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KINETIC, BINDING AND ULTRASTRUCTURAL PROPERTIES OF THE BEEF HEART ADENINE NUCLEOTIDE CARRIER PROTEIN AFTER INCORPORATION INTO PHOSPHOLIPID VESICLES

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Summary

1. ADP/ATP transport has been reconstituted by incorporation of the purified carrier protein in liposomes filled with ATP. The transport was assayed by uptake of [^{14}C]ADP into the liposomes, and by release of ATP as determined by a luminescence technique. [^{14}C]ADP uptake was strictly dependent on internal ATP.

2. The simplest phospholipid system capable of yielding high rates of ADP/ATP transport was a mixture of phosphatidylethanolamine and cardiolipin (92 : 8, w/w).

3. ADP/ATP transport in the reconstituted system proceeded by exchange-diffusion with a 1/1 stoichiometry. The specificity for ADP and ATP was absolute. The capacity and the rate of exchange depended on the concentration of ATP present in liposomes. The rate of transport at 20°C, at 20 mM internal ATP, routinely ranged between 300 and 1000 nmol of nucleotide exchanged per min/mg of added carrier protein. The apparent K_m value for external ADP was around 10 μM.

4. The ADP/ATP exchange in the reconstituted system was rather stable to ageing. It dropped by only 20% after 1 day of ageing at 20°C. Divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}) at concentrations higher than 1 to 2 mM had a deleterious effect on ADP/ATP transport, concomitant with the release of internal ATP and accumulation of multilamellar vesicles.

Abbreviation: LAPAO, 3-laurylamido-*N,N'*-dimethylpropylaminoxide.

5. Atractyloside behaved as a competitive inhibitor and carboxyatractyloside as a non-competitive inhibitor. Bongkrekic acid required a slightly acidic pH to be inhibitory. The data concerning atractyloside, carboxyatractyloside and bongkrekic acid were similar to those obtained with whole mitochondria, suggesting that the carrier protein in liposomes has the same asymmetrical arrangement as in mitochondria.

6. The percentage of competent carrier protein in liposomes was calculated from dose-response data concerning the inhibition of ADP/ATP transport by atractyloside or carboxyatractyloside, and from the amount of bound [^{3}H]-atractyloside removable by ADP. By both methods, 3 to 6% of the added carrier protein was found to be competent in ADP/ATP transport, based on the assumption that the binding of one atractyloside or carboxyatractyloside molecule per 30 000 molecular weight carrier unit results in complete inhibition of transport.

7. Freeze-fracture electron microscopy showed that the ADP/ATP carrier protein-lipid preparations are formed by small vesicles, most of which give rise to smooth fracture faces (probably pure lipid vesicles). Only a small percentage of the vesicles (2 to 4% depending on the amount of carrier protein added) were clearly particulated. About 90% of the particulated vesicles showed no more than 2 particles per vesicle and only 5% more than 5 particles per vesicle. The distribution of the particles between convex and concave fracture faces was asymmetric; about 2/3 of the protein molecules were anchored at the external surface of the vesicles and only 1/3 at the internal one. This asymmetric distribution was not significantly modified after the addition of carboxyatractyloside but changed drastically upon addition of bongkrekic acid, leading to an increased percentage of protein molecules anchored at the internal surface of the vesicles.

8. The above experimental data suggest that the ADP/ATP carrier protein is able to move by translation across the phospholipid membrane. The data are interpreted by assuming that the carrier is stabilized in a conformation more exposed to the inside upon bongkrekic acid binding, and to the outside upon (carboxy)atractyloside binding. A similar translational motion could be involved in ADP/ATP transport.

Introduction

Shertzer and Racker [1,2] recently reported the first successful reconstitution of the mitochondrial adenine nucleotide carrier activity by incorporation of the purified protein into phospholipid vesicles, using a sonication procedure. These reports were corroborated by Krämer and Klingenberg [3,4] with the carrier protein purified by another method. Although the two teams undoubtedly presented evidence for reconstitution of adenine nucleotide transport, some of their data appeared to be conflicting, in particular those concerned with the specificity of phospholipids, the optimum pH, and the effect of metal ions. Furthermore, uptake of [^{14}C]ADP or [^{14}C]ATP was exclusively used as the criterion to assess transport activity, but no evidence was presented to show that the uptake of external adenine nucleotide was compensated by the release

of internal adenine nucleotide as is the case in intact mitochondria.

The aim of the present work was to correlate functional and ultrastructural studies of the ADP/ATP carrier protein incorporated in liposomal membranes. In this particular case, reconstitution experiments offered the possibility to explore in more detail the asymmetry of the ADP/ATP carrier in terms of inhibitory effects and binding of specific inhibitors. In intact mitochondria, atractyloside and carboxyatractyloside, two impermeant inhibitors, bind to the outer face of the inner mitochondrial membrane whereas bongkrekic acid, a pemeant inhibitor attacks the carrier from the inside. The inhibitory and binding properties of these inhibitors have been studied in the reconstituted system. The studies have been coupled to ultrastructural investigation of the incorporated carrier protein, using the freeze fracture electron microscopy.

Materials and Methods

[^{14}C]ADP (Commissariat à l'Energie Atomique, Saclay, France) was purified by chromatography on Dowex AG1X8, 20—50 mesh (Serva). The final radioactivity was $5 \cdot 10^6$ dpm/μmol. Other nucleotides and atractyloside were obtained from Sigma Chemical Co. Carboxyatractyloside was obtained from Boehringer. Bongkrekic acid was prepared according to the method of Limjbach et al. [5] as modified by Lauquin et al. [6]. All other reagents were analytical grade. Beef heart mitochondria were isolated by the method of Smith [7]. Egg phosphatidylethanolamine and phosphatidylcholine were purified according to Lea et al. [8]. Phosphatidylethanolamine and phosphatidylcholine were kept at —80°C under argon and repurified before use by chromatography on a column filled in the upper half with a mixture of silicic acid and Celite 545 (2 : 1, w/w) and in the lower half with cellulose CF11, using the same solvent system as for the preliminary purification. Cardiolipin from beef heart was a gift of Dr. Faure, Institut Pasteur, Paris. Lysophosphatidylethanolamine was prepared as described in Ref. 9. Luciferase was purified from a commercial sample (Lumac Systems, Basel) and freed of traces of adenylate kinase by Sephadex G-100 chromatography as described by Rasmussen and Nielsen [10]. [^{3}H]Atractyloside was prepared as previously described [11]. The detergent used for extraction of the ADP/ATP carrier protein from mitochondrial membrane, 3-lauryl-amido-*N*,*N*'-dimethylpropylaminoxide (LAPAO), was synthetized in two steps. First *N*,*N*-dimethyl-*N*-(3-laurylamidopropyl)amine was made as described by Muzyczko et al. [12]. This compound was then oxidized to form the aminoxide, following the same procedure as that described by Applebury et al. [13]. The ADP/ATP carrier protein from beef heart mitochondria was prepared by chromatography on hydroxyapatite (Biorad) as described by Krämer and Klingenberg [3], except that the detergent used was pure LAPAO instead of aminoxide WS35 which is a mixture of LAPAO and longer chain aminoxides.

In routine preparations, 55 μl of a mixture of 10% LAPAO (w/v), 1 M sodium sulfate, 1 mM EDTA and 100 mM Tricine-KOH pH 7.4 was added to 0.5 ml of a beef heart mitochondria suspension in 0.25 M sucrose containing 30 to 50 mg protein/ml. The suspension was incubated for 5 min at 0°C and the lysate was centrifuged at 20 000 $\times g$ for 5 min. A 300 μl fraction of the super-

natant was placed on a small column of 1.5 cm diameter filled with 4 ml of decanted hydroxyapatite gel. The column was eluted with a medium consisting of 0.5% LAPAO (w/v), 0.1 M sodium sulfate, 0.1 mM EDTA and 10 mM Tricine-KOH, pH 7.4. The elution was monitored by absorbance of ultraviolet light at 254 nm. As already shown by Krämer and Klingenberg using the same chromatographic procedure with aminoxide WS35 [3] or Triton X-100 [4] as detergents, the first elution peak contained the 30 000 molecular weight ADP/ATP carrier protein purified to an extent of about 80%, as assessed by SDS-polyacrylamide gel electrophoresis.

