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RECONSTITUTION OF ($Na^+ + K^+$)-ATPase INTO PHOSPHOLIPID VESICLES WITH FULL RECOVERY OF ITS SPECIFIC ACTIVITY

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(1) ($Na^+ + K^+$)-ATPase from rectal glands of the spiny dogfish has been reconstituted into phospholipid vesicles. The nonionic detergent octaethyleneglycoldodecyl monoether ($C_{12}E_8$) is used to dissolve both the enzyme and the lipids and reconstitution is accomplished by subsequent removal of the detergent by adsorption to polystyrene beads. (2) About 60% of the enzyme incorporates in the right-side-out orientation (r/o). The fraction of molecules in the inside-out orientation (i/o) increases from about 10% to about 30% with a parallel decrease in the fraction of 'non-oriented' (n-o) molecules (both sides exposed) when the protein/lipid ratio decreases from 1:10 to 1:75. (3) The orientation of enzyme molecules detected from vanadate binding is the same as measured from activity, i.e., the turnover of the enzyme molecule in the diffrent orientations is the same. (4) The recovery of the specific activity of the incorporated enzyme increases with an increase in the protein/lipid ratio and is 100% with a protein/lipid ratio of about 1:20 or higher. Full recovery is only obtained provided a proper lipid composition is chosen which includes both negatively charged phospholipids, preferably phosphatidylinositol, and cholesterol. (5) The ATP-dependent, K^+-stimulated Na^+-influx is found to be about 35 μmol Na^+ per mg (i/o)-protein per min at 22°C in 1:10 protein/lipid liposomes. The specific activity corresponds to 3 Na^+ transported per ATP molecule hydrolyzed.

Introduction

Incorporation of the purified ($Na^+ + K^+$)-ATPase into the membrane of closed phospholipid vesicles (reconstitution) with reestablishment of the sidedness of the enzyme [1–3] reveals new possibilities for investigation of the Na^+/K^+-transport system.

In order to exploit this possibility fully reconstitution of the enzyme without loss of activity is required. So far this has not been accomplished with the reported methods used for reconstitution. The recovery of activity of the reconstituted enzyme ranges from 5 to 40%, with a corresponding low active Na^+-flux [4–9]. This may in part be due to an inactivating effect of the detergent used for solubilization and in part to problems with measurements, of the enzyme activity and ion fluxes in the reconstituted system.

In order to measure the recovered activity of the incorporated enzyme it is necessary to reopen the vesicles with the aid of a detergent. However, a

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; $C_{12}E_8$, octaethyleneglycoldodecyl monoether; DOC, sodium deoxycholate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

problem is to find a detergent which ensures the opening of all closed vesicles with no deactivation of the enzyme.

Another problem is to determine the orientation of the incorporated enzyme. The fraction of (i/o)-oriented enzyme molecules can be measured from the activity which is insensitive to external ouabain. However, the inhibition of any 'unreconstituted' enzyme by extracellular added ouabain is not instantaneous but takes 0.5–1 min even with 0.3 mM ouabain at 23°C [4]. With the short test times normally required this means that, unless special precautions are taken to correct for this, the amount of (i/o)-oriented enzyme is overestimated and the Na^+ to ATP hydrolysis ratio is underestimated.

A third problem is the small vesicle volume which means that the amount of internal K^+ very fast becomes rate limiting in measurements of activity and of Na^+-flux caused by (i/o)-oriented enzyme molecules. To overcome this problem it may not be sufficient to add valinomycin as a carrier for back diffusion of K^+. On red cells the addition of a proton carrier increases the valinomycin-dependent K^+-flux [10] and this has been suggested to explain that a proton carrier in addition to valinomycin increases the K^+ and ATP-dependent Na^+-influx in liposomes with reconstituted ($Na^+ + K^+$)-ATPase [8]. The effect is probably due to the short-circuiting effect of the proton flux on the K^+-diffusion potential [8,10].

The ($Na^+ + K^+$)-ATPase can be dissolved in the noninic detergent octaethyleneglycoldodecyl monoether ($C_{12}E_8$) and the solubilization from partly purified membranes from rectal glands of spiny dogfish leads to a purification. The dissolved enzyme is stable at 4°C [11]. $C_{12}E_8$ can easily be removed by adsorption to polysteren beads. By taking advantage of this it is possible as shown in the present paper to reconstitute the enzyme into lipid vesicles. By re-adding the detergent $C_{12}E_8$ to reopen the reconstituted vesicles it can be demonstrated that reconstitution of the enzyme takes place without loss of specific activity provided the proper lipids and protein-to-lipid ratio is chosen.

A method is described which circumvents the problem of delayed ouabain inhibition in the determination of the fraction of (i/o)-oriented enzyme molecules and this is compared to a determination of the number of vanadate binding sites. From this, and by using a combination of a K^+ and a H^+ ionophore [8] to ensure an optimal internal K^+ concentration is it shown that in liposomes with full recovery of enzyme activity the inside-out-oriented enzyme molecules of the closed vesicles have a specific activity which is identical to the specific activity of the solubilized enzyme used for incorporation. The correspondingly K^+-stimulated, ATP-dependent influx is 3 Na^+ per ATP molecule hydrolyzed.

Methods

Materials. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids (bovine) or Supelco (bovine) or Sigma (egg yolk). Asolectin was from Associated Concentrates and used without further purification. Cholesterol was from Sigma. All lipids were stored at −20°C and dissolved in chloroform before an experiment. Octaethyleneglycoldodecyl monoether ($C_{12}E_8$) was obtained from Nikko Chemicals, Tokyo, Japan. Bovine serum albumin was from Behring Institute. Di[1-^{14}C]palmitoyl L-α-phosphatidylcholine (in the following ^{14}C-PC), [^{3}H]inulin, [^{48}V]vanadyl choline, and ^{22}NaCl were obtained from The Radiochemical Center, Amersham. ^{51}Cr-EDTA and *N*-[^{14}C]ethylmaleimide were from New England Nuclear. ^{14}C-labelled $C_{12}E_8$ was obtained from CEA, France.

Lipid extraction. Lipid from the membrane bound enzyme of *Squalus achantias* was extracted essentially following the method of Folch et al. [12]. One part of enzyme was freeze-dried and homogenized with 19 parts of chloroform/methanol (2 : 1, v/v). After addition of water and separation of the methanol and chloroform phases by mild centrifugation, the lipid was collected. The extract was redissolved in chloroform and stored at −20°C.

Thin-layer chromatography (TLC). The lipids were applied in a small volume of chloroform on silica gel plates which were developed in two directions according to the method of Skidmore and Entenman [13]. The lipids were detected using

ninhydrine spray or iodine vapour. Lipid containing areas were scraped into glass tubes and digested for subsequent determination of phosphorus according to Bartlett [14].

Preparation of membrane bound and solubilized enzyme. Membrane bound ($Na^+ + K^+$)-ATPase from rectal glands of the spiny dogfish (*Squalus acanthias*) was purified as described by Skou and Esmann [15] to a specific activity of about 1400 μmol ATP hydrolyzed/mg protein per h at 37°C. The enzyme was prepared in 20 mM histidine, 25% glycerol (pH 7.0) and stored at −20°C. The membrane bound enzyme was solubilized according to Esmann et al. [11] using the detergent octaethyleneglycoldodecyl monoether ($C_{12}E_8$). The detergent was added to the enzyme at room temperature to a concentration of 2 mg/mg protein. After 1 h centrifugation at 280000 × *g* at 10°C the solubilized enzyme was collected from the supernatant. The specific activity was 600–900 μmol P_i/mg protein per h at 22°C and 1600–2400 μmol P_i/mg protein per h at 37°C.

ATPase assay. The ATPase activity of the membrane bound enzyme was tested in a medium containing (mM): 130 Na^+, 20 K^+, 4 Mg^{2+}, 3 ATP, 0.2 EGTA, 20 histidine (pH 7.4) at 37°C and 0.330 mg/ml bovine serum albumin. For solubilized enzyme the pH was 6.8, which is optimum [11], the albumin concentration was 0.66 mg/ml and the assay medium also contained 0.1 or 0.2 mg/ml $C_{12}E_8$ [16]. The reaction was initiated by adding enzyme (approx. 8 μg/ml) to the assay medium and after 0.5–2 min (at 37°C or 22°C) it was terminated by the addition of 50% trichloroacetic acid. The inorganic phosphate (P_i) was determined by the method of Fiske and SubbaRow [17].

The activity of reconstituted solubilized enzyme was tested as described for the solubilized enzyme without or with the addition of the detergent $C_{12}E_8$ to the assay medium according to the purpose of the experiment (see Results). P_i was determined according to Baginsky et al. [18] with addition of 5% sodium dodecyl sulfate (SDS) to the arsenite-citrate reagent. P_i-standards were run in parallel and the absorbance read at 850 nm against a reagent blind. After reconstitution the ATPase activity showed a broad pH optimum between 6.8 and 7.4.

