A Conformational Transition of the Sarcoplasmic Reticulum Calcium Transport ATPase Induced by Vanadate

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Vanadate binding to sarcoplasmic reticulum vesicles results in the loss of the externally located high affinity calcium binding sites of the calcium transport ATPase. Conversely the occupation by calcium of the internally located low affinity sites in the vanadate enzyme complex leads to the release of vanadate. Since the total number of calcium binding sites is not diminished by vanadate binding but slightly increases we conclude that vanadate binding induces a transition of the enzymes external high to internal low affinity calcium binding sites. The transposition of external to internal calcium binding sites is accompanied by a definite change in the structure of the sarcoplasmic reticulum membranes. On vanadate binding the asymmetrically arranged electron dense protein particles become symmetrically distributed.

Introduction

In contrast to many ATP hydrolyzing enzymes, the calcium transport ATPase of native sarcoplasmic reticulum vesicles is inhibited by vanadate only if applied at relatively high concentrations [1, 2]. The observations made by O'Neal *et al.* that in the presence of the calcium ionophore A 23187 vanadate becomes much more effective lead to the suggestion that calcium bound to sites located in the internal membrane leaflet of the veiscles might counteract vanadate inhibition [2]. It is well established that the calcium transport ATPase is equipped with high affinity calcium binding which are located on the external surface of the sarcoplasmic reticulum vesicles [3, 4]. Calcium dependent phosphoprotein formation, ATP hydrolysis and calcium transport depend on the occupancy of these high affinity sites [5–8]. On the other hand, low affinity calcium binding sites have been found on the external as well as on the internal surface of the membrane [9, 10]. Calcium translocation during ATP dependent calcium accumulation has been proposed to be brought about by the transformation of high to low affinity sites in connection with their translocation from outside to inside [11]. The occupation of these internally located sites results in a severe inhibition of calcium transport and ATP hydrolysis [12]. This report deals with the mutual interaction of calcium and vanadate with the calcium transport ATPase. Vanadate binding to different sarcoplasmic reticulum membrane preparations was determined by measuring bound vanadate colorimetrically and by phosphorylating the vanadate free enzyme fraction with [^{32}P-γ-]ATP. The latter methods exploited the slowness of vanadate binding and dissociation as compared to phosphoprotein formation [13, 14]. The results of vanadate binding measurements at equilibrium and under initial rate conditions indicate that vanadate binding leads to a transition of external high to internal low affinity calcium binding sites.

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Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle [15, 16]. Calcium permeable preparations were obtained by supplementing the assay media with 10 µM of the calcium ionophore A 23187. Calcium binding was measured by separating the protein from the solution by filtration through Satorius filters [3]. Vanadate binding was performed in media containing 40 mM KCl, 20 mM imidazole pH 7, 5 mM $MgCl_2$, and 1 mM EGTA. The colorimetric determination of bound vanadate was performed with the metalochromic dye [17]. The vanadate free protein fraction was phosphorylated with [^{32}P-γ-]ATP in the presence of 0.1–0.3 mM free calcium [18]. For the calculation of the concentration of ionized calcium the stability constant of Ca-EGTA given by Schwarzenbach [19] was used.

Electron microscopy studies were performed with ultrathin sections and replicas of control and vanadate treated samples. For the preparation of ultrathin sections, loose pellets of sarcoplasmic reticulum vesicles were fixed in 2.5% glutaraldehyde in 0.06 M sodium cacodylate buffer (pH 7.3) for 2 hours at 4 °C and postfixed in 1% OsO_4 in distilled water for 90 min. Some samples were fixed with a glutaraldehyde solution as above supplemented with 1% tannic acid [20] and postfixed with OsO_4 as above. All specimens were block stained with 0.5% uranyl acetate in distilled water for 1 h, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were stained with 5% acqueous uranyl acetate [21] for 20 min and with lead citrate [22] for 5 to 10 min, or with lead citrate alone.