Reconstitution of ADP/ATP transport with the purified carrier protein consited of two steps. The first one was the preparation of liposomes filled with ATP. In standard assays, a chloroform solution containing 40 mg of phospholipids was dried under argon at 20—30°C. The dried lipid film was Vortex-dispersed in 1 ml of a medium made of 20 mM ATP, 100 mM glycerol, 0.1 mM EDTA and 10 mM Tricine-KOH, pH 7.4. The lipid dispersion was left to stand overnight at 4°C before being subjected to sonication. A Branson sonifier (Model W185D) was used with a titanium microtip. Sonication was carried out at 40—50 W under an argon atmosphere for 10 to 15 min in ice. The second step consisted in the incorporation of the purified carrier protein into the liposomes. A small aliquot of the protein (60 to 100 μg) in 100 to 200 μl of LAPAO solution was added to the liposome suspension and let to incubate for 5 min at 0°C. Then the mixture was submitted to a second sonication for 20—30 s in ice. Proteoliposomes were freed of external ATP, by passage through a column of Dowex AGIX8 (20—50 mesh) of 0.6 cm diameter and 25 cm height equilibrated and eluted with 136 mM glycerol, as described in Ref. 14. Freeze-fracture electron microscopy showed that the preparations contained almost exclusively small vesicles, with a diameter of 60 ± 20 nm, when small amounts of protein-detergent were used for reconstitution. More heterogenous preparations with bigger vesicles (120 ± 60 nm) were obtained when larger amounts of protein-detergent were used.

The amount of the ADP/ATP carrier protein in LAPAO which can be added to liposomes is limited by the deleterious effect of the detergent on the transport activity and the structure of the liposomes (Fig. 1). In the present work, the amount of LAPAO brought with the added carrier to liposomes was between 0.1 and 0.2 mol LAPAO/mol phospholipid. This amount of LAPAO was 4 to 5 times less than the critical amount resulting in 90% inactivation of transport (Fig. 1). A criterion of integrity of ATP-loaded proteoliposomes is the retention of the entrapped ATP. Breakdown of liposomes as assessed by the release of ATP was 3 to 4 times less susceptible to LAPAO than transport activity.

Adenine nucleotide transport was measured by a radioactive assay and by a luminescence technique at 20—22°C. In a large number of experiments, the two methods were used in parallel. In both cases, the medium contained 136 mM glycerol, 0.5 mM $MgCl_2$, 5 mM Tricine-KOH buffer and 0.05 to 0.3 ml of the proteoliposome suspension. The final volume was 0.5 ml; unless stated, the final pH was 7.4. With this medium, isoosmolarity was maintained across the vesicle membrane; this was important to avoid vesicle shrinking or swelling due to osmotic forces. In the radioactive assay, transport was initiated by addition

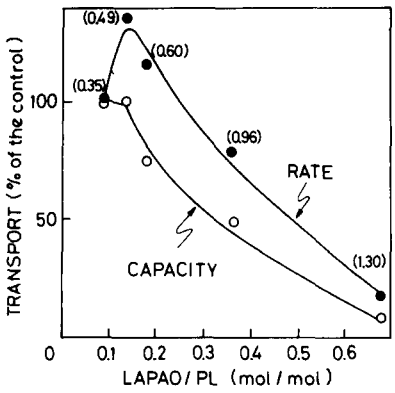


Fig. 1. Effect of LAPAO on the rate and the capacity of transport of the ADP/ATP carrier in reconstituted proteoliposomes. The rate and capacity of ADP/ATP transport were estimated by the radioactivity assay and the luminescence assay. The data plotted are the means of the two assays. The ratio of LAPAO/phospholipid was increased by adding more of the protein preparation in detergent to a fixed amount of phospholipids made of 92% phosphatidylethanolamine and 8% cardiolipin. The following amounts of protein, 19 μg, 28 μg, 38 μg, 76 μg and 142 μg in 40 μl, 60 μl, 80 μl, 160 μl and 300 μl of 1% LAPAO were added to 9 mg of phospholipids in 0.22 ml of medium consisting of 20 mM ATP, and sonicated for 20 s at 0°C. Dowex treatment yielded proteoliposomes with the nominal molar ratios of LAPAO to phospholipid indicated in the abscissa. The values into brackets in the figure refer to concentrations ((LAPAO/PL, M/mmol).

of 10 μl of a solution of [^{14}C]ADP (routine final concentration 50 μM) and terminated by the addition of 15 μl of a mixture of carboxyatractyloside and bongkrekic acid to yield a final concentration of 3 μM for each inhibitor. After termination of transport activity, the mixture was passed through a Dowex column, to eliminate external [^{14}C]ADP (cf. Ref. 14), and the radioactivity of a 2 ml fraction of the eluate was counted in 10 ml of a scintillation fluid [15]. Blanks consisted in addition of [^{14}C]ADP to proteoliposomes preincubated with 3 μM carboxyatractyloside and 3 μM bongkrekic acid for 2 min. In the luminescence assay, the incubation medium was supplemented by 50 μl of a solution of purified luciferase and luciferine. Unless stated, the final pH was 7.4. Efflux of ATP was routinely initiated by addition of 10 μl of a solution of ADP (final concentration 50 μM). Light emission was recorded with the photomultiplier of an Aminco-Chance spectrophotometer. The amount of luciferase-luciferin added to the medium was such that the response rate of the luminescence probe was in large excess of the rate of ATP efflux (at least 1000 times higher); the luminescence response was always proportional to the rate of ATP efflux. The luminescence assay was used either for continuously monitoring ATP efflux from liposomes after addition of ADP, or for assaying, in aliquot samples, the ATP released at different times after termination of the transport reaction by carboxyatractyloside and bongkrekic acid.

Electron microscopy

The samples were incubated with glycerol (25% w/v final concentration) before being rapidly frozen in liquid Freon 22 at −160°C. Platinum-carbon replicas were obtained using a Balzers 301 freeze-etching unit equiped with an electron gun for Pt-C shadowing. The organic material was digested with

chromic acid and the replicas were examined in a Philips 301 electron microscope.

Specific binding of [^{3}H]atractyloside to the reconstituted ADP/ATP carrier

As atractyloside and ADP (or ATP) compete for binding to the ADP/ATP carrier, specific binding of atractyloside to proteoliposomes was quantitated as ADP-inhibitable binding of [^{3}H]atractyloside. Samples of proteoliposomes (0.7 ml) consisting of 5 mg of sonicated phospholipids and the incorporated carrier protein were introduced into two series of 0.8 ml plastic tubes containing increasing concentrations of [^{3}H]atractyloside ($30 \cdot 10^6$ dpm/μmol). The second series of tubes differed from the first one by the presence of 250 μM ADP. The tubes were incubated for 30 min at 20°C for full equilibration of bound and free atractyloside and then inserted with adapters into a SW50-1 Beckman rotor. After 1 h centrifugation at 45 000 rev./min, the tubes were withdrawn, the supernatant fluid removed by aspiration and the interior of the tubes carefully blotted with filter paper strips. Then the bottom of the tubes containing the sedimented proteoliposomes was cut out and introduced into scintillation vials. The sediments were dispersed by stirring for 2 h in 2 ml of ethyl ether, and radioactivity was counted in 10 ml of a scintillation fluid [15].

Results

Effect of the phospholipid composition of liposomes on the rate of ADP/ATP transport in the reconstituted system

The rate of ADP/ATP transport was markedly dependent on the phospholipid composition of the proteoliposomes (Table I). Clearly a mixture made of a large percentage of phosphatidyl ethanolamine (92%) supplemented by a small amount of cardiolipin (8%) was the simplest phospholipid system to yield high rates of transport. A complementary addition of a small amount of lysophosphatidylethanolamine (less than 3%) slightly stimulated the rate of transport. The cardiolipin to phosphatidylethanolamine ratio required to obtain efficient proteoliposomes was found to be critical. Formation of liposomes by sonication of mixtures of the two phospholipids required a minimal concentra-

TABLE I

EFFECT OF THE PHOSPHOLIPID COMPOSITION OF LIPOSOMES ON THE RATE OF ADP/ATP TRANSPORT

The mixtures of phospholipids in chloroform were dried under argon and the liposomes prepared as described under Materials and Methods: Phosphatidylethanolamine (PE); phosphatidylcholine (PC); cardiolipin (CL); lysophosphatidylethanolamine (LPE). The rate of transport was determined by the radioactivity assay as described under Materials and Methods.

PE	PC	CL	LPE	Rate of transport [^{14}C]ADP uptake (nmol/min/mg)
92	0	8	0	745
90.8	0	6.3	2.9	783
80	13.3	6.7	0	556
77.8	12.8	6.4	3.0	313
0	92	8	0	112

tion of 4 to 5% cardiolipin. The most efficient proteoliposomes for ADP/ATP transport were obtained with mixtures of phosphatidylethanolamine and cardiolipin where the cardiolipin percentage amounted to 7–10%. Proteoliposomes made of phosphatidylcholine alone or a large percentage of phosphatidylcholine mixed with other phospholipids had a poor efficiency of transport.

The capacity of transport which is defined as the maximal amount of [^{14}C]-ADP accumulated in 30 min (plateau phase) was roughly proportional to the rate of transport with the different phospholipid species, routinely ranging between 0.7 and 2 μmol ADP/mg added protein for phosphatidylethanolamine/cardiolipin liposomes to values as low as 0.05 μmol ADP/mg protein for phosphatidylcholine liposomes.