Protein. The protein determination followed the method of Lowry et al. [19] as modified by Peterson [20] in which the protein is quantitatively precipitated with sodium deoxycholate plus trichloroacetic acid to remove it from interfering substances such as histidine, and SDS is included to leave the lipids transparant and noninterfering. Bovine serum albumin was run as standard.

Density gradient centrifugation. A 1–3 ml sample was layered on top of a discontinuous gradient of 5–40% sucrose (w/v) in 30 mM histidine. The sample was centrifuged at 98000 × *g* in a Beckman TI70 rotor overnight at 4°C. 2-ml samples were collected from the top by pumping 55% sucrose into the bottom of the centrifuge tubes.

Gel-filtration and isolation of liposomes. The vesicles were sized by gel chromatography on a Sepharose CL-2B column (2 × 35 cm) equilibrated with an appropriate buffer. The flow rate was 12 ml/h, fraction size 2 ml, and temperature 4°C. Vesicles were isolated from the medium by filtration on a small Sephadex G-50 columns (1 × 10 cm). Flow rate was 1 ml/min and fraction size 0.5 ml. Alternatively, the vesicles were spun down by centrifugation in a Beckmann air-fuge for 40 min at 30 lb/inch^2 (100000 × *g*) and resuspended in the actual medium.

Reaction with ^{14}C-labelled N-ethylmaleimide. The membrane bound enzyme was reacted with *N*-[^{14}C]ethylmaleimide as described by Esmann [21]. The enzyme (1 mg/ml in 150 mM NaCl, 3 mM ATP, 5 mM CDTA, 35% glycerol, 20 mM histidine, pH 7.0) was incubated with 0.1 mM *N*-[^{14}C]ethylmaleimide at 37°C for 5 h. The reaction was stopped with 1 mM 2-mercaptoethanol and the enzyme washed repeatedly by centrifugation at 98000 × *g*. The *N*-[^{14}C]ethylmaleimide-reacted enzyme was solubilized as described above. ^{14}C activity was measured by liquid-scintillation counting.

Reaction with [^{48}V]vanadate. Vanadate was used to estimate the orientation of reconstituted ($Na^+ + K^+$)-ATPase, since it binds exclusively to the cytoplasmic site of the enzyme [22]. [^{48}V]vanadylchloride was oxidized and neutralized with KOH or NaOH, to [^{48}V]vanadate. [^{48}V]vanadate was added to reconstituted liposomes in the presence of K^+ to a final concentration of 6 μM and incubated for 5 min at 22°C in the presence of

absence (blank) of 4 mM Mg^{2+}. The bound [^{48}V]vanadate activity was measured in a Gamma-counter after passage of the liposomes through a Sephadex G-50 column at 4°C.

Loading of liposomes with [^{3}H]inulin or ^{51}Cr-EDTA. In order to estimate liposome volume [^{3}H]inulin or ^{51}Cr-EDTA were used as intravesicular markers. Liposomes were prepared with [^{3}H]inulin or ^{51}Cr-EDTA present. After passage through a Sephadex G-50 or Sepharose CL-2B column the intravesicular radioactivity eluted in the void volume was determined. Both ^{3}H and ^{51}Cr activity was measured by liquid-scintillation counting.

Affinity chromatography. Concanavalin A coupled to Sepharose 4B (Con A-Sepharose, Pharmacia) was packed in a small column (1 × 10 cm) and equilibrated with a 30 mM histidine buffer (pH 6.8) which contained 130 mM Na^+ and 4 mM Mg^{2+}. A 1 ml sample of liposomes with reconstituted ATPase was layered on top and eluted at 2 ml/h in 2-ml fractions. The bound fraction was desorbed by eluting with the same buffer solution to which was added 200 mM α-methyl-D-mannoside and 4 mg/ml $C_{12}E_8$.

Preparation of liposomes with reconstituted ($Na^+ + K^+$)-ATPase. Liposomes with different lipid composition and incorporated ATPase were prepared by cosolubilization of lipids, protein and detergent in a weight-ratio of 10 : 1 : 12.5, unless otherwise stated, whereafter the detergent was removed by adsorption to Bio-Beads SM-2 (Bio-Rad, CA, U.S.A.).

8 mg of the appropriate lipids in chloroform was transferred to a 10 ml vial and the $CHCl_3$ evaporated under N_2 while rotating the vial to deposite a thin lipid film. The lipids were then dried under vacuum for 1 h; 1000 μl $C_{12}E_8$ (10 mg/ml) in the appropriate ion/buffer solution was added and the lipid solubilized at room temperature by sonication in a bath-sonicator (Metason, Struers, Denmark). 1500 μl of solubilized enzyme (approx. 0.5 mg/ml) in a buffer was added and the suspension kept on ice. The $C_{12}E_8$ was subsequently removed either by adding 300 mg of Bio-Beads and incubating overnight at 4°C followed by 1 h incubation at 22°C on a tissue-incubator, or alternatively by passing the suspension through a Bio-Bead column (1 × 10 cm) with a flow rate of 7 ml/h at 4°C. Using the first method the beads were removed by centrifugation at 3000 rpm for 10 min at 4°C. Finally, the vesicle suspension was centrifuged for 10 min at 100000 × *g* in a Beckman air-fuge at 4°C and the supernatant collected. The recovery of both lipid and protein after Bio-Beads treatment was better than 80%.

Na^+-transport. In the transport assay 100 μl of reconstituted liposomes containing 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+} in 30 mM histidine (pH 7.0) was diluted 10-times into the incubation medium containing the same concentrations of ions plus 3 mM ATP, $3 \cdot 10^{-6}$ M valinomycin, $3 \cdot 10^{-4}$ M carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 1 mM ouabain and 5.0 μCi/ml of ^{22}Na. For passive transport measurements ATP was omitted.

50 μl samples were withdrawn at different times after mixing and added to a 0.5 cm × 2.5 cm Bio-Rex 70 (Bio-Rad) column and eluted under slight pressure with 1 ml of 150 mM histidine buffer, pH 7.0. The elution time was 10 s. The eluate was collected and counted directly in a gamma-counter. A 50 μl sample taken before the addition of liposomes but otherwise processed as described above was taken as a blank.

Before use the Bio-Rex 70 cation-exchange resin (100–200 mesh, sodium-form) was converted to the Tris-form, pH 7.0, 1/2 ml of this was transferred to a small column, 1/2 cm in diameter, and equilibrated with 150 mM histidine buffer, pH 7.0 before the sample was added.

Results

Characterization of lipid vesicles with reconstituted ($Na^+ + K^+$)-ATPase

The detergent $C_{12}E_8$ is used to dissolve both the enzyme and the lipids and reconstitution is accomplished by removal of $C_{12}E_8$ by adsorption to polystyrene beads as described in Methods.

The standard lipids used for reconstitution were, unless otherwise stated, a mixture of highly purified phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylinositol (PI), and cholesterol in a weight ratio of 60 : 14 : 2 : 24 which is about the ratio found for the lipids bound to the solubilized enzyme [17].

The solubilization of the lipids in detergent was facilitated by sonic oscillation using a bath sonicator. In this way is was possible to dissolve 1 part of lipid in 1 part of detergent (wt./wt.). Using 1 : 10 protein to lipid liposomes this result in a 1 : 10 protein to detergent weight ratio which is higher than initially used for solubilization of the enzyme. However, even a 7.5-times further increase in detergent concentration had no deleterious effect on the enzyme activity.

The enzyme was dissolved in $C_{12}E_8$ as described in Methods and mixed with the likewise dissolved lipids. The removal of the detergent $C_{12}E_8$ from the mixed micelle preparation of detergent/lipid/protein with Bio-beads SM-2 resulted in the formation of lipid vesicles with reconstituted $(Na^+ + K^+)$-ATPase. The elimination of detergent from the mixed micelle suspension with Bio-Beads was followed by using ^{14}C-labelled $C_{12}E_8$. After 18–20 h a plateau was reached corresponding to 0.75 g detergent/g protein. Gel filtration on Sepharose CL-2B column of samples treated with Bio-beads for 1 h did not reduce this value further. This fraction of detergent is of the same size as has been previously shown to be

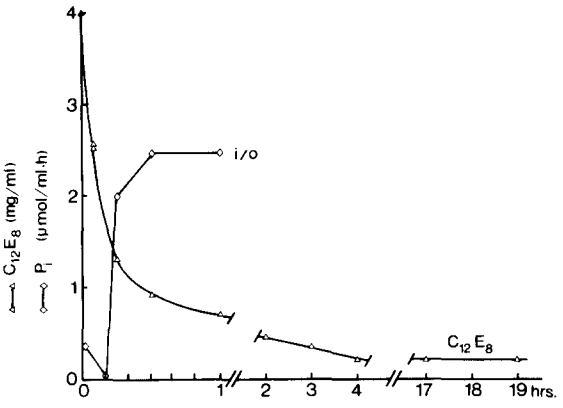

Fig. 1. The time-course for removal of the detergent $C_{12}E_8$ from a mixed protein/lipid/detergent micelle suspension and for the formation of closed vesicles. $C_{12}E_8$ was removed by adsorption to Bio-Beads SM-2 at 4 °C. The presence of ouabain insensitive activity from (i/o)-oriented enzyme was used as an indication of formation of closed vesicles (see text). Initially, 0.33 mg protein, 3.3 mg lipid and 4 mg detergent was present in 1 ml suspension. Lipid composition was PC/PE/PS/cholesterol = 60 : 14 : 2 : 24 (wt.%). About 300 mg of Bio-Beads were added to 2.5 ml liposome suspension. The level of detergent reached after about 4 h at 4 °C corresponds to 0.73 g/g protein.

