experiment B) and to an enzyme preparation that had 81% of its ouabain-binding sites occupied (curve II = experiment C). The data were obtained in the presence of 1 mM K^+ exactly as described under Methods.

of K^+, show differences in nucleotide binding properties (cf. cases (a) and (b) in Scheme I), and we use the three experiments described under Methods:

(A) ADP binding to normal enzyme (lower curve, Fig. 6).

(B) ADP binding to an enzyme preparations with 100% of its ouabain-binding sites occupied (curve I, Fig. 4).

(C) ADP binding to an enzyme preparation with 81% of its ouabain-binding sites occupied (curve II, Fig. 4).

The hypothesis being tested predicts that enzyme preparation of experiment C should consist of a mixture of only two types of ADP-binding sites. (i) 19% of the sites should be on enzyme

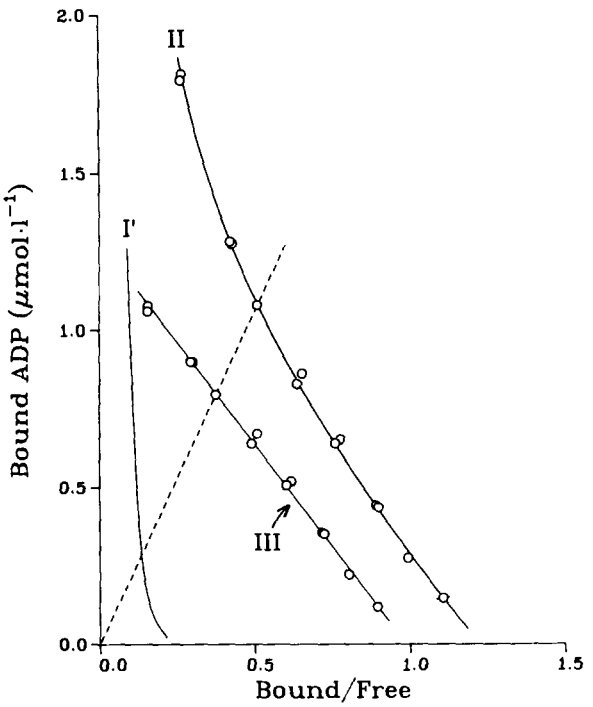


Fig. 5. A graphical representation of the method used for finding the difference between two isotherms in Scatchard plots. Curve I': isotherm fitted to the data obtained in experiment B (binding of ADP to an enzyme preparation with 100% of its ouabain-binding sites occupied), recalculated so as to correspond to an enzyme concentration equal to 81% of that used in the experiment of curve II. Curve II: Scatchard plot of the binding data obtained in experiment C (binding of ADP to an enzyme preparation with 81% of its ouabain-binding sites occupied. The two curves have the same concentration of ouabain sites that have bound ouabain. The slope of any straight line through the origin (e.g. the dashed line) represents a given concentration of free ADP, and the intercepts of different binding isotherms with any such line give values of bound ADP at the same concentration of free ADP. It is along such lines, therefore, that subtractions of values of one isotherm from another can be made [20]. If from the ADP bound at each point on isotherm II one subtracts the ADP bound on isotherm I' at the same concentration of free ADP, a series of points is obtained through which isotherm III was obtained by linear regression. Isotherm II was then calculated as the sum of isotherms I' + III.