For the preparation of replicas, sarcoplasmic reticulum vesicles added with 30% glycerol were frozen in liquid Freon-22, fractured, etched for 1 min at −100 °C, and unidirectionally or rotary shadowed at angles varying from 25° to 40° with platinum carbon and carbon, using a Balzers BAF 301 device equipped with a rotary cold stage. Pictures were taken in a Siemens Elmiskop 101 electron microscope at magnifications ranging from X 20000 to 40000. Membrane thickness, and size and distribution of electron dense membrane particles were first evaluated by conventional visual inspection at a 400000-fold magnification of 40 ultrathin sections from control and vanadate treated preparations.

Subsequently the membrane of 5 representative perfectly cross cut control and 5 vanadate treated vesicles were scanned using a digital TV-Camera connected to a light microscope (Mess-Camera, Bosch, Darmstadt).

Photographic plates obtained by electron microscopy at an original magnification of ×40000 were first enlarged 25 fold in the light microscope, and the 2 fold with the above television camera; density profiles of the membrane layers were measured at a final magnification of about ×2000000, using a digital image evaluation system [23].

To obtain reasonable average of membrane thickness each of the selected vesicles was scanned at 10 different regions.

Results and Discussion

The titration of the enzyme's calcium binding sites in the presence and absence of vanadate reveals the involvement of the enzyme's high affinity binding sites in its reaction with vanadate. In the presence of 0.5 mM vanadate the high affinity sites completely disappear (Fig. 1).

Conversely, vanadate is displaced from the enzyme when calcium is added to the vanadate binding assay containing 0.1 mM vanadate and 1 mM EGTA. Half desaturation is reached at approximately 1 μM free calcium indicating the occupation of the enzyme's high affinity sites (Fig. 2). Calcium and vanadate binding in these experiments were performed under equilibrium conditions, *i.e.* after an incubation time of 20–30 min at room temperature. These conditions must prevail in order to demonstrate the involvement of the enzyme's high affinity calcium binding sites in its interaction with vanadate. The measurement of bound vanadate by the applied colorimetric procedure (Fig. 2, ●—●) is made difficult by the fact that vanadate in contrast does not form an acid stable complex with the enzyme and therefore cannot be trapped by acid quenching. This difficulty has been circumvented by measuring the vanadate free enzyme fraction by phosphorylating it with [^{32}P-γ-]ATP in the presence of 0.1–0.3 mM calcium. This indirect procedure could be applied due to the fact that the rate of phosphorylation is much higher than the rate with which vanadate is displaced from the enzyme by calcium [18].

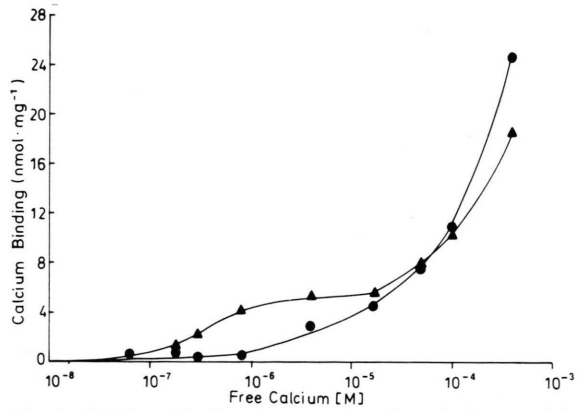


Fig. 1. Calcium binding to sarcoplasmic reticulum vesicles in the absence and in the presence of 0.5 mM vanadate. The assay contained 40 mM KCl, 10 mM imidazole pH 7, 5 mM $MgCl_2$ and 0.27 mg protein/ml. After an incubation period of 20 min with $^{45}CaCl_2$ at concentrations as indicated on the abscissa. Calcium binding was determined at room temperature, 20±2 °C, by filtration as described in "Material and Methods". (▲) Calcium binding in the absence of vanadate; (●) calcium binding in the presence of 0.5 mM vanadate.