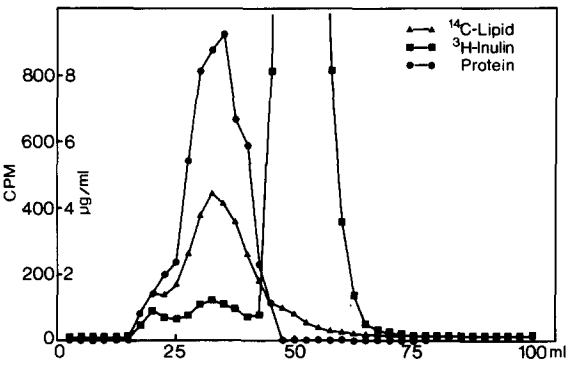

Fig. 2. Gel filtration of a liposome suspension on Sepharose Cl-2B. Lipid composition was PC/PE/PS/cholesterol = 79 : 9 : 1 : 15 (wt.%) and the protein/lipid ratio was 1 : 20. Liposomes were prepared in the presence of ^3H-inulin and ^{14}C-labelled phosphatidylcholine. The lipid and protein elutes together with a small fraction of ^3H-inulin, which represents the intravesicular trapped inulin. A major ^3H-inulin peak representing free inulin is eluted in the total volume of the column (see Methods).

bound to $C_{12}E_8$-solubilized enzyme [16]. It is apparently fairly tight bound to the enzyme and was found not to exchange with unlabelled $C_{12}E_8$ in the bulk solution on a time scale of several hours. The elimination of the detergent $C_{12}E_8$ down to the plateau value could be described as a double exponential with time constants of 8–15 min and 3–5 h, respectively. The formation of closed vesicles was detected by measuring the activity of (i/o)-oriented enzyme, Fig. 1 (see below). Closed liposomes formed when the detergent concentration was lowered from the initial value of about 4.0 mg/ml to a value between 1.5–2.0 mg detergent/ml in samples containing 2 mg/ml lipid and 0.3 mg/ml protein. This was obtained about 10–30 min after addition of Bio-beads (Fig. 1).

Fig. 2 depicts the elution profiles from a gel filtration experiment where liposomes prepared in the presence of ^{14}C-PC and ^3H-inulin were run on a Sepharose CL-2B column. As seen the lipid (^{14}C cpm) is eluted in a major peak in the void volume of the column. All protein is eluted together with the lipid. That vesicles have been formed is demonstrated by the presence of a fraction of 'trapped' ^3H-inulin that co-elutes with the lipid and protein, while a much greater fraction is eluted as a second peak in the total volume as free inulin.

The internal volume of reconstituted 1 : 20 protein/lipid liposomes was calculated from experi-

ments like the one shown in Fig. 2 to be 10–13 μl/mg of lipid.

Density gradient centrifugation of the reconstituted liposomes showed that all the protein was found together with the lipid in the upper part of the gradient at the 5%–10% sucrose boundary. In a parallel experiment using unreconstituted solubilized enzyme labelled with *N*-[^{14}C]ethylmaleimide as a marker the enzyme was recovered in the 30% sucrose layer. Since no protein was detected at this position using reconstituted enzyme it can be concluded that all protein is lipid associated in the liposome preparation, in agreement with the results shown in Fig. 2.

Symmetry of incorporation of ($Na^+ + K^+$)-ATPase into liposomes

There are three different possible orientations of the reconstituted enzyme: inside-out (i/o); right-side-out (r/o); and finally in a mode showing no orientation (n-o). The latter includes unincorporated enzyme, enzyme adsorbed to lipid vesicles (internally or externally) or enzyme incorporated into non-closed (leaky) vesicles.

The orientation of the enzyme incorporated into the liposomes was monitored using four different approaches:

a. The ouabain / detergent method. The total activity (A_{tot}) of the enzyme incorporated in a sample of liposomes was determined after the liposomes were reopened by addition of an appropriate amount of saponin or $C_{12}E_8$. In parallel samples without addition of detergent, the activity was measured without and with added ouabain, i.e., the activity of (i/o + n-o)-oriented (A_2) and of (i/o)-oriented (A_1) enzyme molecules, respectively.

Since the existence of unreconstituted (free) enzyme can be excluded from the sucrose density gradient experiments as previously mentioned and no enzyme is found adsorbed inside the liposomes (see below) the relative frequencies (f) of enzyme orientations after reconstitution can be calculated as follows:

$$f_{(i/o)} = A_1/A_{tot}$$

$$f_{(r/o)} = (A_{tot} - A_2)/A_{tot}$$

$$f_{(n\text{-}o)} = (A_2 - A_1)/A_{tot}$$

A reagent blank is subtracted from all activities. The blank is either a sample where trichloroacetic acid is added before the enzyme to the test medium; or the ouabain or digitoxigenin (see below) insensitive activity of a sample of the opened liposomes.

Several conditions must be fullfilled, however, for the outlined above to apply:

First, the detergent used for opening the closed vesicles must be effective without interfering with the enzyme activity. This is important because many detergents are known to denature the enzyme in the concentrations needed for opening of closed vesicles [23]. Moreover, certain detergents are only effective in the presence of specific lipids (see below).

The closed liposomes were opened by readdition of the detergent $C_{12}E_8$. Optimum effect on the activity was obtained with 0.35 mg detergent per mg lipid for 5 min at 22°C; there was no inactivating effect of increasing the incubation time up to 30 min and/or increasing the detergent concentration to 0.70 mg per mg lipid which showed that the concentration of $C_{12}E_8$ necessary for reopening has no inactivating effect on the enzyme activity. In liposomes containing cholesterol, the detergent saponin in a concentration of 0.8 mg per mg lipid gave specific activities of the enzyme identical to that obtained with $C_{12}E_8$. Saponin was only fully effective provided cholesterol was present in the liposomes.

Second, ouabain must not be able to penetrate the lipid barrier and enter closed liposomes and the delay in the inhibition by ouabain of the (n-o)-oriented enzyme molecules must be accounted for (see below).

Third, the intravesicular K^+-concentration must not be limiting: It is essential that an optimal K^+-concentration inside the liposomes can be continously ensured during activity measurements which normally requires 0.5–2 min. In accordance with previous findings [8] this was achieved by adding the K^+-ionophore valinomycin ($3 \cdot 10^{-6}$ M) in combination with the H^+-ionophore CCCP ($3 \cdot 10^{-4}$ M). Valinomycin increases the K^+-permeability of the lipid membrane thus permitting K^+ to leak back into the liposomes once it is being pumped out. However, since valinomycin transports K^+ electrogenically, CCCP is also added in order to establish a rapid exchange of a counterion

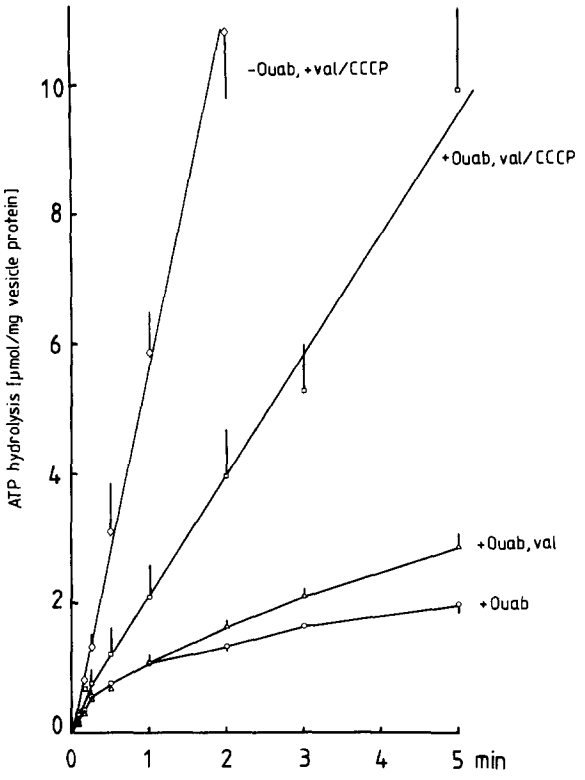

Fig. 3. Time-course of ATP hydrolysis at 22 °C of reconstituted ($Na^+ + K^+$)-ATPase. The three lower curves represent measurements in the presence of 1 mM ouabain and with either valinomycin ($3 \cdot 10^{-6}$ M) or a combination of valinomycin and carbonylcyanide *m*-chlorophenylhydrazone (CCCP, $3 \cdot 10^{-4}$ M) present. The upper curve (◇) represents the measurements in the absence of ouabain but with valinomycin + CCCP. Lipid composition of liposomes was PC/PE/PS/cholesterol (60:14:2:24) and the protein/lipid ratio was 1:10. Liposomes were produced in K^+ (150 mM)/Mg^{2+} (4 mM)/histidine (30 mM) (pH = 6.8). Test medium, see Methods.