Demonstration of a 1/1 exchange-diffusion process. Kinetics and specificity of transport

All of the following experiments were carried out with proteoliposomes in which the phospholipids consisted of a mixture of 92% phosphatidylethanolamine and 8% cardiolipin. Kinetics of transport were followed both by [^{14}C]-ADP uptake and ATP release, using proteoliposomes loaded with 20 mM ATP. Transport was initiated by addition of [^{14}C]ADP to the proteoliposomes suspension. No release of ATP was detected by the luciferase reaction in proteoliposomes in the absence of external ADP, or in liposomes loaded with ATP, but devoid of carrier protein.

The experiment illustrated in Fig. 2 consisted in sampling the proteoliposome suspension at different periods after addition of [^{14}C]ADP, and assaying for [^{14}C]ADP incorporation and ATP release. The amount of ADP incorporated was equal to that of ATP released at any time of the transport process.

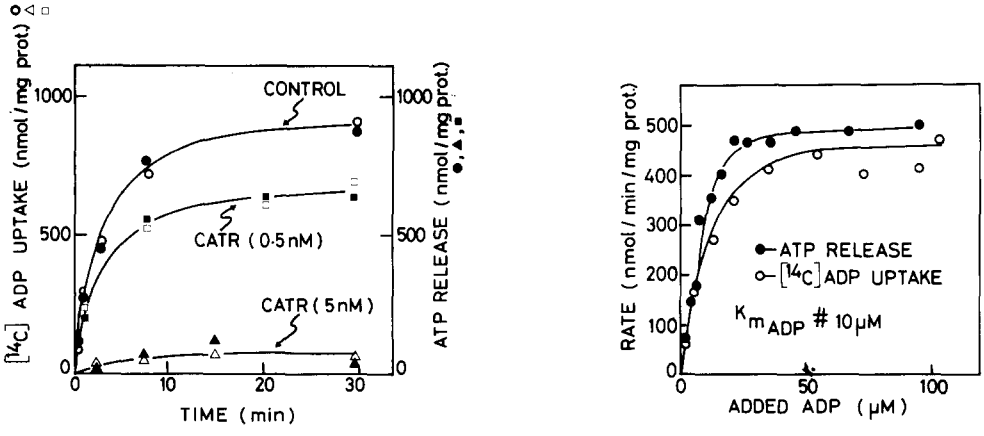


Fig. 2. Time course of ADP/ATP exchange assessed by radioactivity and luminescence assays. Assays were performed as described under Materials and Methods with an amount of proteoliposomes corresponding to 0.45 μg protein. The activity was corrected for the carboxyatractyloside- and bongkrekic acid-insensitive rates (see Materials and Methods). The ATP released by ADP/ATP exchange was assayed in samples incubated for the times indicated. CATR, carboxyatractyloside.

Fig. 3. Effect of increasing concentrations of ADP on the rate of ADP/ATP exchange assessed by the radioactivity and luminescence assays. Same conditions as in Fig. 2.

A one to one stoichiometry was also observed under conditions of partial and nearly full inhibition by carboxyatractyloside. Under standard conditions, the initial kinetics was first order. The maximal rates of transport routinely observed at 20°C ranged between 300 and 1000 nmol of ADP exchanged per min and per mg of added protein; a few preparations had an activity up to 2000 nmol of exchanged ADP/min/mg of added protein. These data pointed to large variation in the percentage of competent carrier protein incorporated in liposomes. Beside possible alteration of the carrier protein, another major critical factor of transport activity appeared to be the purity of the phospholipid used.

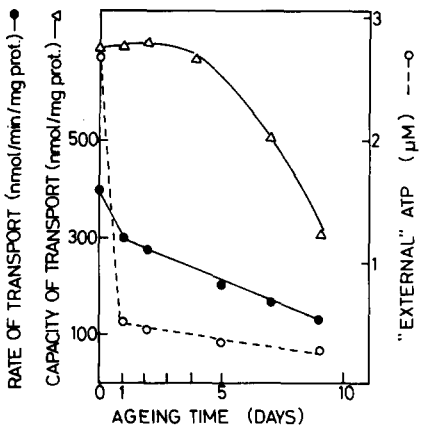
The K_m value for external ADP was determined both by [^{14}C]ADP uptake and ATP efflux (Fig. 3). In both assays, the mean K_m value (±S.D.) calculated from 8 experiments was 10 ± 2 μM, which was very close to the K_m value found for ADP transport in intact mitochondria. The constancy of the K_m value can be contrasted with the variation in maximum rates of transport suggesting that successful reconstitution is an all or non phenomenon.

The effect of internal ATP on the rate and the capacity (plateau) of ADP_{ex}/ATP_{in} exchange was also tested with liposomes loaded with varying concentrations of ATP. In the absence of internal ATP, there was no measurable [^{14}C]-ADP uptake. The capacity of exchange increased monotonically with increasing concentrations of internal ATP. The rate of exchange, using a saturating concentration of external ADP (100 μM), increased also with concentration of ATP to reach an apparent plateau at 20 mM ATP. It is probable that the concentration in internal ATP determines the required gradient which provides the driving force favorable for ADP/ATP exchange.

Increasing concentration of proteoliposomes proportionally increased both the rate and the total capacity of transport. The specificity for ADP and ATP was quite strict. The luminescence technique of assay of ADP/ATP transport, which is much more sensitive than the radioactivity method, could not reveal any release of internal ATP upon addition of AMP, IDP, CDP, GDP and UDP.

Effect of ageing

Proteoliposomes were kept at 20°C for 24 h and then at 4°C (Fig. 4). The transport activity dropped by about 20% after 24 h at 20°C and then slowly decreased upon standing at 4°C. The half time of inactivation at 4°C was 5 to 6 days. The transport capacity was stable at 4°C for 4 days. The data in Fig. 4 show the presence in proteoliposome suspensions of ATP which can be assayed by the luminescence technique in the absence of added ADP. It must be recalled that the proteoliposomes had been already passed on a Dowex column to eliminate the excess of external ATP. In spite of Dowex chromatography, the proteoliposome suspension still contained some residual external ATP, accessible to added luciferase. Repeated Dowex treatments hardly lowered the residual external ATP. This ATP does not arise by the adenylate-kinase-dependent transphosphorylation of ADP. It was present in large amounts immediately after preparation of proteoliposomes, therefore precluding accurate determination by the luminescence assay of initial kinetics of ADP/ATP exchange; its concentration markedly dropped after a few h of standing at room temperature; it then remained virtually stable for several days at 4°C. After 1 day at 20°C, less than 15% of residual external ATP initially present was left (Fig. 4).



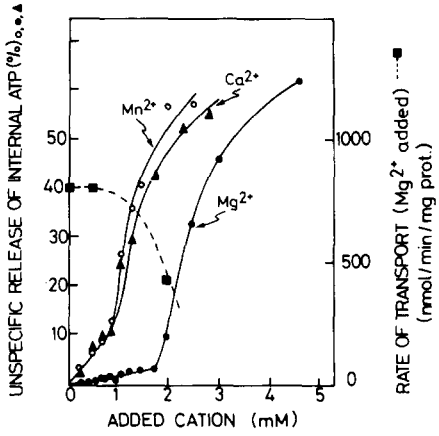


Fig. 4. Effect of ageing proteoliposomes on the rate and capacity of ADP/ATP transport and on the concentration of externally bound ATP. The proteoliposomes were kept for 1 day at 20°C and for the rest of the time at 4°C. Kinetics of transport was measured by the luminescence method (cf. Materials and Methods). The protein present in proteoliposomes was 0.42 μg.

Fig. 5. Effect of cations on ADP/ATP transport activity and leakage of proteoliposomes. Transport assay was carried out by the luminescence method as described under Materials and Methods. The amount of protein in added proteoliposomes was 0.45 μg. Unspecific ATP release in the absence of added ADP was also assessed by the luminescence method; the percentage of ATP released was calculated on the basis of total internal ATP, which was assayed after lysis of proteoliposomes by 0.1% Triton X-100 (final concentration).

On the other hand the rate of transport was decreased by only 15% after 1 day at 20°C. These data clearly indicate that a short period of ageing is beneficial before assaying kinetics of transport, at least by the luminescence technique; in most of the experiments reported in this paper, the proteoliposomes were aged for 16 h at 20°C before assay of transport activity. Although no satisfactory explanation can be given at present, it appears that the physical state of the lipid bilayer may play a critical role in this process.

Effect of Mg^{2+} and other divalent cations

Mg^{2+} is required for the luciferase reaction. As no deleterious effect of 0.5 mM Mg^{2+} on ADP/ATP transport was revealed by the [^{14}C]ADP uptake assay, Mg^{2+} was routinely added at that concentration. Above 2 mM $MgCl_2$, transport activity dropped abruptly. This deleterious effect of Mg^{2+} on ADP/ATP transport is probably related to the release of internal ATP from liposomes (Fig. 5). In fact, freeze etching of liposomes treated by millimolar concentrations of Mg^{2+} revealed a large number of fused multilamellar vesicles. Ca^{2+} and Mn^{2+} at concentrations higher than 1 mM also resulted in the release of ATP from liposomes (Fig. 5).