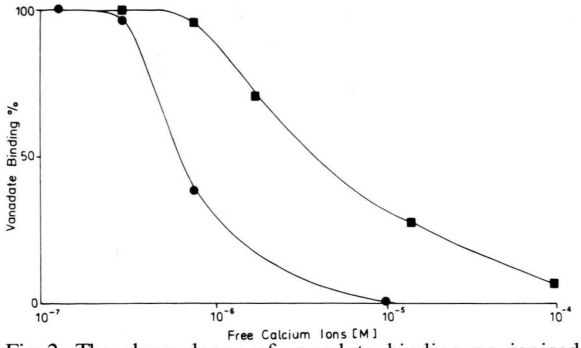


Fig. 2. The dependence of vanadate binding on ionized calcium as determined by colorimetry and phosphorylation of the vanadate free protein. 0.2 mg protein/ml of sarcoplasmic reticulum vesicles were incubated for 20–30 min at room temperature in standard media containing 0.1 mM sodium vanadate and free calcium as indicated on the abscissa. Bound vanadate was determined by the colorimetric procedure (●) as well as by phosphorylating the vanadate free protein fraction (■) by the simultaneous addition of 0.1 mM [^{32}P-γ-]ATP and 0.1–0.3 mM $CaCl_2$. Phosphorylation was terminated after 2 s.

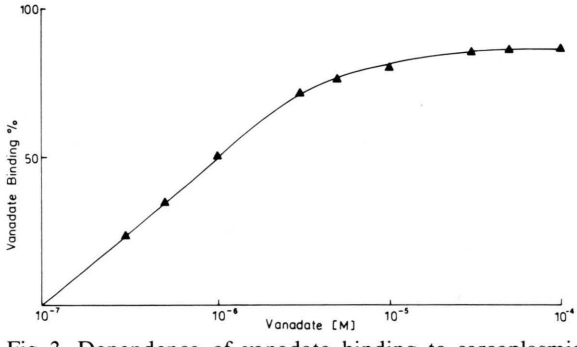


Fig. 3. Dependence of vanadate binding to sarcoplasmic reticulum vesicles on the concentration of vanadate at equilibrium. 0.2 mg protein/ml of sarcoplasmic reticulum vesicles were incubated for 20 min at room temperature in standard media containing 10 μM A 23187 and vanadate concentrations as indicated on the abscissa. 1.3 mM $CaCl_2$ and 0.1 mM [^{32}P-γ-]ATP were added simultaneously. Phosphorylation was terminated after 1–2 s by acid quenching.

Fig. 2 (■—■) shows the displacement of vanadate from the enzyme by calcium monitored by the phosphorylating procedure. – It yields somewhat higher calcium concentration for vanadate displacement than the colorimetric method.

Phosphorylation allows not only to monitor vanadate binding under equilibrium conditions but also to follow the time course of vanadate binding as well as vanadate displacement. Under equilibrium conditions the binding isotherm shown in Fig. 3 was obtained. The analysis of the data yielded an affinity

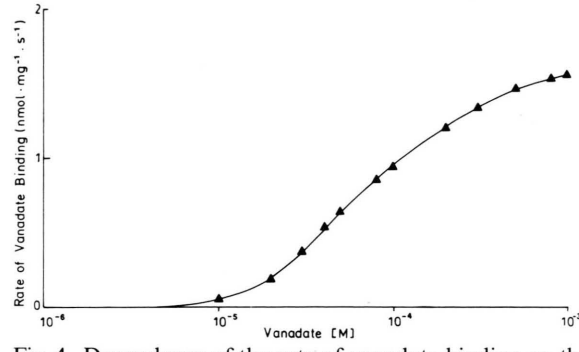


Fig. 4. Dependence of the rate of vanadate binding on the concentration of vanadate to calcium permeable sarcoplasmic reticulum vesicles. 0.2 mg protein/ml of sarcoplasmic reticulum vesicles were assayed in standard media containing 10 μM A 23187 at room temperature. Vanadate binding was started by the addition of vanadate as indicated on the abscissa and terminated after 3 s by the simultaneous addition of 1.3 mM $CaCl_2$ and 0.1 mM [^{32}P-γ-]ATP. The phosphorylation was then terminated after 2 s by acid quenching.