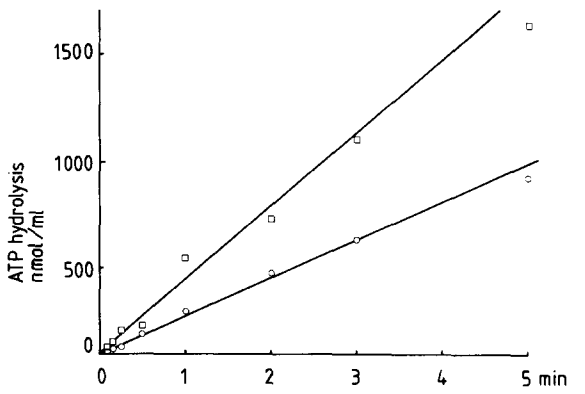

Fig. 4. Time-course of ATP hydrolysis at 22 °C by inside-out oriented reconstituted ($Na^+ + K^+$)-ATPase. The enzyme was reconstituted in a histidine buffer (30 mM, pH 6.8) containing Mg^{2+} (4 mM) and P_i (1 mM). The protein/lipid ratio was 1:10 and the lipid composition was PC/PE/PS/cholesterol = 60:14:2:24. After reconstitution the enzyme was preincubated in Mg^{2+} (4 mM), P_i (1 mM), ouabain (1 mM) and histidine (30 mM) (pH = 6.8), for 5 min in order to inhibit (n-o) and (r/o)-oriented enzyme. After the preincubation with ouabain the activity was tested as a function of time after addition of 130 mM Na^+, 20 mM K^+, and 3 mM ATP (upper curve, □). Alternatively, the liposome suspension was washed by centrifugation at 4 °C at 100000 × *g* for 30 min followed by a resuspension in 130 mM Na^+, 20 mM K^+ and 4 mM Mg^{2+} before measurement of activity. After addition of 3 mM ATP the hydrolysis was followed as a function of time (lower curve, ○). The recovery of protein after the wash was about 50%. In all the test solutions valinomycin ($3 \cdot 10^{-6}$ M) and CCCP ($3 \cdot 10^{-4}$ M) were added.

(namely H^+) for the extravesicular K^+ thus avoiding decellerating the K^+ back diffusion caused by the K^+ diffusion potential.

In Fig. 3 is shown the effect of valinomycin and CCCP on the activity of the ($Na^+ + K^+$)-ATPase incorporated in liposomes containing 150 mM K^+. The reaction is started by addition of the liposomes to the test medium which contains 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+} and 3 mM ATP. With ouabain in the test medium there is a progressive decrease in hydrolysis of ATP as a function of time. Addition of valinomycin to the test medium slightly prevented the decrease in activity and this effect of valinomycin is considerably enhanced in the presence of the uncoupler CCCP. With valinomycin, CCCP and ouabain present the hydrolysis of ATP as a function of time seems to become rectilinear 0.5–1 min after the reaction is started. The decrease in activity during the first 0.5–1 min disappears when ouabain is omitted from the test solution suggesting that it is due to a delay in the inhibition of the (n-o)-oriented enzyme molecules by ouabain, as has been previously described by Goldin [4].

To overcome a delay in the inhibition by ouabain the (n-o)-oriented enzyme molecules were inhibited by ouabain before the measurement of the enzyme activity (Fig. 4). A reconstitution of enzyme was performed in a buffer containing Mg^{2+} and P_i and the liposomes preincubated with 1 mM ouabain for 5 min at 22 °C. Then the activity of the (i/o)- and (n-o)-oriented enzyme

was tested either directly or after removal of the P_i by a cold centrifugation (4 °C, 40 min, 100 000 × *g*) followed by resuspension in cold P_i-free medium. The protein recovery after the centrifugation is about 50%. Control experiments with membrane bound enzyme demonstrated that during this washing procedure the ouabain inhibition persisted. To the standard test solution was added valinomycin, CCCP and ouabain. As seen from the figure, the ATP hydrolysis as a function of time now becomes rectilinear supporting the view that the decrease in slope seen in Fig. 3 in the presence of valinomycin + CCCP is due to a delay in the ouabain inhibition. The fraction of enzyme activity due to (i/o)-oriented enzyme calculated from the slopes of the straight lines in Fig. 4 and correcting for protein recovery is 10% of the total activity of the reopened vesicles.

The rectilinear curves found when preincubation with ouabain is performed (Fig. 4) furthermore demonstrate that there is no inhibition of the (i/o)-oriented enzyme due to a penetration of ouabain into the liposomes. If the two more lipid soluble digitaloids digitoxin and digitoxigenin were employed instead of ouabain no enzyme activity remained uninhibited suggesting that these digitaloids readily penetrates the liposomes.

The possible existence of internally adsorbed or trapped protein was excluded by performing the following experiment. Reconstitution was performed in a histidine buffer (30 mM, pH = 6.8) containing Mg^{2+} (4 mM) and P_i (1 mM) and the liposomes incubated in the same medium with 1 mM ouabain for 5 min at 22 °C to inhibit all enzyme with the extracellular side exposed (i.e., r/o and n-o). The liposomes were then washed free of non bound ouabain and P_i as explained above and tested for ATPase activity after opening with detergent. The measured activity was 10% of total ATPase activity corresponding to the fraction of (i/o)-oriented enzyme molecules measured in the parallel experiment referred to in Fig. 4. This indicates that there is no intravesicular trapped or adsorbed enzyme.

b. The ouabain / ionophore method. Another type of experiments (from here on referred to as the 'ouabain/ionophore method') overcoming the problem of delayed ouabain inhibition was the following. Reconstituted liposomes were produced in a histidine buffer without K^+ but with Na^+ (130 mM) and Mg^{2+} (4 mM). A sample of these 'Na-liposomes' was incubated in a buffer adjusting the ion composition outside the liposomes to K^+ (20 mM), Na^+ (130 mM) and Mg^{2+} (4 mM). Finally, the P_i-liberation versus time was determined with an without added ouabain (1 mM) in two parallel experiments, one in which the samples were preincubated with valinomycin and CCCP to obtain ion equilibration and one without the addition of ionophores. Thus, each experiment results in four curves as seen in Fig. 5 (plus a curve which is the blank where digitoxigenin is present in the test solution). Each curve reflects the enzyme activity for different enzyme orientations in the liposomes: curve 1 (−ouabain, +val/CCCP) results from enzyme incorporated in both the (i/o)-orientation and the (n-o)-orientation. Curve 2 (+ouabain, +val/CCCP) reflects the sum of (i/o)-orientations plus a fraction of enzyme activity resulting from (n-o)-oriented enzyme that initially is active due to delayed ouabain inhibition. Curve 3 (−ouabain, −val/CCCP) results from (n-o)-orientation of enzyme alone, since K^+ is absent inside the liposomes, and finally curve 4 (+ouabain, −val/CCCP) which reflects the fraction of (n-o)-oriented enzyme that initially is active in the presence of ouabain. The net difference between curves 1 and 3, and also between curves 2 and 4 is therefore due to the activity of enzyme incorporated in the (i/o)-orientation. A replot of the data resulting from the two curve-subtractions should result in two straight lines with identical slopes proportional to the activity of the (i/o)-oriented enzyme. As seen from the inset in Fig. 5 these requirements seem to be fulfilled. Calculations using the slopes show that the enzyme activity of the (i/o)-oriented enzyme molecules is about 10% of the activity of the reopened vesicles.