Effect of pH

The rate of transport did not critically depend on pH. A broad optimum activity was found between pH 6.5 and 7.8 with a maximum around pH 7. The transport capacity was maximum between pH 7.2 and 7.6.

Sidedness of the ADP/ATP carrier protein in liposomes. Inhibition by atractyloside, carboxyatractyloside and bongkrekic acid

In the following experiments, inhibitors were preincubated with proteoliposomes for 2 min at 20°C. This preincubation period was sufficient for equilibration with the carrier protein. Furthermore, for better accuracy, the inhibition data utilized for kinetic analysis were obtained by the luminescence method of evaluation of transport rates. Control assays carried out by the radioactivity method were in good agreement with the luminescence assays.

As shown in Fig. 6, atractyloside at increasing concentrations decreased progressively the rate of transport; however the total capacity of transport remained unchanged. In contrast, carboxyatractyloside decreased both the rate and the capacity of transport. This can be explained by the nature of the inhibition, reversible in the case of atractyloside, irreversible for carboxyatractyloside, and also by the small amount of carrier protein incorporated in phospholipid vesicles (see Discussion, Section 3). Nearly complete inhibition (85–90%) by carboxyatractyloside was achieved at concentrations as low as 10 nM. However, at 100 nM carboxyatractyloside, 5 to 10% of the transport activity was still present; this residual activity was totally inhibited by further addition of 100 nM bongkrekic acid. It has been established by studies with whole mitochondria that atractyloside and carboxyatractyloside are non-penetrant inhibitors whereas bongkrekic acid is a penetrant inhibitor at slightly acidic pH [16]. In keeping with these data, it may be inferred that the ADP/ATP carrier

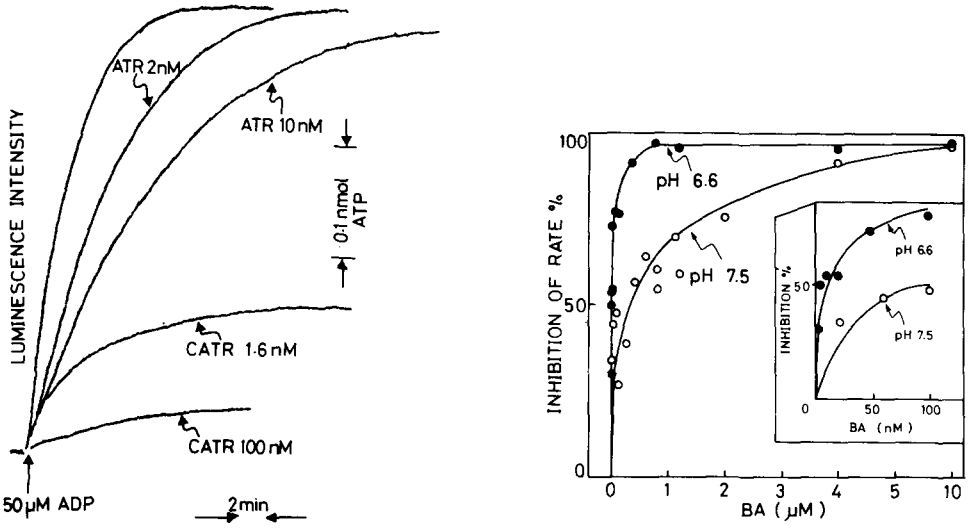


Fig. 6. Inhibition of ADP/ATP transport by atractyloside and carboxyatractyloside. Atractyloside (ATR) and carboxyatractyloside (CATR) were preincubated at the indicated concentrations for 2 min at 20°C with proteoliposomes (0.42 μg protein) before addition of ADP. The transport was followed by the luminescence method as described under Materials and Methods.

Fig. 7. Inhibition of ADP/ATP transport by bongkrekic acid. Bongkrekic acid (BA) was preincubated at the indicated concentrations for 2 min at 20°C either at pH 6.6 or pH 7.5 with proteoliposomes (0.46 μg protein) before addition of ADP. The transport was followed by the luminescence method as described under Materials and Methods. Inhibitions at nM concentrations of BA are represented in the insert.

protein in proteoliposomes is accessible to atractyloside and carboxyatractyloside from the outside and to bongkrekic acid from the inside like in mitochon. dria. This view is corroborated by further data concerning the inhibition of ADP/ATP transport in the reconstituted carrier by bongkrekic acid.

As already stated, inhibition of ADP/ATP transport by bongkrekic acid is pH-dependent, the inhibition being much lower above pH 7 [17]. The pH effect is interpreted by assuming that bongkrekic acid attacks the ADP/ATP carrier from the matrix face of the inner mitochondrial membrane; consequently the inhibitor has to be protonated to penetrate the lipid core of the mitochondrial membrane [17,18]. The same observation holds for the effect of bongkrekic acid on ADP/ATP transport in reconstituted proteoliposomes. As illustrated by Fig. 7, bongkrekic acid at pH 7.5 was 10 times less efficient than at pH 6.6.

Kinetic data on inhibition of ADP/ATP transport by atractyloside and carboxyatractyloside. Titration of competent ADP/ATP carrier protein by dose response measurement

A criterium of competency for the reconstituted ADP/ATP carrier is the ability to recognize and to be sensitive to specific inhibitors. In the present case, atractyloside and carboxyatractyloside were selected as inhibitory ligands because of their rapid and nearly complete inhibitory effect on the reconstituted transport. Two types of assay were conducted to titrate competent ADP/ATP carrier in proteoliposomes. One was based on the kinetic evaluation of inhibition of transport, the other was a direct titration of specific atractyloside binding sites with the radioactive ligands.

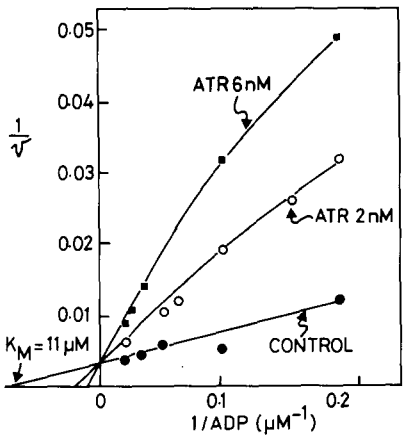
Preliminary experiments (Figs. 8 and 9) indicated that atractyloside and carboxyatractyloside behave as competitive and non-competitive inhibitors respectively. However, examination of the Lineweaver-Burk plots showed that, for atractyloside inhibition, the slopes departed from linearity at low concentrations of ADP. This particular situation occurs with tightly bound inhibitors, since the concentration of free inhibitor varies markedly with small changes in the concentrations of substrate and catalyst [19]. In the case of non-competitive inhibition, the amount of catalyst, capable of binding a high affinity inhibitor, can be derived from the equation:

$$\frac{I_t}{\left(1 - \frac{v_i}{v_0}\right)} = K_i \left(\frac{v_0}{v_i}\right) + E_t$$

where E_t is the total concentration of catalyst, I_t the total concentration of inhibitor, v_0 and v_i the velocity without and with inhibitor respectively (cf. Ref. 19). In the case of competitive inhibition, a slightly different equation applies:

$$\frac{I_t}{\left(1 - \frac{v_i}{v_0}\right)} = E_t + K_i \left(\frac{A_t + K_M}{K_M}\right) \frac{v_0}{v_i}$$

where A_t is the total concentration of substrate (cf. Ref. 19). Plots of $I_t/(1 - (v_i/v_0))$ vs. v_0/v_i for different concentrations of atractyloside or carboxy-



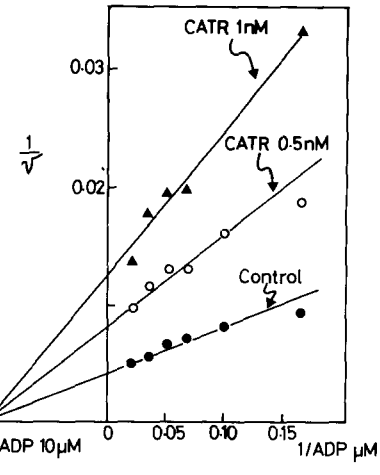


Fig. 8. Lineweaver-Burk plots of inhibition of ADP/ATP transport by atractyloside (ATR). Transport was followed by the luminescence method (cf. Materials and Methods). Proteoliposomes contained 0.40 μg protein, and ADP concentration was varied from 5 to 50 μM. Atractyloside was preincubated with proteoliposomes for 2 min prior addition of ADP. Blanks were made at the different concentrations of ADP in the presence of 6 μM atractyloside.