of the enzyme for vanadate of 1.6×10^6 M^{-1} [18]. On the other hand, from the rate concentration relation of vanadate binding an apparent dissociation constant of 0.1 mM results which is more than two orders of magnitude higher than that found under equilibrium condition (Fig. 4). Evidently vanadate like phosphate interact with the enzyme in a multistep reaction sequence in which the final complex is considerably more stable than that initially formed. The dissociation of the vanadate complex induced by calcium was followed in time by phosphorylating the vanadate free enzyme. Addition of calcium at 1 μM to 0.1 mM concentrations as they are needed to saturate the enzyme's high affinity sites only induces a very slow release of vanadate. The rate of vanadate release starts to increase considerably when the calcium concentration added to the medium exceed 0.1 mM. Fig. 5 shows that the rate concentration dependence displays saturation kinetics which is characterized by an apparent dissociation constant of 2 mM. This constant is characteristic for the enzyme's low affinity calcium binding sites. Its value only little depends on the temperature while the rate of release characterized by an higher temperature coefficient of 3.5. Fig. 5 further demonstrates that vanadate is much more slowly displaced from closed than from calcium permeable vesicles or ATPase preparations. This finding suggests that the calcium binding sites which must be occupied in the initial step of the elimination reaction are not accessible

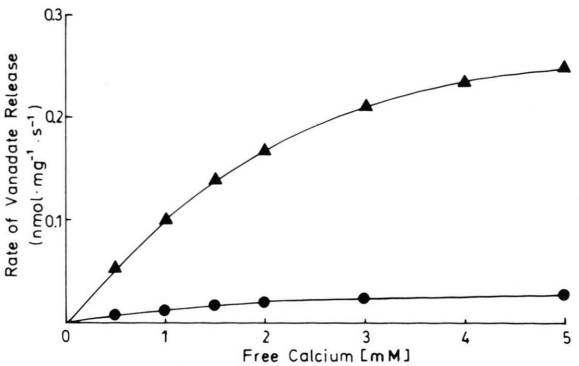


Fig. 5. Dependence of the rate of vanadate release from open and closed sarcoplasmic reticulum vesicles on the concentration of calcium at 0 °C. 0.2 mg protein/ml of sarcoplasmic reticulum vesicles were allowed to react with 0.1 mM sodium vanadate in standard media at room temperature for 2 min. The vesicular suspension was then cooled to 0 °C for 10 min and vanadate release was initiated by the addition of calcium at concentrations as indicated on the abscissa. After 10 s phosphorylation was started with 0.1 mM [^{32}P-γ-]ATP and terminated after 2 s by acid quenching. (●) Closed native vesicles; (▲) native vesicles in the presence of 10 μM A 23187.