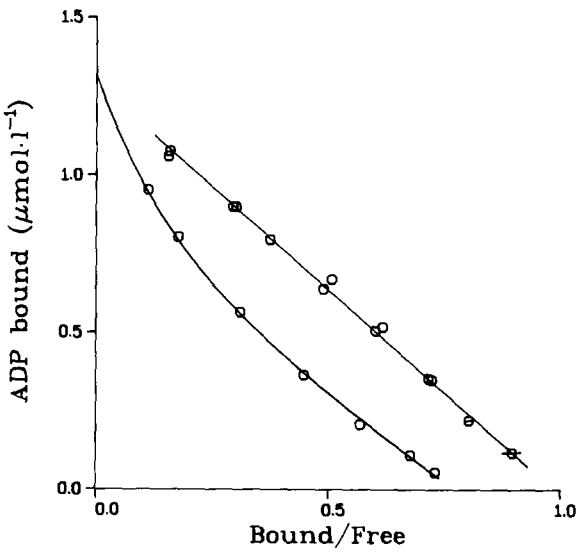


Fig. 6. Testing the hypothesis that preparations of (Na^+ + K^+)-ATPase consist of mixtures of nucleotide-binding site populations differing in nucleotide binding properties. Lower curve: the ADP-binding isotherm in the presence of 1 mM K^+ for enzyme free of ouabain (i.e. experiment A). The actual experiment was carried out with enzyme that had never seen ouabain, at an enzyme concentration and other experimental conditions identical to those used in the two experiments of Fig. 4. The curve obtained was reduced, arithmetically, to 19% of its original value: according to the hypothesis being tested this should correspond to the contribution of ouabain-free enzyme in an enzyme preparation that has 81% of its ouabain-binding sites occupied. The upper curve is a reconstruction made by starting with the ADP bound to a preparation with 81% of its ouabain sites occupied and subtracting from it the ADP bound to sites whose corresponding ouabain-binding sites were occupied by ouabain; it equals isotherm III in Fig. 5. According to the hypothesis being tested the two curves in this figure should both represent isotherms for the binding of ADP to an enzyme preparation that has not bound ouabain. The two curves extrapolate to the same value for total ADP binding capacity, but they are obviously not identical. This invalidates the hypothesis.

units where the corresponding ouabain site is free: these sites can be expected to behave exactly as the ADP-binding sites of experiment A. (ii) The remaining 81% of the ADP-binding sites should be on enzyme units where the corresponding ouabain site is occupied: they must behave exactly as the ADP-binding sites of experiment B. Hence, if from the values of bound ADP obtained in experiment C one subtracts the ADP binding contributed by (ii), one will obtain an isotherm that according to the model describes the binding of ADP to enzyme sites whose corresponding ouabain sites are free. This isotherm should, therefore, have an identical shape to that of experiment A, but with a total site concentration that is 19% of that obtained in experiment A. Fig. 5 shows how this subtraction can be carried out and the consequences of this manoeuvre are shown in Fig. 6. Since the two lines in Fig. 6 are not identical, a hypothesis with independent sites with various properties can herewith be considered as having been disposed of.

That the two curves in Fig. 6 extrapolate to the same value on the ordinate, thus giving the same total concentration of ADP-binding sites, is a clear demonstration that the experimental procedures used in establishing the binding isotherms in experiments A–C, as well as the subsequent arithmetic manipulations can only be encumbered with very small errors.

Further evidence for interaction within a molecule of ATPase: the apparent heterogeneity of ouabain-binding sites in the presence of Na^+

Straight-lined Scatchard plots of ouabain binding to $(Na^+ + K^+)$-ATPase such as the one shown in Fig. 3 are not representative of the results obtained under all conditions. Addition of Na^+ to the medium used for the binding assay causes Scatchard plots to become curved (Fig. 7). It must be significant that Na^+ can induce an apparent heterogeneity in the ouabain-binding site population while having no such effect on nucleotide binding [21].

Under the assumption that the curved Scatchard plot shown in Fig. 7 is due to the presence of two types of sites only, a mathematical analysis of the results gives as best fit a system with either (i) two identical-sized populations of independent sites or