Fig. 9. Lineweaver-Burk plots of inhibition of ADP/ATP transport by carboxyatractyloside (CATR). Same conditions as in Fig. 8. Proteoliposomes contained 0.40 μg protein. Blanks were made at the different concentrations of ADP in the presence of 4 μM carboxyatractyloside.

atractyloside were linear in agreement with the above equations (Fig. 10). The intercept of the slopes with the ordinate yielded the concentration of competent carrier protein. This value was equivalent to 3—4% of the added protein, when recalculated in terms of percentage, for both atractyloside and carboxyatractyloside inhibitions. The calculation was based on the assumption that the binding of one inhibitor per 30 000 molecular weight carrier protein resulted in complete inhibition of transport. If the carrier consisted of two subunits with a half site reactivity, the percentage of added protein behaving as competent carrier would be 6—8% (see Discussion).

The slopes of the lines in Fig. 10 gave apparent K_i values, valid only for the concentration of ADP used in that experiment (50 μM). Assays similar to that represented in Fig. 10 were repeated at different fixed concentrations of added ADP. They yielded for atractyloside different slopes and for carboxyatractyloside only one slope, in agreement with the competitive and non competitive nature of the inhibition by atractyloside and carboxyatractyloside, respectively. Plots of these slopes for the two inhibitors vs. ADP concentration (insert of Fig. 10) yielded two different curves; their intercepts with the ordinate gave the true K_i values which were approximated to 2 nM and 1 nM for atractyloside and carboxyatractyloside inhibitions, respectively. The K_m value for ADP calculated from the atractyloside slope was 12 μM.

Titration of atractyloside-inhibitable ADP/ATP carrier in proteoliposomes by (^3H)atractyloside

In the above section, the percentage of competent ADP/ATP carrier protein

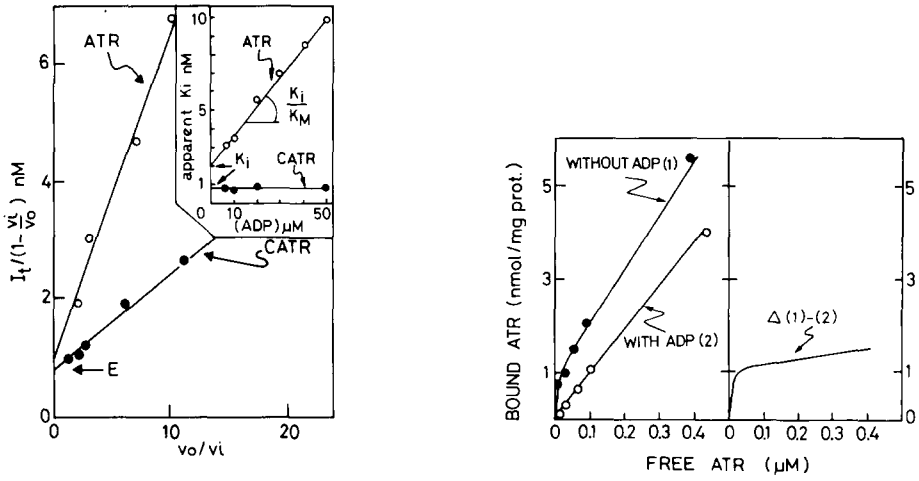


Fig. 10. Graphical determination of the concentration of carrier protein in proteoliposomes and of the K_i of atractyloside and carboxyatractyloside. ADP/ATP transport was followed by the luminescence technique as described under Materials and Methods. I_t is the total concentration of the inhibitors used, i.e. atractyloside (ATR) and carboxyatractyloside (CATR). The inhibitor was preincubated with proteoliposomes (0.40 μg protein) for 2 min before addition of ADP (50 μM final concentration). E_t is the total concentration of competent carrier protein defined by its ability to bind and to be inhibited by atractyloside or carboxyatractyloside. v_0 and v_i are the rates in the absence and presence of inhibitor, respectively. The inhibition data plotted according to the equation of Henderson (see Results) allow the determination of the concentration of the competent carrier protein. Insert. Slopes from Henderson plots at different fixed concentrations of ADP and variable concentrations of inhibitor were replotted vs. ADP concentrations. The intercept with the ordinate yields K_i values for atractyloside and carboxyatractyloside; the slope for atractyloside inhibition gave the K_i/K_m ratio from which a K_m value of 12 μM for ADP could be derived.

Fig. 11. Binding of [^{3}H]atractyloside to proteoliposomes. Effect of ADP. 0.7 ml of proteoliposomes containing 5 μg protein were incubated in 0.8 ml centrifuge tubes with increasing concentrations of [^{3}H]-atractyloside (ATR), in the presence and in the absence of 250 μM ADP. The bound radioactivity was determined as described in Materials and Methods. The two binding curves in the presence and absence of ADP are represented in the left hand side. The difference between the two binding curves is represented in the right hand side. It corresponds to the ADP-sensitive atractyloside binding and to the high affinity region of curve 1 in the left hand side. The bound atractyloside, sensitive to ADP, is approximated to 1 nmol/mg of added protein (present in proteoliposomes). On the basis of a mol. wt. of 30 000 for the ADP/ATP carrier and assuming a binding stoichiometry of 1 to 1, it can be calculated that 3% of the added protein is capable of binding [^{3}H]atractyloside with high affinity.

in proteoliposomes was calculated from kinetic data. Because of the competitive nature of inhibition of ADP/ATP transport by atractyloside, the amount of competent ADP/ATP carrier in proteoliposomes can also be calculated from data of ADP/ATP-sensitive binding of atractyloside (difference in bound atractyloside in the absence and presence of excess ADP).

The control [^{3}H]atractyloside binding curve (without ADP) in Fig. 11 shows a high affinity region corresponding to 1 nmol of bound atractyloside per mg of added carrier protein and a low affinity region with no apparent saturation. Low affinity binding is probably due to the amphiphilic nature of atractyloside [16] and thus to the ability of this inhibitor to bind non specifically to the lipid core of proteoliposomes. As expected, when [^{3}H]atractyloside binding was carried out in the presence of excess ADP (250 μM ADP), a typical, non saturable, low affinity binding curve was obtained. The difference between the