The 'ouabain/ionophore method' enables one to calculate the activities of enzyme molecules oriented either (i/o), (r/o) or (n-o) as a fraction of the total activity of the enzyme of the reopened liposomes. A problem is, however, can the fractions of activities be taken as an indication of the fractions of the molecules oriented in the different ways? In other words is the turn-over of the enzyme molecules incorporated in the different orientations the same? In order to test this the

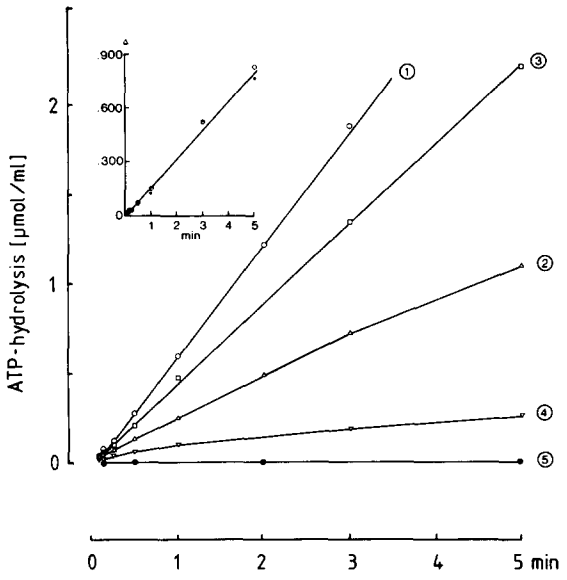

Fig. 5. Time-course of ATP hydrolysis at 22 °C of reconstituted liposomes made in a histidine buffer (30 mM, pH = 6.8) containing Na^+ (130 mM) and Mg^{2+} (4 mM). The protein/lipid ratio was 1:10 and the lipid composition was PC/PE/PS/cholesterol = 60:14:2:24. Test medium, see Methods. Curves 1 and 3: −ouabain; curves 2 and 4: +1 mM ouabain. Curves 1 and 2 measured after preincubation with 20 mM K^+ in the presence of valinomycin ($3 \cdot 10^{-6}$ M) and CCCP ($3 \cdot 10^{-4}$ M). The lower curve (●) represents the blank with added digitoxigenin. Inset shows the difference between curves 1 and 3 (○), and between curves 2 and 4 (●). The identical slopes of the resulting two curves are proportional to the activity of (i/o)-oriented enzyme, as explained in Results.

orientation of the molecules was determined from the binding of vanadate which binds in a 1:1 stoichiometry to the enzyme molecules.

c. The vanadate binding method. Vanadate has been found to bind to $(Na^+ + K^+)$-ATPase exclusively from the cytoplasmic side and only in the presence of Mg^{2+}. The binding of vanadate is facilitated by extracellular K^+, whereas ATP, Na^+ and P_i oppose its binding [22,24].

With the proper ligands present vanadate binding to solubilized ATPase and to enzyme reconstituted into 1:10 protein to lipid liposomes which have been reopened by the addition of detergent is completed within a few minutes. The total number of vanadate binding sites are found to be 3.8 ± 0.2 nmol/mg protein, (mean ± S.D., six experiments).

As demonstrated in Fig. 6 the ligand-dependent binding of vanadate to the enzyme can be used to evaluate the symmetry of reconstitution. The liposomes containing enzyme were prepared and incubated in the presence of 150 mM Na^+ and 4 mM Mg^{2+}. These 'Na-liposomes' served as a blank, since vanadate binding is prevented under these conditions (curve □——□). However, if [^{48}V]vanadate is added together with K^+ (final concentration 20 mM) to overcome the 'Na inhibition', vanadate binding occurs (curve ○——○). Under the conditions chosen only enzyme which exposes both the intracellular side (the vanadate binding site) and the extracellular side (the side from which K^+ has to act) can bind the vanadate, i.e., the (n-o)-oriented enzyme, since K^+ penetrates

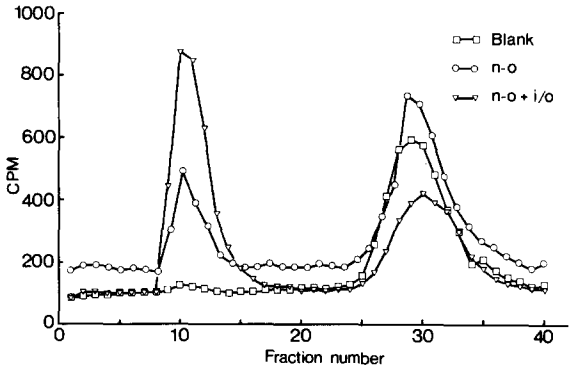

Fig. 6. Vanadate binding to liposomes with reconstituted ATPase. Two liposome preparations were used. One is produced in 130 mM Na^+, 4 mM Mg^{2+}, 30 mM histidine (pH 6.8) (○, □) and another in 150 mM K^+, 4 mM Mg^{2+}, 30 mM histidine (pH 6.8) (▽). The liposomes produced in sodium and magnesium were incubation for 5 min at 22 °C in a histidine buffer (30 mM, pH 6.8) containing 130 mM Na^+, 4 mM Mg^{2+} and about 6 µM [^{48}V]vanadate (□). They served as a blank. Another batch of liposomes produced in sodium was incubated for 5 min in a histidine buffer (30 mM, pH 6.8) containing 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+} and about 6 µM [^{48}V]vanadate (○) allowing vanadate to bind to (n-o)-oriented enzyme. After incubation with vanadate a sample of the liposome suspension was passed through a Sephadex G-50 column (1 × 10 cm) at 4 °C with the same buffer but without vanadate in order to separate liposomes with bound vanadate from free vanadate. The same binding experiment was carried out using the liposomes produced in the histidine buffer containing K^+ (150 mM) and Mg^{2+} (4 mM) allowing vanadate to bind to (i/o + n-o)-oriented enzyme. After incubation for 5 min in a histidine buffer (30 mM, pH 6.8) containing 150 mM K^+, 4 mM Mg^{2+} with [^{48}V]vanadate at 22 °C the liposomes were separated from the medium on a Sephadex G-50 column eluted with the same buffer without vanadate and the radioactiity in the eluate was counted (▽).

TABLE I

THE ORIENTATION OF RECONSTITUTED ($Na^+ + K^+$)-ATPase IN 1 : 20 PROTEIN/LIPID LIPOSOMES DETERMINED FROM MEASUREMENTS OF ACTIVITY USING THE 'OUABAIN/IONOPHORE' METHOD AND 'VANADATE BINDING' METHOD (see text)

Method	Symmetry of reconstitution (%, mean ± S.D.)			No. of expts.
	i/o	r/o	n-o	
Ouabain/ ionophore	19.4 ± 2.9	55.8 ± 2.5	24.8 ± 5.2	3
Vanadate	19.6 ± 6.3	61.1 ± 7.3	19.3 ± 2.9	3

the liposomes extremely slowly compared to the time of incubation with vanadate (5 min). Calculation of the number of binding sites gives in this case 1.4 nmol/mg protein.

The experiment was repeated with liposomes made up in the presence of 20 mM K^+, 130 mM Na^+ and 4 mM Mg^{2+} (curve ▽——▽). Under these circumstances enzyme incorporated in both the (n-o)-orientation and the (i/o)-orientation binds the vanadate and calculation of the number of binding sites gives for this case 1.8 nmol/mg protein. The number of (i/o)-oriented molecules is thus 0.4 nmol/mg protein. The total number of vanadate binding sites was 3.8 nmol/mg protein, i.e., the symmetry of reconstitution is in this case: (i/o) : (n-o) : (r/o) = 0.4 : 1.4 : 2.0 corresponding to 10% : 37% : 53%.

In Table I is shown the symmetry of reconstitution determined from a number of vanadate binding experiments using liposomes with a protein/lipid ratio of 1 : 20 which gives a higher fraction of (i/o)-orientations than with 1 : 10 protein/lipid liposomes (see below). For comparison is shown the symmetry detected from determination of activity using the ouabain/ionophore method. The agreement between the two methods shows that the enzyme incorporated in the different orientations has the same turnover number which means that the fraction of activity of enzyme in a given orientation also expresses the fraction of enzyme molecules in that orientation.

None of the described methods descriminates between a situation where the different possible orientations of the enzyme incorporated are all present in one and the same vesicle or in different vesicles. To elucidate this the following experiments were carried out.

d. The concanavalin A method. The β-subunit of the ($Na^+ + K^+$)-ATPase is a glycoprotein with an extracellular directed sugar moiety [25]. $C_{12}E_8$-