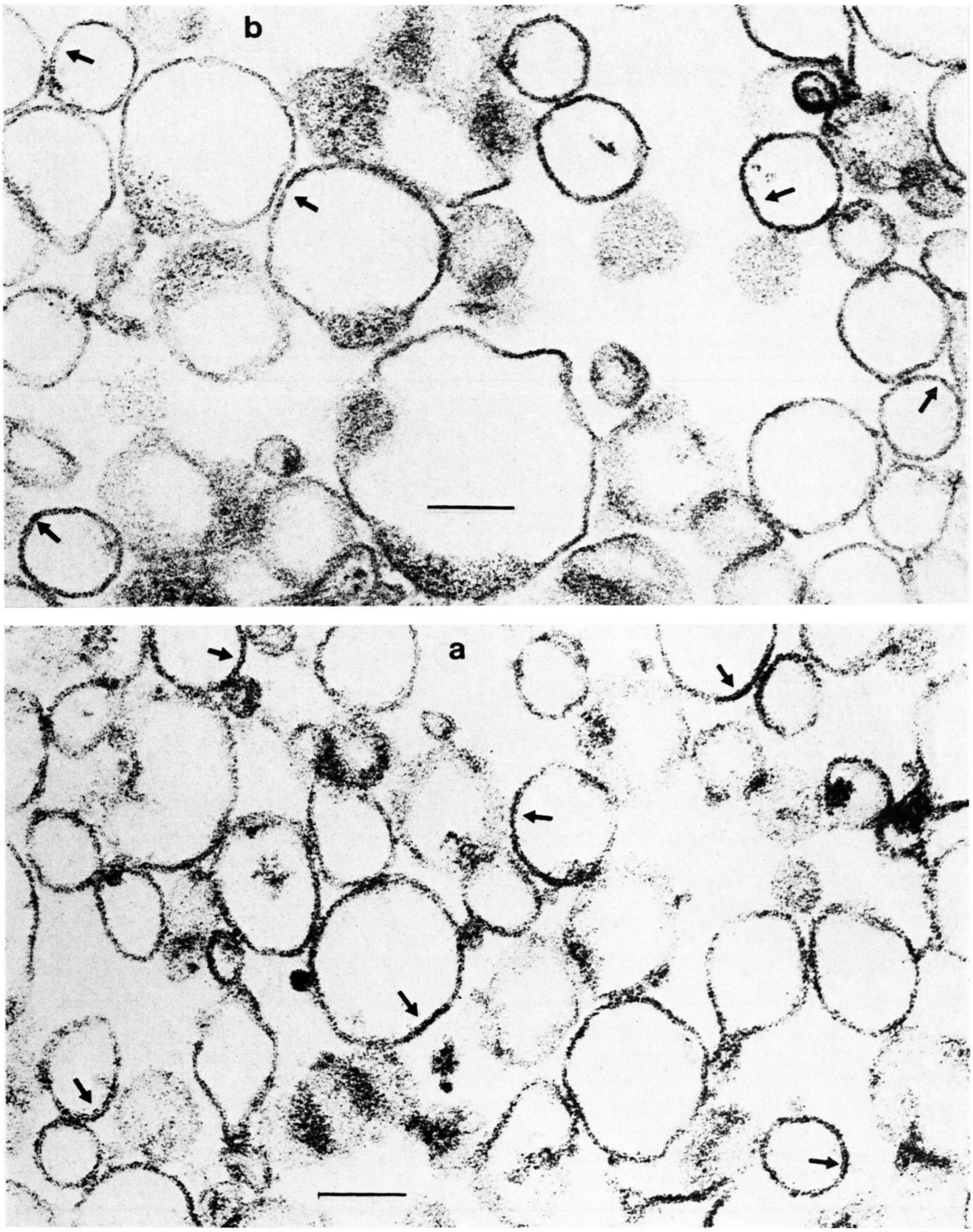
from the external medium. If the displacement of vanadate from closed vesicles is started by simultaneously adding calcium and the calcium ionophore A 23187, the rates of displacement reach values indistinguishable from those of preparations made calcium permeable in advance. The fact that in the absence of the ionophore calcium applied to closed sarcoplasmic reticulum vesicles is only little effective is consistent with the vanadate induced disappearance of the external high affinity sites. Their transposition to internal low affinity sites following calcium depletion and vanadate binding is a most attractive hypothesis. It is in line with the mostly applied models assuming a calcium dependent conformational transition of the free enzyme as an essential step of the reaction mechanism. However, we cannot exclude that the internal low affinity calcium binding sites, the occupation of which is followed by vanadate dissociation, belonged to the sites of low affinity which are not directly involved in calcium translocation but which are also located at the internal section of ATPase molecule. Yet, the notion that the high affinity sites are transformed into low affinity sites is supported by the finding that in media containing 0.5 mM calcium, vanadate binding does not diminish but rather enhances calcium binding (Fig. 1). This indicates that the high affinity sites which had been abolished by vanadate binding emerge at least partially, as additional low affinity sites.