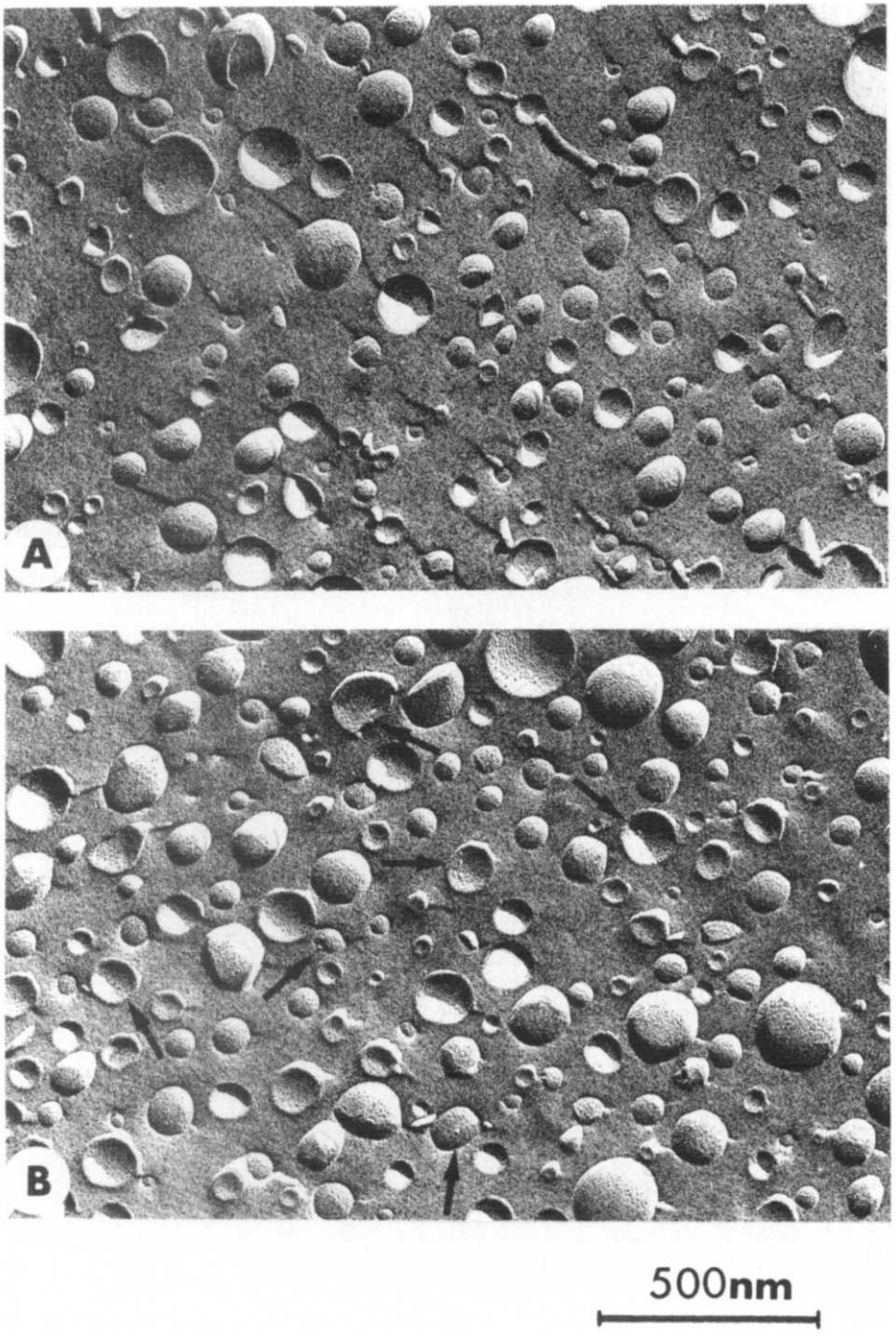


Fig. 12. Freeze-fracture micrographs. (A) Sonicated liposomes (92% phosphatidylethanolamine and 8% cardiolipin) incubated with the same amount of detergent (LAPAO) as that used for the reconstitution of ADP/ATP carrier protein proteoliposomes (see Materials and Methods). Note the presence of smooth concave and convex fracture faces, typical of fractured pure lipid vesicles. (B) ADP/ATP carrier protein proteoliposome preparation. The carrier protein in LAPAO was concentrated by filtration on Amicon PM10 membrane to 0.6 mg/ml, and incorporated into ATP-loaded liposomes, as described in Materials and Methods; about 36 μg of carrier protein were added to 10 mg phospholipid (twice the amount used in routine experiments of transport). Note the presence of a few lightly particulated fractured vesicles (arrows). The analysis of a large number of such vesicles indicates that about 2/3 are concave and 1/3 convex (see Results).

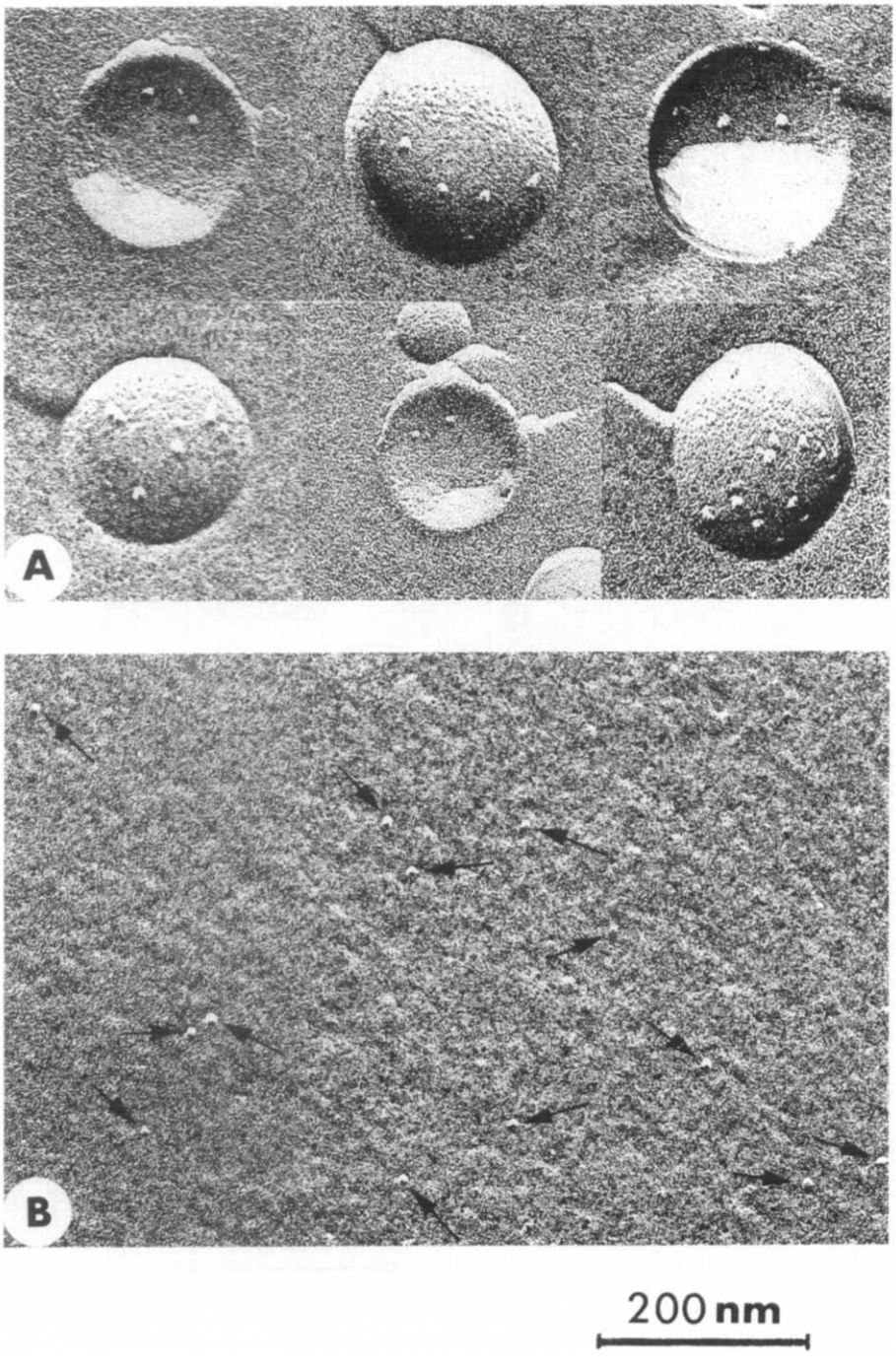


Fig. 13. Composite freeze-fracture micrographs. (A) Particulated fractured vesicles from ADP/ATP carrier protein proteoliposome preparations. (B) LAPAO-ADP/ATP carrier protein micellar solution. Arrows point to some typical protein particles.

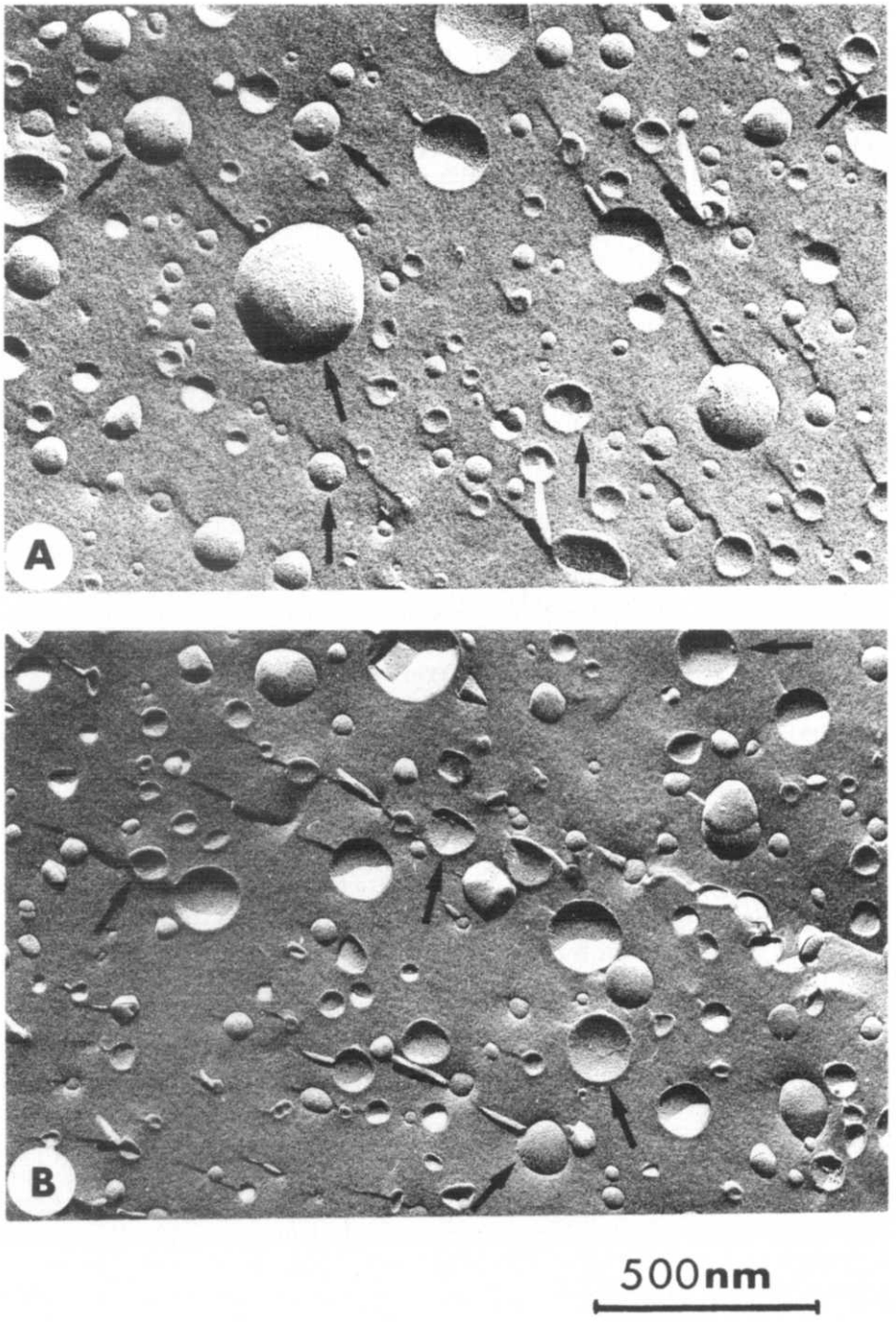


Fig. 14. Freeze-fracture micrographs. (A) ADP/ATP carrier proteoliposomes incubated with 10 μM bongkrekic acid, final pH 7.4. The analysis of a large number of particulated fractured vesicles such as those indicated by arrows shows that about half of them are convex and half are concave. (B) ADP/ATP carrier proteoliposomes incubated with 10 μM carboxyatractyloside, final pH 7.4. The analysis of a large number of particulated fractured vesicles, such as those indicated by arrows shows that 71% of them are concave and 29% convex.