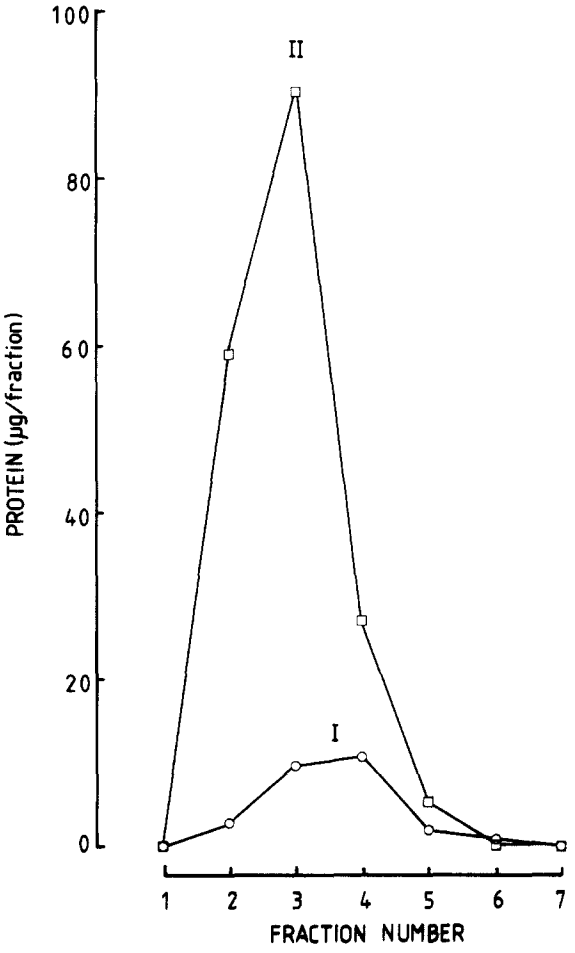

Fig. 7. Binding of reconstituted liposomes to concanavalin A. 1.0 ml reconstituted liposomes were chromatographed on a small Con A-Sepharose column (1 × 10 cm) at 2 ml/h. Fraction size was 2 ml. Liposomes were produced in 130 mM Na^+, 4 mM Mg^{2+}, histidine (30 mM, pH 6.8), the protein/lipid ratio was 1 : 10 and the lipid composition was PC/PE/PS/cholesterol = 60 : 14 : 2 : 24. Peak I represents the protein which does not bind to the column when the liposomes are eluted with 40 ml of histidine buffer (30 mM, pH 6.8) containing Na^+ (130 mM) and Mg^{2+} (4 mM). Peak II represents the protein which is desorbed by elution with the same buffer solution but added 200 mM α-methyl-D-mannoside and 4 mg/ml $C_{12}E_8$.

solubilized ($Na^+ + K^+$)-ATPase has been shown to bind to concanavalin A [26]. It is therefore expected that after incorporation into liposomes the enzyme with (i/o)-orientation is unable to bind to concanavalin A.

If the different orientations of the enzyme are present in one and the same vesicle then all liposomes with reconstituted enzyme will bind to a Con A-Sepharose column. If, however, each liposome contains only one of the possible enzyme orientations then all liposomes with enzyme in the (i/o)-orientation should remain unbound to the Con A Sepharose column.

Fig. 7 shows the elution profiles of reconstituted liposomes from a Con A-Sepharose column. Peak I is the fraction of the liposome protein added to the column which is not bound. Peak II is the fraction which is bound but eluted with a 30 mM histidine buffer (pH 6.8) which contains 200 mM α-methyl-D-mannoside and 4 mg/ml of detergent to desorb the bound protein. The inclusion of detergent in concentrations higher than 3 mg/ml was necessary to obtain quantitative desorption of the bound enzyme.

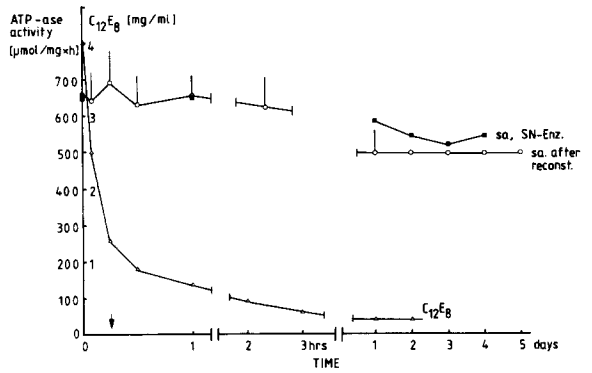

Fig. 8. The specific enzyme activity at 22°C of reconstituted enzyme as a function of the time after Bio-Beads addition. 300 mg of Bio-Beads were added to 2.5 ml liposome suspension. The liposome suspension was stored at 4°C. The detergent concentration was measured using ^{14}C-labelled $C_{12}E_8$. Arrow indicates time of appearance of closed liposomes (see Fig. 1). The protein/lipid ratio was 1:10 and the lipid composition was PC/PE/PS/cholesterol = 60:14:2:24. The activity of the reconstituted enzyme was measured after the liposomes had been opened by the addition of $C_{12}E_8$. Test medium, see Methods. For comparison is shown the specific activity (sa) of $C_{12}E_8$-solubilized enzyme (SN-Enz) which was tested and stored under the same conditions.

Peak I corresponds to about 13% of the added reconstituted protein while Peak II corresponds to about 87%. The liposomes in peak I showed no ouabain sensitivity and there was no increase in activity when the liposomes were opened by addition of detergent. This indicates that they contain protein exclusively incorporated in the (i/o)-orientation. In agreement with this it was found that the fraction of i/o molecules determied by the 'ouabain/ionophore method' of the same sample of liposomes was 12% compared with the 13% in peak I. 30% was n-o and 58% r/o oriented.

Recovery of specific enzyme activity after reconstitution

Using the above described methods it was tested how the recovered specific activity of reconstituted enzyme and the orientation of the enzyme molecules depend on a number a variables.

Varying the temperature between 4 and 37°C during the reconstitution, i.e., during removal of the detergent by Bio-beads had no significant effect. Neither was there an effect of the presence of mM concentrations of Ca^{2+} or Mg^{2+} (results not shown).

a. Stability of reconstituted enzyme. In Fig. 8 is shown the specific enzyme activity measured at 22°C as a function of storage at 4°C of solubilized enzyme and of enzyme incorporated into 1:10 protein/lipid liposomes with a lipid composition PC/PE/PS/cholesterol = 60:14:2:24; the activity of the reconstituted enzyme was measured after reopening of the vesicles. The figure also shows the removal of $C_{12}E_8$ from the membrane. About 90% of the activity of the solubilized enzyme was retained after storage for one week at 4°C. The specific activity of the reconstituted enzyme was initially identical to that of the solubilized enzyme and after 3–5 days storage at 4°C it still retained about 90% of that of the solubilized enzyme.

b. Effect of lipid composition and of protein / lipid ratio on recovery of specific activity and orientation. The lipid composition of the liposomes was varied by variation of the initial composition of the mixed micelle suspension. The initial composition of lipids was identical to the final composition as checked by two-dimensional TLC.

In Fig. 9 is shown the effect of a variation in

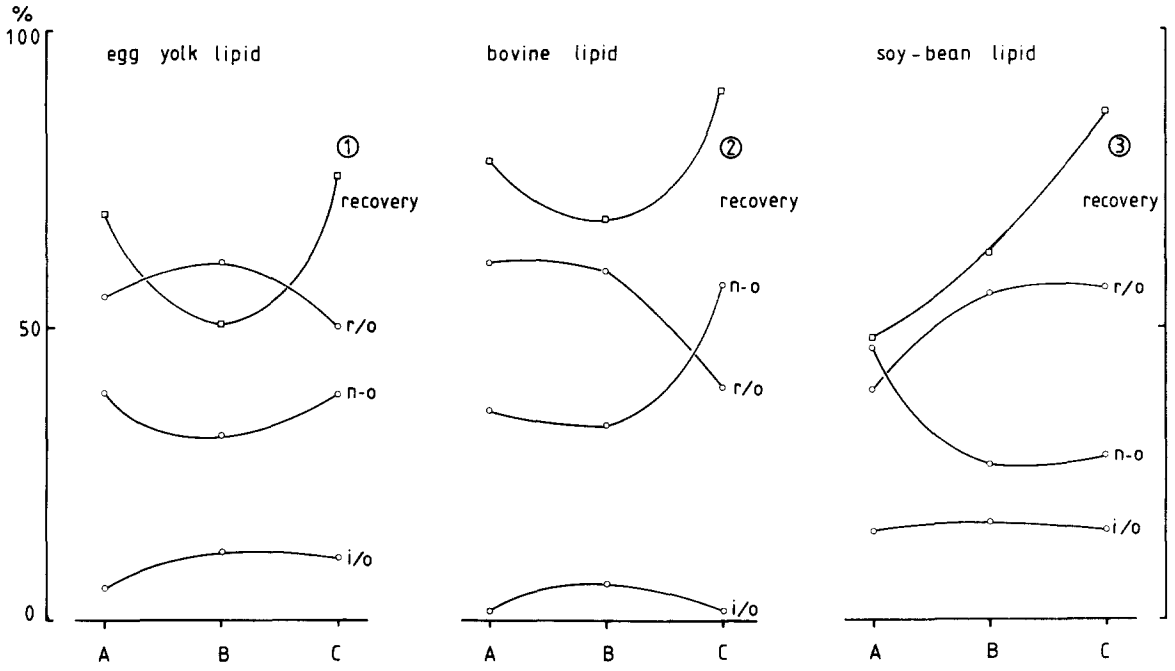

Fig. 9. The effect of lipid composition and of lipid source on the symmetry of reconstitution and on the recovery of the specific enzyme activity (□) after reconstitution. Three different lipid compositions were compared: A, pure PC vesicles; B, PC/PE/PS vesicles (78:20:2); and C, PC/PE/PS/cholesterol (60:14:2:24). Lipids were from three sources as indicated, egg yolk (panel (1)); bovine (panel (2)) and soybean (panel (3)). All liposomes were prepared in Na^+ (130 mM), Mg^{2+} (4 mM) and histidine (30 mM), pH = 6.8. The symmetry of reconstitution was determined using the ouabain/ionophore method. The recovery of specific activity is determined after reopening of the liposomes with $C_{12}E_8$. Test medium, see Methods. The specific activity is given in % of the specific activity of the solubilized enzyme used for the reconstitution and stored under the same conditions as the liposomes. The results are the mean of two experiments where deviations between the results were less than 5%. In each experiment the measurements were carried out in triplicate.

the lipid composition on the orientation and the specific activity. The protein/lipid weight ratio was 1:10 and the lipids used were: PC alone; PC/PE/PS (78:20:2); or PC/PE/PS/cholesterol (60:14:2:24). Lipids were of bovine, egg and soybean origin, respectively. The same general effects are found for bovine and egg-yolk lipids, while lipids from soybean gave somewhat different results.