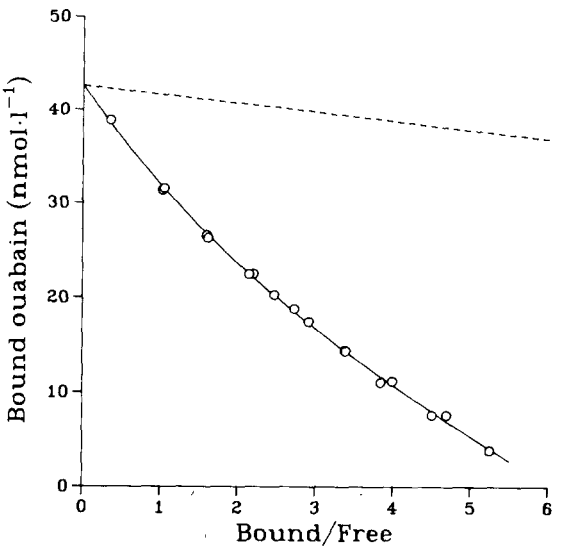


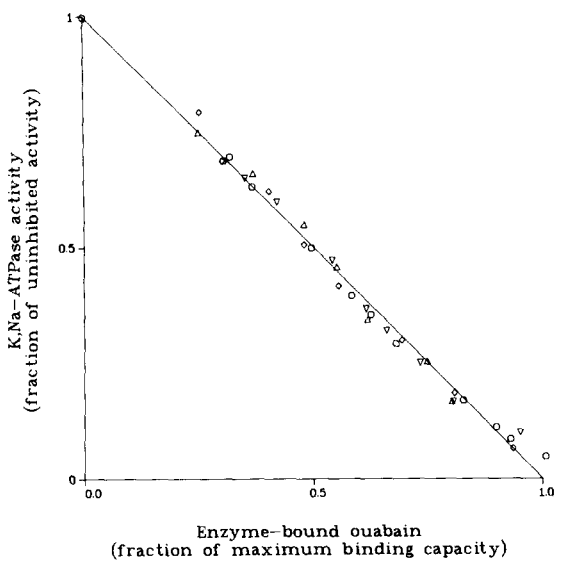
Fig. 7. Scatchard plot of ouabain-binding data obtained under the same conditions as used for the experiment of Fig. 3, except for the addition of 25 mM Na^+ to the binding medium. The curvature in the isotherm induced by Na^+ is covered by the same arguments used to explain the curved Scatchard plots of nucleotide binding in the presence of K^+. The curved line has been fitted to the data using Eqn. 2: there is a perfect fit to a model that assumes an enzyme with two identical ouabain-binding sites that show apparent negative cooperativity. The dashed line is a redrawing of a small section of the curve obtained when measuring ouabain binding in the absence of added Na^+.

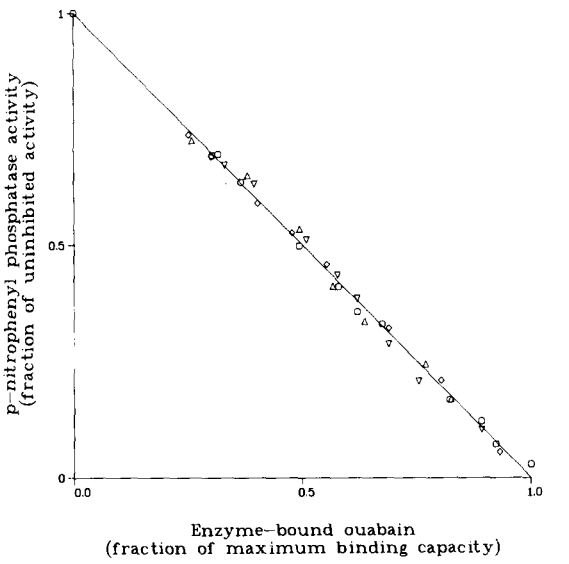
(ii) a population of molecules with two sites that show apparent negative cooperativity. Since we have shown that nucleotide sites are not independent of each other, and since nucleotide and ouabain sites show pairwise interaction [4], the conclusion that cooperativity is operative between structural units containing, not only a nucleotide site each, but also a ouabain-binding site, is strengthened.

Evidence that all hypothetical units containing one ouabain site each have identical molar activities towards ATP and p-nitrophenyl phosphate

Figs. 8 and 9 show that the inhibition of the hydrolytic activity of $(Na^+ + K^+)$-ATPase towards ATP and *p*-nitrophenyl phosphate is directly proportional to the fractional occupancy of ouabain-binding sites on the enzyme with ouabain.