If this explanation is valid, vanadate binding would stabilize a reaction intermediate which permanently arises and breaks down during active calcium transport. The stabilization of an active enzyme intermediate could facilitate the detection of structural changes attributable to enzyme activity. Recently, Blasie *et al.* succeeded in detecting changes in the enzyme's structure when highly ordered membrane preparations were successively activated by light induced liberation of ATP from caged ATP included into the specimen [24]. The minute changes in the small angle diffraction diagram were interpreted as a deeper immersion into the lipid bilayer of the enzyme accompanied by a small lateral expansion. These structural changes remain below the resolution limit of the electron microscope. In contrast, vanadate binding induces changes in the membrane structure that can be recognized in the electron microscope.

The electron micrographs of thin sectioned preparations show that the characteristic asymmetric arrangement of the 25–30 Å electron dense membrane particles on the external leaflet of conventional preparations (Fig. 6 a) disappears when the vesicles were treated with 0.1–0.3 mM vanadate in the presence of 0.1 mM EGTA (Fig. 6 b), so that the membrane displays a rather symmetric feature. At higher magnification (Fig. 7 b–d, arrows) the external and internal vesicular leaflet of these preparations appear equally and uniformly made up with electron dense globules about 15–20 Å in diameter; they are smaller than those of the outer leaflet and larger than those of the internal leaflet of control vesicles (Fig. 7 a, arrow and arrowhead, respectively). At some places dots of inner layer appear even larger than in the outer one (Fig. 7 b and c, double arrows), probably depending on orientation of sectioning. Membranes treated with vanadate appear thinner than in the control.