Addition of PE + PS from bovine and egg yolk decreased the recovered specific enzyme activity and increased the fraction of (i/o)-orientations compared to pure PC liposomes, whereas PE + PS from soybean increased both the specific enzyme activity recovered and the fraction of (i/o)-orientations. Addition of 50 mol% cholesterol to PC/PE/PS liposomes increased the recovered specific enzyme activity for all lipid sources used (see Fig. 9). The most significant difference between the effects of lipids from bovine and from egg yolk is the higher recovery of specific enzyme activity and the lower fraction of (i/o)-orientation using bovine lipids. Compared to animal lipids soybean lipids gave a higher fraction of (i/o)-orientation (about 15% compared to the 5% for bovine and 10% for egg-yolk lipids). Another difference between animal and plant lipids was that with plant lipids the specific enzyme activity deteriorated rather quickly. Much the same results were obtained if non-purified soybean lipid (asolectin) was used.

Fig. 10 depicts the effect of a variation in the protein/lipid ratio on the specific activity and on the orientation; the lipid composition was PC/PE/PS/cholesterol (60:14:2:24). The general picture is that the fraction of enzyme in

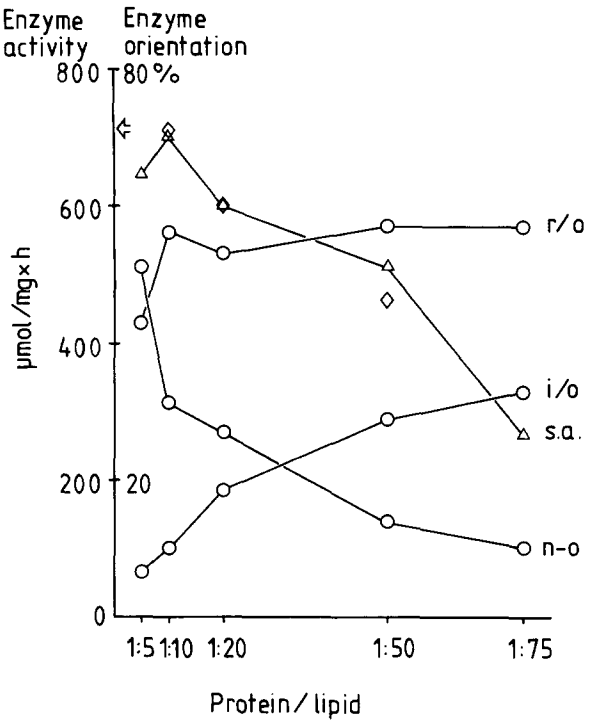

Fig. 10. Effect of the protein/lipid ratio on the orientation and on the recovery of the specific enzyme activity (sa) of reconstituted enzyme. The lipid composition was PC/PE/PS/cholesterol = 60:14:2:24. Ionic composition of the liposomes as in Fig. 9. The specific enzyme activity (△) was determined 24 h after reconstitution at 22°C. The specific activity of the solubilized enzyme used for incorporation was 713 μmol P_i/mg protein per h at 22°C (arrow in the figure). Diamonds (◇) represent enzyme activity calculated from ATP-dependent Na^+-influx experiments where a 3:1 Na^+/ATP ratio was used as described in the text.

(i/o)-orientation increases, the fraction of (n-o)-orientation decreases and the recovery of the specific activity decreases when the protein/lipid ratio is decreased from 1:5 to 1:75.

In Table II are given some results concerning the effect on recovery of specific enzyme activity and symmetry of reconstitution of variation in the lipid source, the proportion of the different lipids in the mixture and of changing the protein to lipid weight ratio.

Under certain conditions namely when PS or better PI is present and with an 1:10 protein/lipid ratio it is possible to obtain about 100% recovery of the specific enzyme activity 24 h after reconstitution. (The vesicles can be stored frozen at −70°C for weeks with no loss of activity.) In these experiments about 10% of the enzyme molecules are oriented i/o. With the same lipids but a lower protein/lipid ratio, 1:20, the fraction of (i/o)-oriented molecules is increased to about 19% but the recovery of the specific activity decreases to about 80%. With an 1:50 protein/lipid ratio the fraction of (i/o)-oriented molecules increased to 25% but the recovery of specific enzyme activity was only 60%.

Table II also shows that dioleoylphosphatidylcholine, which is an unsaturated PC, with an 1:10 ratio of protein/lipid give a higher fraction of (i/o)-oriented enzyme molecules, 16%, than with the purified egg yolk PC/PE/PS/cholesterol (60:14:2:24) lipid mixture but the recovery of the specific enzyme activity is lower, about 63%.

The results thus show that it is possible to obtain full recovery of the specific activity of the incorporated enzyme by choosing a proper combination of lipids and by using a relatively high protein to lipid ratio. But the fraction of molecules which are incorporated in the (i/o)-orientation is relatively low, about 10%. Conditions which give a higher fraction of inside-out oriented molecules leads to a decrease in recovery.

Active and passive ^{22}Na influx

In Fig. 11 is shown a typical passive ^{22}Na influx experiment using liposomes with a protein to lipid ratio of 1:20. The liposome medium was 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+}, 30 mM histidine (pH 7). The time-course of isotopic equilibration could be adequately described as monoexponential as indicated in the lower part of the figure. The rate constant deduced from the log plot was 0.016 min^{-1}.

Fig. 12 shows the K^+ + ATP-dependent Na^+ influx. The lipids were PC/PE/PS/cholesterol (60:14:2:24) with a 1:20 protein/lipid ratio. The initial rate of the Na^+ flux is fast compared to the time it takes for the sampling and filtration of the liposomes on the ion exchange column. This makes it difficult to get an exact measure of the initial Na^+ influx, but using the straight line shown in Fig. 12 as the initial slope, the flux is 30 μmol Na^+ per mg (i/o)-oriented protein per min at 22°C. The 'ouabain/ionophore' method was used to detect the orientations. The catalytic activity

TABLE II

SYMMETRY OF INCORPORATION AND RECOVERY OF THE SPECIFIC ENZYME ACTIVITY AFTER RECONSTITUTION INTO VESICLES WITH DIFFERENT LIPID COMPOSITIONS

The orientation was measured with the 'ouabain/ionophore' method (see text). The specific enzyme activity was measured after reopening the liposomes by addition of $C_{12}E_8$ and is given in % of the specific activity of the solubilized enzyme used for reconstitution and stored under the same conditions.

Lipids (weight ratio)	Protein/lipid (weight ratio)	Symmetry of reconstitution (i/o : n-o : r/o) (%, mean ± S.D.)	Recovery of specific activity after 24 h in % of specific activity of solubilized enzyme used for reconstitution (mean ± S.D.)	No. of independent expts. (n)
Natural lipids				
Asolectin	1:10	18±4/33±17/49±13	72±12	3
S. acanthias lipid	1:10	14/43/43	71	1
Purified lipids				
PC/PE/PS/chol (60:14:2:24)	1:10	10±1/34±3/56±2	95± 4	5
PC/PE/PI/chol (60:14:2:24)	1:10	10±2/37±2/53±1	99± 3	3
PC/PE/PS/chol (60:14:2:24)	1:20	19±3/25±5/56±3	81± 3	3
PC/PE/PS/chol (75:9:1:15)	1:20	18±5/23±4/60±6	45± 1	3
PC/PE/PS/chol (36:44:5:15)	1:20	16±3/23±1/61±4	30± 6	2
PC/PE/PI/chol (60:14:2:24)	1:20	16±1/24±4/60±4	87± 6	3
PC/PE/PS/chol (60:14:2:24)	1:50	24±5/11±2/65±6	62± 8	4
PC/PE/PI/chol (60:14:2:24)	1:50	26±6/10±1/64±6	59± 7	3
Synthetic lipid				
DOPC	1:10	16±1/27±1/57±1	63±10	3

was 9.3 μmol ATP hydrolysed per min per mg protein at 22°C. This gives a stoichiometry of 3.2 Na^+ transported per ATP hydrolysed.