Two somewhat different situations have to be





Figs. 8 and 9. Relationship between the fraction of total enzymatic activity remaining and the fractional occupation of total number of ouabain-binding sites. Fig. 8: $(Na^+ + K^+)$-ATPase activity; Fig. 9: *p*-nitrophenylphosphatase activity. The binding of ouabain was supported either by Mg^{2+} + inorganic phosphate with concentrations of Na^+ varying between 0 and 30 mM, or by $Mg^{2+} + Na^+ +$ ATP [13]. The different symbols refer to different binding conditions. The activities per ouabain site derived from these experiments were $(1.42 \pm 0.02) \cdot 10^3$ min^{-1} for *p*-nitrophenylphosphatase and $(7.26 \pm 0.14) \cdot 10^3$ min^{-1} for ATPase.

considered. One in which ouabain binds randomly to all ouabain-binding sites in a preparation, as it probably does in the experiments with straight-lined Scatchard plots; another in which ouabain binds non-randomly as in the experiment represented in Fig. 7.

A moment's reflection should show that when all ouabain-binding sites in a population are identical and their occupation occurs randomly, inhibition of enzymic activity vs. occupancy of sites will, with one exception, always show direct proportionality. The exception will arise with enzyme preparations containing one or more species of molecules with more than one binding site for ouabain where the inhibition by ouabain of the activity of each individual molecule is not proportional to the fractional occupation of the molecule's ouabain-binding sites.

When ouabain is bound to enzyme non-randomly, directly proportional changes in enzyme activity and ouabain site occupancy will arise only when all the groups of ouabain-binding sites in a preparation (each group having its specific affinity for ouabain), have identical average molar activities towards substrate.

In experiments in which ouabain was bound to the enzyme in either random or non-random fashion (representing data in Figs. 8 and 9) there is strict proportionality between site occupancy and inhibition of enzyme activity. It thus seems that each ouabain-binding site in the preparation is associated with a unit having both ATPase and *p*-nitrophenylphosphatase activities and that the molar activity of all such units is identical. No conclusions whatsoever can be drawn from these last experiments concerning the number of sites on each molecule of enzyme.

A cautionary note

Similar results relating pump rate to ouabain-site occupancy in erythrocytes have been used as proof for the argument that each pump molecule must consist of a unit containing a single ouabain-binding site [22]. On the basis of what has been said above, however, these results are not amenable to such categorical statements, even

though they may still yield some useful information about the system being studied.

Discussion

Under appropriate experimental conditions, both impure preparations of membrane-bound $(Na^+ + K^+)$-ATPase [18,21] as well as highly purified enzyme show an apparent heterogeneity in the properties of their nucleotide-binding sites. Since we have shown above that this apparent heterogeneity cannot be due to the presence in the preparations of a mixture of different and independent nucleotide-binding site populations, the only possible alternative requires that each molecule of $(Na^+ + K^+)$-ATPase contain (a minimum of) two nucleotide-binding sites that, in appearance or in fact ((c), (d) or (e) in Scheme I), show interaction with each other. It must be stressed once again that all these sites have a high affinity for nucleotide and that each is accompanied by one ouabain-binding site.

Under appropriate experimental conditions all nucleotide sites in our preparations behave as if they were identical (Fig. 1) and so do all ouabain-binding sites (Fig. 3); it appears, furthermore, that the rate of hydrolysis of substrates provoked by each of the structural units containing one ouabain site is identical. These findings give reasonable support to the idea that the enzyme preparations we have used consist of identical units each of which possesses one nucleotide site, one ouabain site and is capable of hydrolysing ATP and *p*-nitrophenyl phosphate. So as to accommodate two nucleotide sites, therefore, a molecule of the enzyme would contain at least two such units.

An obvious structural consequence of the above is that the molecular weight of the enzyme must be sufficiently large to accommodate two nucleotide-binding sites and two ouabain-binding sites.

The enzyme's capacity for binding nucleotide and ouabain and for phosphorylation from ATP has recently been claimed to have been increased to 5.6 nmol per mg of protein [23], if the protein is measured by amino acid analysis. This would make the molecular weight of the protein associated with one nucleotide and one ouabain site equal to 179 000 or less. Since the protein molecular weight of the α- and β-peptide components of the enzyme from pig kidney are about 94 000 and 32 000 [24], and since in $(Na^+ + K^+)$-ATPase there is at least one β-peptide per α-peptide [1,25,26], there must be a minimum of one nucleotide site per α-peptide. (Using these molecular weights for the α- and β-peptides and if an $(\alpha\beta)$-unit binds nucleotide, the expected nucleotide-binding capacity of the enzyme would be 7.9 nmol per mg protein; this would make the best preparations available today about 70% pure). The minimum weight of protein in a molecule of $(Na^+ + K^+)$-ATPase that can accommodate the two nucleotide sites demanded by our results is, therefore, about 250 kDa. Values very near this have been obtained with ultracentrifugal measurements made on highly purified, detergent-solubilized enzyme from shark salt gland [27] and with radiation inactivation of impure, membrane-bound enzyme from pig kidney and brain [28,29]. Further evidence for the possible dimeric structure of the enzyme has come from electron micrography and from protein cross linking studies. The former has shown that highly purified, fully functional and membrane embedded enzyme always appears as clusters of two or more units considered to represent α-peptides [30]. The latter have demonstrated the proximity between pairs of α-peptides in membrane-bound ATPase [31–33].