These changes observed by conventional visual inspection of electron micrographs were documented by results of scanning at very high magnification of perfectly cross-cut ultrathin sections.

Tracings of membrane thickness represented in Fig. 8 definitely show the equal and symmetric distribution of electron dense material within the two osmiophile leaflets and the reduced thickness of vesicle membrane after treatment with vanadate.



Figs. 6–8. Electron micrographs of ultrathin epon sections through pellets of sarcoplasmic reticulum vesicles fixed with glutaraldehydeosmium. Uranyl acetate and lead citrate stain. Bars mean 0.1 nm.

Fig. 6. Survey pictures of a control (a) and a vanadate treated preparation (b). The tripled layered asymmetric feature of control vesicular membrane (arrows) is lacking on vanadate treated vesicles, the membrane of which displays a rather symmetric arrangement (arrows) × 140000.

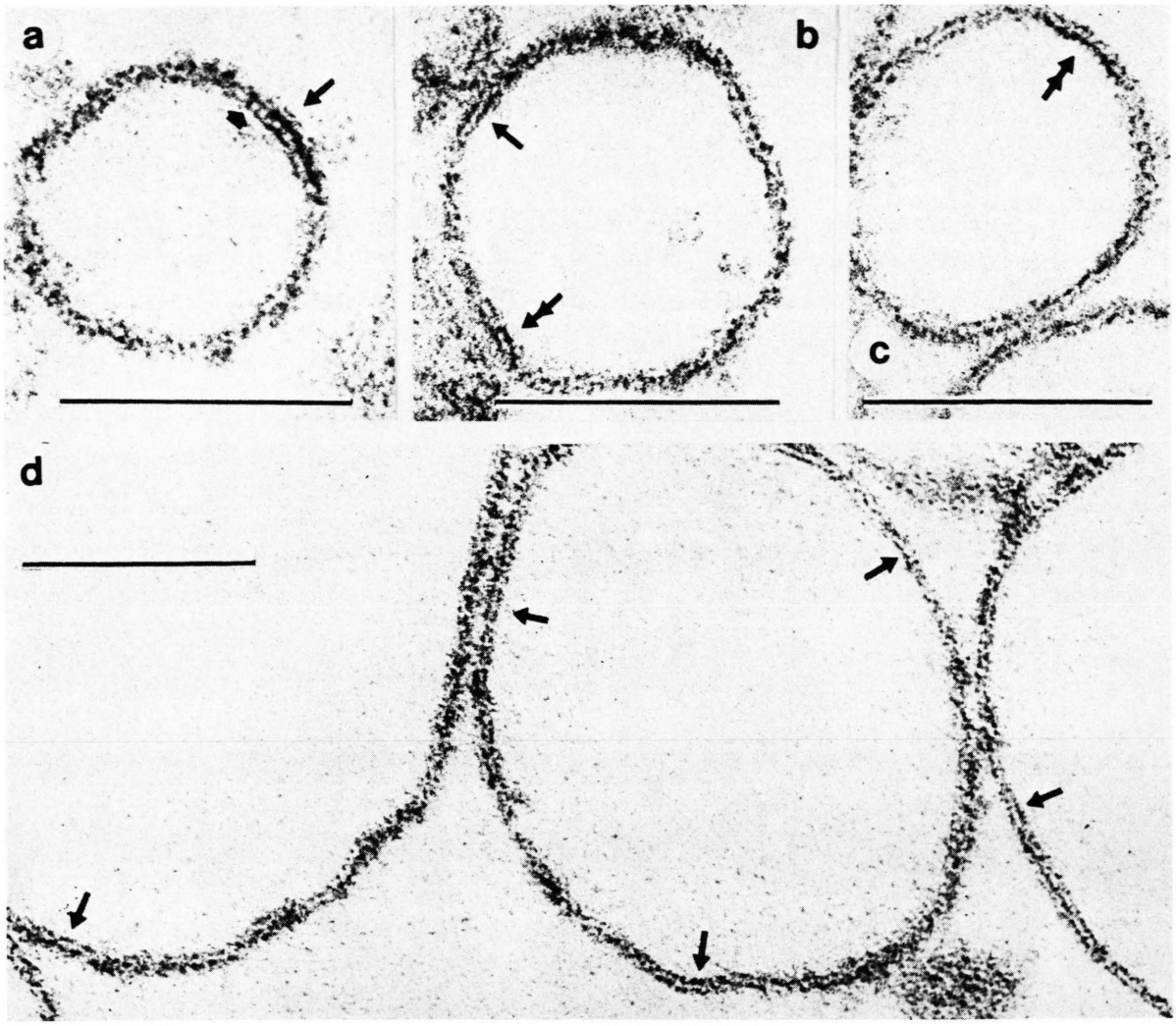


Fig. 7. Higher magnification of representative micrographs of control (a) and vanadate treated vesicles (b–d). The membrane of the control vesicle (a) shows a clear asymmetry due to the presence of dense spots about 25–30 Å in diameter on the outer rim arrow and of smaller dots about 10–15 Å in diameter on the inner leaflet (arrowhead). After treatment with vanadate (b–d) both outer and inner leaflet of vesicle membrane, which at this high magnification appear definitely symmetric, display dense dots about 15–20 Å in diameter (arrows); at some places dots of inner leaflet are even larger than in the outer one (double arrows). Vanadate treated membrane appears somewhat thinner than in the control. a–c: ×400000; d: ×320000.

The widths of membrane trilayer measured from the inside to the outside of the vesicles on 50 tracings like those represented in Fig. 8 were about 22, 20, 38 Å (*i.e.* a total membrane thickness of about 80 Å) in the control, and respectively 25, 20, 25 Å (*i.e.* a total membrane thickness of about 70 Å) after treatment with vanadate. These values would correspond to a reduction of membrane width by vanadate of about 12%. This is probably due to a partial dislocation of membrane protein from the external to the internal leaflet as well as to a more regular distribution of part of it within the outer leaflet after disaggregation of original electron dense globules by vanadate.

Since the width of the electron thin layer within the unit membrane appeared unchanged after application of vanadate, the symmetry of the membrane was also proved by measurement of its inner