two binding curves (without and with ADP) yielded a saturable curve with a plateau value of 1.2 nmol of bound [^{3}H]atractyloside per mg of added carrier protein, which corresponds to 0.04 mol of specifically bound atractyloside/mol of added carrier protein, assuming a binding stoichiometry of 1 mol atractyloside/1 mol carrier (mol. wt. 30 000). In four different assays, this value ranged from 0.03 to 0.06, indicating that 3 to 6% of the added carrier protein is able to bind atractyloside in such a way that the bound atractyloside is displaced by ADP.

Freeze-fracture electron microscopy

Sonicated pure liposomes containing the same amount of detergent as reconstituted proteoliposomes showed only the presence of vesicles displaying smooth fracture faces (Fig. 12A). The same type of picture was obtained for liposomes incubated in the presence of carboxyatractyloside or bongkrekic acid (not shown).

The reconstituted ADP/ATP carrier protein-lipid proteoliposome preparations showed the presence of a small number of lightly particulated, fractured vesicles (Fig. 12B and Fig. 13A). The amount of particulated vesicles depended on the amount of protein added. For the reconstituted material described in this work, the number of fractured vesicles showing particles was close to 3% of the total number of fractured vesicles. Analysis of 270 particulated vesicles indicated that 70% of them contained only one particle, 18% two particles, 4% three particles and 8% more than three particles. The dimensions of the particles in proteoliposomes were quite close to those observed after fracturing micellar solutions of carrier protein in detergent (Fig. 13N).

The distribution of the protein particles between convex and concave fracture faces of the reconstituted proteoliposomes was not equal; much more particulated vesicles (68%) were concave than convex. If one takes into account the fact that for a large number of fractured vesicles, the number of convex and concave fracture faces is identical, the observed asymmetry of particle distribution means that more protein molecules are anchored at the outer surface of the vesicles (concave fractures correspond to the external half of the fractured vesicles) than at the inner one. This asymmetric distribution of the particles remained practically unaltered after addition of carboxyatractyloside to proteoliposomes (71% of particulated concave fracture faces for 260 particulated vesicles analysed, Fig. 14B), but changed drastically after addition of bongkrekic acid (51% of particulated concave fracture faces for 242 particulated vesicles analysed, Fig. 14A). The bongkrekic acid induced change of orientation was rapid; it reaches a plateau in a few min after addition of bongkrekic acid at pH 7.4 and was stable for at least 15 h. Due to the specific recognition of bongkrekic acid by the ADP/ATP carrier protein, one may wonder whether the change in orientation observed upon addition of bongkrekic acid (which probably corresponds to an immobilization of the carrier on the inner face of the vesicles) is not the basis of the inhibitory effect of bongkrekic acid on ADP/ATP transport.

Discussion

1. Methodological aspects of reconstitution

In the present work, the ADP/ATP carrier protein was purified in presence of synthetic LAPAO, which is a well defined detergent with a chain length of 12 carbon atoms contrary to the commercial aminoxide WS35, which consists of approximately 50% LAPAO and 50% aminoxides with higher chain lengths up to 18 carbon atoms. The long chain aminoxides are less soluble at 0°C than LAPAO, which may lead to some difficulties in the preparation of the ADP/ATP carrier protein in aminoxide WS35 as described by Krämer and Klingenberg [3].

A mixture of phosphatidylethanolamine and cardiolipin in the proportion of 92 and 8% was found to be the simplest phospholipid system to be efficient in reconstitution assays of ADP/ATP transport. In agreement with Shertzer and Racker [1], but in contrast with Krämer and Klingenberg [3], we found that proteoliposomes made of phosphatidylcholine had a very poor efficiency. Extensively purified phosphatidylethanolamine was required for high transport activity; minor contaminants (possibly neutral lipids) had to be carefully removed (cf. Methods).

At concentrations lower than 0.5 mM, Mg^{2+} did not change the rate of transport. At concentrations of Mg^{2+} higher than 2 mM, ATP present in liposomes was released to the outside. The decreased concentration of internal ATP could be the cause of the decreased rate of transport. It may also be possible that the true substrates for the ADP/ATP carrier are the free forms of ADP and ATP [20,21]; consequently at high concentration of Mg^{2+}, the decrease in the concentration of free external ADP might limit the rate of transport. Concentrated solutions of Mg^{2+} also induced fusion of liposomes with formation of multilamellar vesicles (cf. Ref. 22), thereby hampering efficient transport.

A last comment on methodology concerns the accuracy of determination of rates of transport based on [^{14}C]ADP uptake. A non negligible amount of [^{14}C]ADP was found to bind to proteoliposomes treated by carboxyatractyloside and bongkrekic acid prior to [^{14}C]ADP addition (background radioactivity). The amount of [^{14}C]ATP specifically transported by the carrier was taken as the difference between the incorporated [^{14}C]ADP in the absence of inhibitors and the background radioactivity. In our experiments, the maximal amount of [^{14}C]ADP transported in the first 10 sec (first determination) was at least twice higher than the background radioactivity, and the maximal capacity of transport corresponded to a radioactivity 5 to 15 times higher than the background. In inhibition assays where the rate of transport is artificially decreased, only values of transported [^{14}C]ADP which amounted to at least 30% of the background radioactivity were used for calculation of transport rates; this corresponded to rates of at least 20 nmol/min/mg of added protein. Lower values were considered to be meaningless. For this reason, low values of inhibited ADP transport which have been recently reported in literature (cf. Ref. 3, Fig. 6) are doubtful, and conclusions drawn from these experiments must be accepted with reservation.

A particular advantage of the luminescence assay of ADP/ATP transport described in the present work is the possibility to determine initial kinetics of

ADP/ATP exchange by continuously monitoring the ADP-induced release of internal ATP. This method combined with the [^{14}C]ADP uptake assay was also useful in verifying the 1/1 stoichiometry of the ADP/ATP exchange in the reconstituted system. Finally, the luminescence method is very sensitive and can be used with an amount of proteoliposomes at least 5 times lower than the minimal amount necessary for a reliable assay by the radioactivity method.

2. Morphological aspects of reconstitution. Effect of carboxyatractyloside and bongkrekic acid on the orientation of the carrier protein in liposomes

Freeze-fracture electron microscopy of ADP/ATP carrier of proteoliposome preparations revealed the presence of a small number, 3%, of vesicles displaying mostly one or two particles per fracture face. If one takes into account the fact that only a small part of the whole vesicles is exposed after fracture and also the fact that the protein can be anchored either on the external or on the internal surfaces of the vesicle, the approximate amount of particulated vesicles can be estimated to be of the order of 15—20%. It is important to know whether the observed particles correspond to the totality of added protein (representing individual protein molecules or their oligomers) or only to the competent carrier protein. The calculation of the number of protein molecules per vesicle in the reconstituted material gives a value between 2 and 3 times larger than that deduced from the freeze-fracture images. This excludes the possibility that the particles represent only the competent carrier protein (3—6% of added protein) and suggests that each particle may be composed of more than 1 carrier protein molecule.

The size distribution of the particles in reconstituted proteoliposomes (Fig. 15A) and in protein-detergent micellar solution (Fig. 15B) is quite large and the mean value of the diameters of particles is close to 75 Å in both cases. These results indicate that in the micellar solution of carrier protein and in the reconstituted material the protein is probably in an oligomeric state (most probably dimers), since the monomeric form (mol. wt 30 000) should show much smaller particles. In the case of globular water soluble proteins, similar size distributions are observed for the proteins of molecular weight larger than 60 000 (Le Maire, M. and Gulik-Krzywicki, T., unpublished data). It is, however, difficult to draw more precise conclusion about the nature of oligomers without knowing the shape of the protein.