Since at present there is no evidence of heterogeneity in the α-peptide component of mammalian kidney ATPase, the results of the present report are compatible with (but do not claim to prove) the following structural and functional properties of $(Na^+ + K^+)$-ATPase from pig kidney.

(1) Enzyme prepared according to the procedures used in this study as well as in the much purer preparations of Jørgensen [8], are homogeneous with respect to the molecules of $(Na^+ + K^+)$-ATPase they contain.

(2) Each enzyme molecule contains two subunits, each containing one α-peptide and each containing one nucleotide-binding site and one ouabain-binding site. This is a minimum but absolute requirement of the present results.

(3) The microscopic affinity constants for the binding of nucleotide to the two sites on an enzyme molecule are, a priori, identical. Under appropriate conditions, the occupation of either site with nucleotide decreases the apparent affinity

constant for nucleotide of the remaining site. Such an effect of ligand site occupancy on ligand affinity would be diagnosed as 'negative cooperativity'. However, the term is used here only to imply the existence of an interaction between the subunits containing the nucleotide sites, without further specification as to the type of interaction that is involved. This subject will be dealt with in a separate communication.

(4) The microscopic affinity constants for the binding of ouabain to the two sites on an enzyme molecule are, a priori, identical. Under appropriate conditions, the occupation of either site with ouabain decreases the affinity constant for ouabain of the remaining site. This 'negative cooperativity' is defined within the same bonds used under point 3.

(5) Enzyme that has bound two molecules of ouabain is still capable of binding two molecules of nucleotide, but does so with much reduced affinity *.

(6) All subunits hydrolyze ATP at identical rates. All subunits hydrolyze *p*-nitrophenyl phosphate at identical rates.

(7) Binding of ouabain to one site on an enzyme molecule decreases the rate of the hydrolysis of substrates by the enzyme molecule by 50%.

Although we have used pig kidney ATPase, the above conclusions will probably be valid also for enzyme from other sources. Enzyme from ox brain grey matter [13,18,21] and from dogfish rectal glands [34], can also show upward concave. Scatchard plots with both nucleotide binding and ouabain binding. Since the enzyme prepared from tissue obtained from whole brain has been shown to be heterogeneous with respect to protein composition [6], at least a part of the site heterogeneity observed in binding experiments carried out with grey matter may be real rather than only apparent.

If the structure of $(Na^+ + K^+)$-ATPase in preparations like those used in the present work is dimeric, one might ask whether such a structure is inevitable. The answer, apparently, is: no. The molecular weight of enzyme dissolved in detergent and retaining its enzymatic activity has been found to be 170 000, or less [35]. This value cannot accommodate a dimer. Also, recent electronmicrographic studies of (presumably enzymatically inactive) two-dimensional crystalline structures in membranous preparations of pure $(Na^+ + K^+)$-ATPase have shown that both monomeric [36] and dimeric [37] unit cells exist.

From what has been said under points 6 and 7 above, each subunit in our preparations appears to have enzymatic activities independently of the other subunit in a molecule of enzyme. This, added to the suggested existence of both monomeric and dimeric enzymatically active forms of the enzyme prompts another question: Does the dimeric structure of the membrane have a purpose? Some implications of such a structure in the function of transport proteins have recently been discussed at length by Klingenberg [38], but if the model proposed in the present communication is to have functional significance for $(Na^+ + K^+)$-ATPase, it will be crucial to come up with a specific answer to this question.