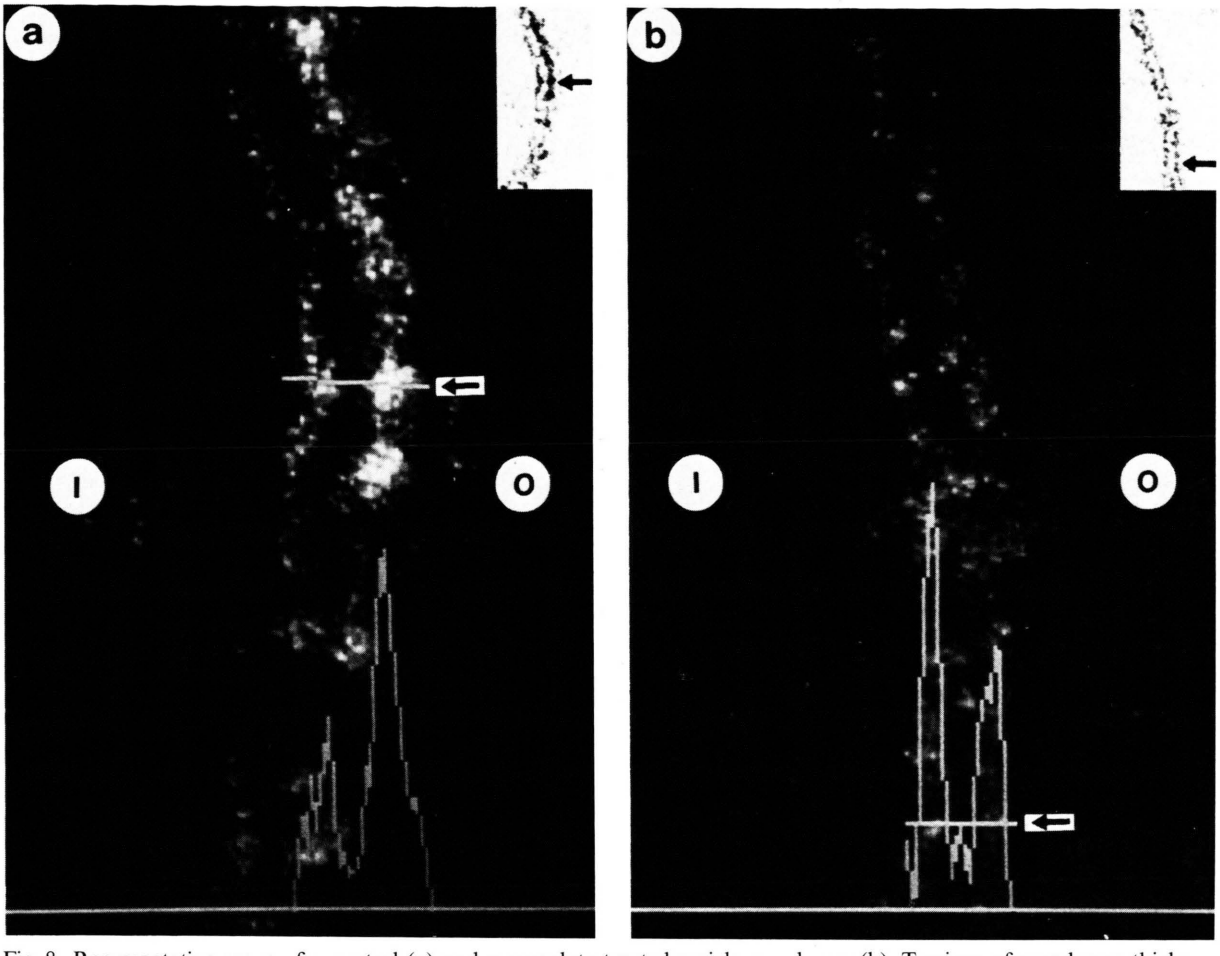


Fig. 8. Representative scans of a control (a) and a vanadate treated vesicle membrane (b). Tracings of membrane thickness are shown on negative photographs as obtained at very high magnification from the densitometer. Scanned regions of cross sectioned membranes are indicated by arrows on negative (main pictures) and corresponding positive (insets at lower magnification) membrane prints. Scans are shown from the inside (I) to the outside (O) of the vesicles. Asymmetric and symmetric structure of control (a) and respectively vanadate treated membrane (b) are well outlined. Reduction of membrane thickness in the latter is also evident. Magnification of negatively printed photographs is about ×2000000; insets: ×400000.

and outer part from the centre of the electron thin layer. In control vesicles the thickness of the inner half of the membrane (inner opaque leaflet plus half of the light layer) is only about 65 ± 12% of that of the outer counterpart (peripherical opaque leaflet plus half of the light layer); in vesicles treated with vanadate the inner half of the membrane has a width quite equal (100 ± 12%) to that of the outer half. The electron dense in the native and in the vanadate treated membrane most likely represent the transport enzyme molecule since it comprises 70–80% of the membrane's protein matrix and the globular pattern is preserved after lipid deprivation [25]. The asymmetric distribution of the transport protein was first seen in osmium fixed sectioned material [26]. Subsequently, this arrangement was confirmed by applying various other electromicroscopic techniques as well as small angle diffraction analysis [27, 28]. The proteinaceous nature of the particles protuding from the external membrane surface was established by their reaction with various electron dense protein reagents and by their susceptibility to trypsin digestion [29]. The latter treatment results in the removal of the membrane

particle seen in negatively stained preparations and to the appearance of a symmetric bilayer in sectioned material. In this case, symmetry arises from the partial removal of protein from the membrane and not from a change in its protein structure. The vanadate induced change in the distribution of the membranes' particles are not related to the appearance of two dimensional membrane crystals observed in vanadate treated membranes [30]. Crystal formation requires prolonged incubation of the membrane in media containing high concentration of vanadate. This procedure results in an irreversible inactivation of the enzyme. The expectation that the vanadate induced membrane rearrangement could also be seen in freeze fraction preparations was not met. In vanadate treated preparations we found only occasionally an increase in the number of particles on the inner leaflet of the membranes.

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