The specific activity of the reconstituted enzyme decreases with a decrease in the protein to lipid ratio, Fig. 10 and Table II. This is also the case for the active Na^+-influx which amounts to 36, 30, and 23 μmol Na^+ per mg (i/o)-protein per min at 22°C in the 1 : 10, the 1 : 20, and the 1 : 50 protein/lipid liposomes, respectively. With 3 Na^+ transported per ATP hydrolyzed these figures correlate to specific activities of 12, 10 and 7.7 μmol P_i per mg (i/o)-protein per min at 22°C; or as shown in Fig. 10 (◇) to 713, 600, and 463 μmol P_i per mg (i/o)-protein per h at 22°C. As also seen from Fig. 10, assuming the 3 Na^+ : 1 ATP stoichiometry as above, the active Na^+ flux decreases parallel to the decrease in specific activity when the protein/lipid ratio is decreased. The specific activity of the solubilized enzyme used for the reconstitution was 713 μmol P_i per mg protein per h at 22°C (arrow in Fig. 10); the recovered specific activity as well as the 'recovered' active Na^+-influx was thus 100%, 84%, and 65% with the 1 : 10, the 1 : 20, and the 1 : 50 protein/lipid liposomes, respectively, see also Table II. The recovery of the enzyme activity is given as recovery of specific activity. However, since all the added protein is incorporated and as the specific activity of the enzyme in the different orientations is the same, the recovery of specific activity in percent of the specific activity of the solubilized enzyme used for incorporation also expresses the recovery of total activity.

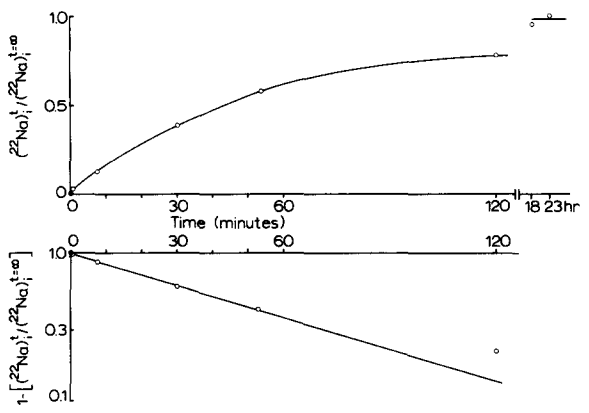

Fig. 11. Passive ^{22}Na-tracer influx into reconstituted liposomes in the absence of ATP at 22 °C. The liposomes were produced and tested in the presence of 130 mM Na^+, 20 mM K^+ and 4 mM Mg^{2+}. Lipid composition of the liposomes was PC/PE/PS/cholesterol = 60:14:2:24, the protein/lipid ratio was 1:20.

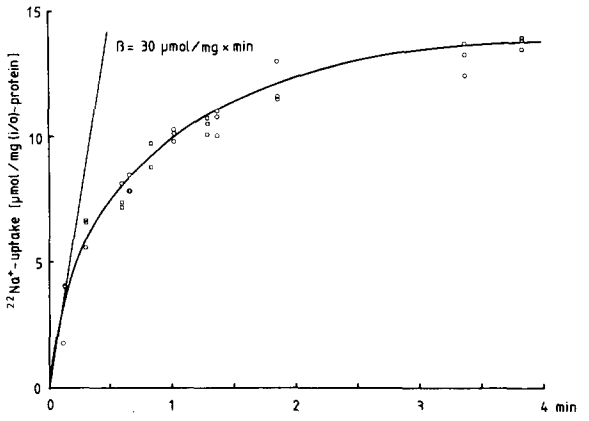

Fig. 12. ATP-dependent, K^+-stimulated Na^+-influx into 1:20 protein/lipid vesicles at 22 °C. The lipid composition was PC/PE/PS/cholesterol = 60:14:2:24. Two independent experiments are shown (○ and □). The liposomes were produced in a histidine buffer (30 mM, pH = 6.8) containing Na^+ (130 mM) and Mg^{2+} (4 mM) and preincubated for 5 min in the same buffer with addition of 20 mM K^+, $3 \cdot 10^{-6}$ M valinomycin and $3 \cdot 10^{-4}$ M CCCP to equilibrate with K^+. The initial Na^+-influx is calculated from the slope of the straight tangent line indicated. The specific ATPase activity of the starting material was 713 μmol/mg per min at 22 °C and 599 μmol/mg per h after reconstitution measured on reopened liposomes. The symmetry of orientation was $f_{(i/o)} = 18.6\%$; $f_{(n-o)} = 27.6\%$; $f_{((r/o)} = 53.8\%$.

Discussion

As seen from the present paper the recovery of specific enzyme activity after reconstitution is found to be critically dependent on the protein/lipid ratio. At a given lipid composition a decrease in the protein/lipid ratio from 1:10 decreases the recovery. The concomittantly increase in the detergent/protein ratio necessary to obtain the mixed protein/lipid/detergent micelles does not explain the decrease in recovery. An increase by 7.5-times in the concentration of $C_{12}E_8$ in the 1:10 protein/lipid vesicles corresponding to the detergent/protein ratio in 1:75 protein/lipid liposomes has no effect on recovery which shows that the decrease in activity is not due to an inactivation effect of the detergent. Another possibility is that it may be necessary to have a certain number of enzyme molecules present per membrane area in the vesicles either to allow cooperation between the molecules or in order to lower the transition temperature of the protein/lipid mixture.

Using active Na^+ transport as an indication Anner et al. [5] finds that a protein/lipid ratio of 1:14 is optimal. This is at variance with other reports in the literature [6,8] that the optimal ratio is around 1:40. The sodium flux is given as micromoles per mg vesicles protein per min. In the present experiment the initial active Na^+ flux per mg vesicle protein per min increases with a decrease in the protein/lipid ratio from 1:5 to 1:50. However, the fraction of protein which is responsible for the active influx, the (i/o)-oriented enzyme molecules, also increases with a decrease in the protein/lipid ratio. Taken together this means that the Na^+ flux per mg (i/o)-oriented enzyme protein per min decreases when the protein/lipid ratio is decreased.

The highest initial active Na^+ flux so far reported is 1.6 μmol per mg vesicle protein per min at 22°C in 1:40 protein/lipid liposomes [8]. Vanadate inhibition of passive rubidium flux indicated a random orientation of the enzyme molecules [27] suggesting an initial Na^+-flux of 3.2 μmol per mg (i/o)-oriented protein per min at 22 °C. This corresponds to a low percentage of the catalytic activity of the starting material which was 18–24 μmol P_i per mg protein per min at 37 °C. In

the present experiments the initial Na^+-flux in 1 : 50 protein/lipid liposomes is about 23 μmol Na^+ per mg (i/o)-oriented protein per min at 22°C increasing to about 35 μmol Na^+ per mg (i/o)-oriented protein per min at 22°C with 1 : 10 protein/lipid liposomes. With 3 Na^+ transported per ATP hydrolyzed the 35μmol per mg (i/o)-oriented protein per min corresponds to the specific catalytic activity of the solubilized enzyme used for the incorporation.

At a given protein : lipid ratio the recovery of enzyme activity after reconstitution depends on the lipid composition of the liposomes. Inclusion of acidic phospholipids, preferably PI, and of cholesterol increases the recovery.

With the method described in this paper the volume of the lipid vesicles is about 10–13 μl per mg lipid. This is much larger than the trapping capacity of liposomes obtained by the cholate dialysis technique or by the sonication techniques [28], and comparable to the trapping capacity of liposomes produced by the freeze-thaw sonication technique [8]. One advantage of having bigger liposomes is that at a given protein/lipid ratio the number of molecules incorporated per unit of volume is lower.

A fraction of about 30% of the enzyme molecules is reconstituted in a fashion where both cytoplasmic and extracellular side are exposed to the extravesicular medium (n-o). A similar or greater fraction whose catalytic activity can be inhibited by external ouabain has previously been reported [2,4,29,30] and attributed to unincorporated enzyme. The (n-o)-oriented enzyme molecules bind to the concanavalin A which suggests either that it is right-side out oriented molecules inserted into leaky vesicles or that it is enzyme adsorbed to the external side of the vesicles with both sides exposed. The observation that the fraction of (n-o) decreases in parallel to an increase in the fraction of (i/o) while the (r/o) stays constant when the protein/lipid ratio is decreased may support the last view.

Asymmetric incorporation of ($Na^+ + K^+$)-ATPase into liposomes with up to 85% of the enzyme in the right-side-out orientation has previously been reported [31] with a reconstitution procedure rather similar to the one described here except for the use of Triton X-100 instead of $C_{12}E_8$. However, the recovered ATPase activity was only 10% of initial, the preparation could not be activated by addition of detergents, and the protein/lipid ratio used was higher than the highest used in the present study.

So far no explanation for the asymmetric insertion of the ATPase into liposomes can be offered. The observation that random orientation is found when cholate is used for reconstitution [4,27] may indicate that parameters such as the detergent employed or the rate of detergent elimination is of importance.

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