Acknowledgements

We wish to acknowledge the painstaking technical assistance of Edith B. Møller, the computation help of Toke Nørby and the criticism of Jens Nørby. A part of this investigation was funded by the Danish Medical Research Council.

References

1 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53–67
2 Jensen, J. and Nørby, J.G. (1979) in Na, K-ATPase, Structure and Kinetics (Skou, J.C. and Nørby, J.G., eds.), pp. 443–452, Academic Press, London
3 Hansen, O., Jensen, J., Nørby, J.G. and Ottolenghi, P. (1979) Nature 280, 410–412
4 Hansen, O., Jensen, J. and Nørby, J.G. (1971) Nature New Biol. 234, 122–124
5 Nørby, J.G. (1983) Curr. Top. Membranes Transp., in the press
6 Sweadner, K.J. (1979) J. Biol. Chem. 254, 6060–6067
7 Klotz, I.M. and Hunston, D.L. (1979) Arch. Biochem. Biophys. 193, 314–328
8 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52

* Enzyme that has bound one molecule of ouabain has two nucleotide-binding sites that are different. When these sites are both unoccupied, one site had an affinity for nucleotide similar to that of sites on ouabain-free and nucleotide-free enzyme while the other site has an affinity similar to that of sites on nucleotide-free but ouabain-saturated enzyme (unpublished observation).

9 Nørby, J.G. (1971) Acta Chem. Scand. 25, 2717–2726
10 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 258–273
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
12 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116
13 Hansen, O. (1976) Biochim. Biophys. Acta 433, 383–392
14 Tobin, T. and Sen, A.K. (1970) Biochim. Biophys. Acta 198, 120–131
15 Joiner, C.H. and Lauf, P.K. (1975) J. Membrane Biol. 21, 99–112
16 Colowick, S.P. and Womack, F.C. (1969) J. Biol. Chem. 244, 774–777
17 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234–5240
18 Schoner, W., Pauls, H. and Patzelt-Wenczler, R. (1977) in Myocardial Failure (Riecker, G., Weber, A. and Goodwin, J., eds) pp. 104–119, Springer-Verlag, Berlin
19 Jensen, J. and Ottolenghi, P. (1983) Curr. Top. Membranes Transp., in the press
20 Rosenthal, H.E. (1967) Anal. Biochem. 20, 525–532
21 Nørby, J.G. and Jensen, J. (1974) Ann. N.Y. Acad. Sci. 242, 158–167
22 Joiner, C.H and Lauf, P.K. (1978) J. Physiol. 283, 155–175
23 Peters, W.H.M., Swarts, H.G.P., de Pont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1981) Nature 290, 338–339
24 Freytag, J.W. and Reynolds, J.A. (1981) Biochemistry 20, 7211–7214
25 Craig, W.S. and Kyte, J. (1980) J. Biol. Chem. 255, 6262–6269
26 Peterson, G.L. and Hokin, L.E. (1981) J. Biol. Chem. 256, 3751–3761
27 Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim. Biophys. Acta 567, 410–420
28 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203
29 Ottolenghi, P., Ellory, J.C. and Klein, R. (1983) Curr. Top. Membranes Transp., in the press
30 Deguchi, N., Jørgensen, P.L. and Maunsbach, A.B. (1977) J. Cell. Biol. 75, 619–634
31 Kyte, J. (1975) J. Biol. Chem. 250, 7443–7449
32 Giotta, G.J. (1976) J. Biol. Chem. 251, 1247–1252
33 Liang, S.M. and Winter, C.G. (1977) J. Biol. Chem. 252, 8278–8284
34 Jensen, J. and Ottolenghi, P. (1976) Biochem. J. 159, 815–817
35 Brotherus, J.R., Møller, J.V. and Jørgensen, P.L. (1981) Biochem. Biophys. Res. Commun. 100, 146–154
36 Skriver, E., Maunsbach, A.B. and Jørgensen, P.L. (1981) FEBS Lett. 131, 219–222
37 Hebert, H., Jørgensen, P.L., Skriver, E. and Maunsbach, A.B. (1982) Biochim. Biophys. Acta 689, 571–574
38 Klingenberg, M. (1981) Nature 290, 449–454