The fact that much more particles are associated with the concave fracture faces can be explained if one considers that in the reconstitution procedure, the protein-detergent micelles are put in contact with the preformed closed lipid vesicles. If the protein is amphipatic and asymmetric (as far as the distribution of its polar and apolar parts is concerned), it will associate with liposomes, in such a way that its most polar part will protrude from the external surface of the vesicles. During short sonication of the mixture some vesicles may be turned inside-out leading to the observed distribution of the incorporated particles (roughly 68% exposed to the outside and 32% exposed to the inside). The addition of carboxyatractyloside to the proteoliposomes does not modify significantly this distribution, but the addition of bongkrekic acid changes it drastically, increasing significantly the percentage of incorporated particles exposed to the inside (from 32% to 49%). The percentage difference (17%) is

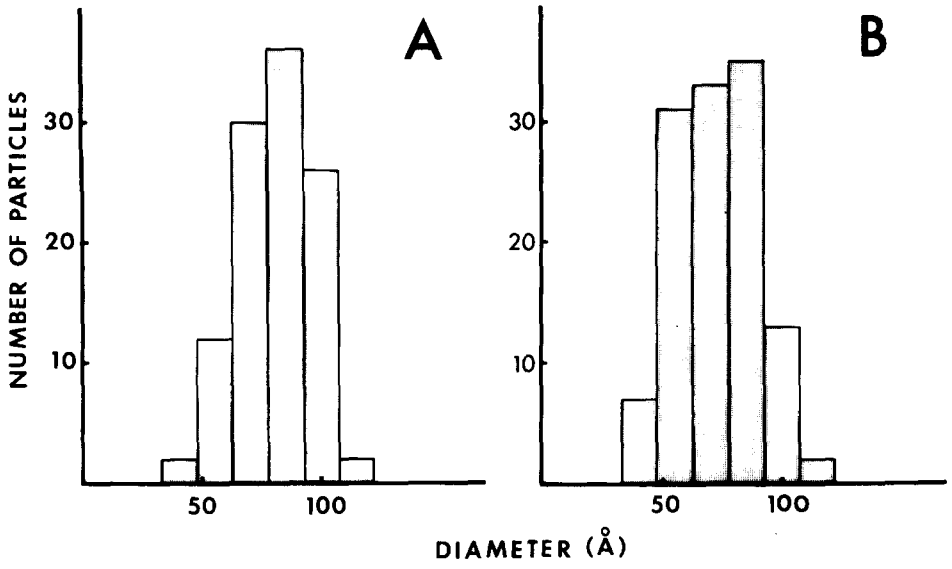


Fig. 15. Size distribution of the protein particles, such as those shown in Fig. 13, measured perpendicularly to the direction of their shadows. (A) ADP/ATP carrier protein proteoliposomes. (B) LAPAO-ADP/ATP carrier protein micellar solutions.

highly significant.

The change in distribution of particles upon addition of bongkrekic acid could be due to the bongkrekic acid induced fusion of the small vesicles leading to redistribution of the carrier protein molecules on both sides of the vesicles. This should lead to the increase in the mean diameter of the vesicles. Although such an increase was not observed experimentally, we cannot exclude entirely that possibility because of the poor precision in measurement of that parameter in our heterogenous vesicle preparations. Another hypothesis (that we favour) to explain the effect of bongkrekic acid is to assume that bongkrekic acid induces a transversal movement of the carrier protein across the lipid bilayer: this motion could be specific of the carrier molecules which are able to recognize the inhibitor. This alternative hypothesis is entirely consistent with some unexplained effects of atractyloside and bongkrekic acid on the labeling by ^{125}I of a membrane protein of molecular weight 30 000, probably the ADP/ATP carrier, the labeling being increased by atractyloside and decreased by bongkrekic acid [23].

The asymmetrical distribution of the reconstituted ADP/ATP carrier, as revealed by freeze cleavage of proteoliposomes is consistent with binding and inhibition data. The maximal inhibition of transport by carboxyatractyloside, a non-penetrant inhibitor, amounted to 90%, which means that 90% of the competent carrier proteins have their carboxyatractyloside site exposed to the outer medium, as in mitochondria. The 10% remaining competent carrier proteins are 'inside-out' and not accessible to carboxyatractyloside. The asymmetry of insertion of the ADP/ATP carrier is corroborated by inhibition data with bongkrekic acid. Bongkrekic acid was much more efficient at slightly acid pH; since bongkrekic acid has to be protonated to penetrate phospholipid

bilayers [17,18], the increased inhibition at acidic pH strongly suggests that bongkrekic acid attacks the reconstituted carrier protein from the inside of the vesicles, i.e. on the opposite face with respect to the binding site of carboxyatractyloside. This behaviour of bongkrekic acid is similar to that observed in mitochondria [16]. In line with these data, evidence has been recently reported for the atractyloside and bongkrekic acid binding sites being different in mitochondria [24]. This was interpreted in terms of an asymmetric channel, spanning the inner mitochondrial membrane, with two preexisting opposite binding sites, one for atractyloside (or carboxyatractyloside), facing the outside, and the other for bongkrekic acid, facing the inside.

The morphological and kinetic data reported here may suggest the following mechanism for the inhibition of ADP/ATP transport by specific ligands (Fig. 16). In the absence of inhibition, the carrier is inserted asymmetrically in the membrane in such a way that more of the polypeptide chain protrudes from the outer face than from the inner face of the membrane ('outer conformation'). Upon addition of bongkrekic acid, the carrier is attracted to the inside of the vesicle with now a portion of its inner surface emerging from the inner face of the membrane ('inner conformation'). This inside-directed movement might be due to the bongkrekic acid-induced unmasking of polar groups in the inner region of the carrier and possibly also to the masking of polar groups at the opposite side of the carrier. These located changes of polarity may lead to a slight translational motion of the carrier towards the inside of the vesicle. Binding of carboxyatractyloside (or atractyloside) to the outer region of the carrier stabilizes the carrier in its 'outer conformation' and therefore has an opposite effect to that of bongkrekic acid on the orientation of the carrier. Additional experiments are in progress to test the validity of this model. This inhibition mechanism differs radically from that advocated by Klingenberg [25], in which (carboxy)atractyloside and bongkrekic acid were postulated to

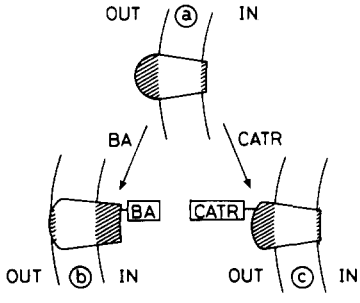


Fig. 16. Hypothetical scheme illustrating the carboxyatractyloside and bongkrekic acid-dependent movement of the reconstituted carrier in liposomes. (a) control; (b) effect of bongkrekic acid (BA); (c) effect of carboxyatractyloside (CATR). The carrier in the absence of ligand (control) is assumed to be inserted asymmetrically in an 'outer conformation'. This external orientation of the carrier is linked to a higher percentage of polar groups (hatched regions) on the outside than on the inside face of the carrier. Binding of bongkrekic acid induces conformational changes, leading to unmasking of polar groups on the inner face and conversely to the masking of polar groups on the outer face; this results in the translational motion of the carrier to the inside ('inner conformation'). Carboxyatractyloside stabilizes the carrier in its outer conformation.

bind to the same site. A translational motion similar to that induced by bongkrekic acid might be involved in ADP/ATP transport.

3. The kinetic competence of the carrier protein

It has been shown by different methods that only 3 to 6% of the added protein behave as efficient catalyst of ADP/ATP transport (competent carrier). These methods were based on: (1) the determination of the number of high affinity sites for [^{3}H]atractyloside, (2) the kinetic determination of the percentage of catalysts of ADP/ATP transport sensitive to atractyloside or carboxyatractyloside. The turnover of the reconstituted ADP/ATP transport expressed on the basis of the concentration of competent carrier protein ranged between 500 and 1000 per min at 20—25°C assuming a molecular weight of 30 000 for the carrier, and between 1000 and 2000, assuming that the competent carrier is organized as a dimer of mol. wt. 60 000, capable of binding only one residue of carboxyatractyloside. These latter values are of the same order of magnitude as those found with intact mitochondria [16,25]. Suggestive evidence for a dimer structure of the ADP/ATP carrier protein first came from binding data obtained with the purified carrier and either [^{3}H]atractyloside [11] or carboxy [^{35}S]atractyloside [26], which indicated that at saturation, only one mole of inhibitor is bound to a mass of carrier protein of 60 000 dalton, i.e. to two elementary subunits of mol. wt. 30 000. The fact that the K_m value for ADP of the reconstituted transport is similar to that found for intact mitochondria ($\leqslant 10$ μM) strongly suggests that reconstitution is an all or none process.

As suggested by freeze-fracture data, a non-negligible part of the reconstituted vesicles contained only one or a very small number of protein particles; yet mono- or pauciparticulated vesicles may be active in ADP/ATP transport. Interaction of carboxyatractyloside, a virtually irreversible inhibitor, with these vesicles is expected to result in full inhibition of transport. This explains why carboxyatractyloside decreases both the rate and capacity of transport, at the difference of atractyloside, a reversible inhibitor (Fig. 6).

Acknowledgements

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