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## Retarded diffusion of ADP in cardiomyocytes: possible role of mitochondrial outer membrane and creatine kinase in cellular regulation of oxidative phosphorylation

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Possible reasons for retarded intracellular diffusion of ADP were investigated. The isolated skinned cardiac fibers were used to study apparent kinetic parameters for externally added ADP in control of mitochondrial respiration. Participation of myosin-ATPase in binding of ADP within cells as it was supposed earlier (Saks, V.A., Belikova, Yu.O. and Kuznetsov, A.V. (1991) *Biochim. Biophys. Acta* 1074, 302–311) was completely excluded, since myosin-deprived skinned cardiac fibers ('ghosts') displayed the same kinetic parameters as intact ones ( $K_m^{app}$  for ADP about 300  $\mu$ M). Significantly lower apparent  $K_m$  values were obtained for fibers with osmotically disrupted outer mitochondrial membrane (25–35  $\mu$ M), which was close to that observed for isolated heart mitochondria. The data obtained are in favor of limitation of ADP movement via anion-selective low-conductance porine channels in the outer membrane of mitochondria. It is proposed that the permeability of this membrane is controlled by some unknown intracellular factor(s). In the presence of saturating concentrations of creatine (25 mM) the apparent  $K_m$  for ADP significantly decreases due to coupling of creatine kinase and oxidative phosphorylation reactions in mitochondria. This coupling is not observed in KCl medium in which mitochondrial creatine kinase is detached from the membrane. It is concluded that in the cells in-vivo ADP movement between cytoplasm and intramitochondrial space is controlled by low-conductivity anion channels in the outer membrane. Thus, the mitochondrial creatine kinase reaction coupled to the adenine nucleotide translocase is an important mechanism in control of oxidative phosphorylation in vivo due to its ability to manifold amplify these very weak ADP signals from cytoplasm.

### Introduction

The mechanism of the regulation of oxidative phosphorylation in the cells in vivo is still unsolved [1–5]. Several hypotheses have been proposed to explain very effective feedback regulation between cardiac work and oxygen consumption. Giesen and Kammermeier [6] and Gibbs [7] analyzed the possible involvement of cytoplasmic phosphorylation potential in this phenomenon but further studies using NMR showed remarkable constancy of phosphocreatine/ATP ratio and phosphorylation potential value over a wide range of cardiac work [2,3,8–10]. This led to the development of the alternative hypothesis of regulation of oxidative phosphorylation by calcium-induced activation of Krebs cycle dehydrogenases and changes in the degree of

NADH reduction [11–13]. However, for better understanding of NMR data and of the role of cellular ADP, it is necessary to solve the problem of ADP diffusion between different cellular compartments: it may be that changes of concentration of adenine nucleotides in some small cellular compartments may induce significant regulatory effects without observable changes in their total cellular contents and in apparent value of phosphorylation potential calculated from tissue contents of these substrates. Very recently, we have shown by using permeabilized cardiomyocytes that intracellular diffusion of ADP in the range of its physiological concentration is very significantly retarded in the cells in vitro, but small fluxes of ADP are manifold amplified by activation of the coupled creatine kinase reaction in mitochondria [14,15].

The purpose of this study was an investigation of possible reasons of retarded intracellular diffusion of ADP in cardiac cells. Two possible mechanisms were considered: intermediate binding to myosin filaments and limitation of ADP movement via porin channels in

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the outer membrane of mitochondria. Permeabilised cell technique was used in combination with selective extraction of myosin or hypoosmotic swelling of mitochondria to disrupt its outer membrane. The results show that there may be multiple sites of retardation of the ADP diffusion but one of the major sites of this type is the outer mitochondrial membrane.

## Materials and Methods

### *Preparations of skinned myocardial fibers*

White male Wistar rats were narcotized with ethyl ether, hearts were excised and placed into a cooled Krebs-Henseleit solution. Bundles of fibers, 0.3–0.4 mm in diameter and 5–7 mm in length, were isolated from endocardial surface of left ventricle and transferred into relaxing solution with EGTA, 7–8 bundles with a total weight of 4–6 mg were incubated for 30 min in 1.8 ml of solution A (see below), containing 50  $\mu$ g/ml of saponin [16]. After that bundles were washed for 10 min in solution B without high energy phosphates to remove saponin. All procedures are carried out at +4°C with intensive stirring.

The extent of the removal of sarcolemma was estimated by determining the activity of lactate dehydrogenase in preparations (see below).

Skinned fibers from musculus soleus skeletal muscle of the rat were prepared in the similar way as those from cardiac muscle.

### *Isolation of rat heart mitochondria*

Isolation of rat heart mitochondria was carried out as described earlier [17]. Liver mitochondria were isolated as described by Schneider [18]. Final mitochondrial preparation was resuspended in a minimal volume of isolation medium (100–200  $\mu$ l, giving a final protein concentration of 50–80 mg/ml) with bovine serum albumin (2 mg/ml) and stored on ice.

'Ghost fibers', deprived of the myosin were produced according to the protocol described by Kakol et al. [19]. Saponin-skinned fibers were washed in the medium containing 50 mM potassium-Hepes (pH 7.0), 10 mM  $\text{MgCl}_2$ , 10 mM ATP, 0.5 mM dithiothreitol, 20 mM taurine, 80 mM Mes, divided into two equal parts and incubated for 30 min in: (1) control fibers in the medium indicated above; (2) for myosin extraction fibers in the medium containing 800 mM KCl, 50 mM potassium-Hepes (pH 7.0), 10 mM  $\text{MgCl}_2$ , 10 mM ATP, 0.5 mM dithiothreitol, 20 mM taurine. After incubation the fibers were washed in medium 'B' (see below) and the kinetic parameters of respiration were determined. At all steps of the experiment in the extracts and washing media the protein concentration was determined according to the Coomassie method (Bio-Rad). We found that after saponin treatment about 7–8% of total protein was solubilized and after

KCl extraction the fibers lost about 18–20% of their protein.

### *Osmotic shock of skinned fibers*

Osmotic shock of skinned fibers was carried out with the purpose of disruption of the outer mitochondrial membrane. The preparation of skinned fibers was washed in medium 'B' and transferred into 1.8 ml of the hypoosmotic medium according to the description by Stoner and Sirak [20]. The hypoosmotic medium contained for 40 mosM 5 mM Hepes, 13.65 mM KCl. After incubation of these fibers for 30 min in the medium indicated, the fibers were taken off and transferred into the oxygraph medium for determination of the respiratory parameters of the mitochondria.

### *Cytochrome c test*

Cytochrome *c* test for estimation of the extent of the mitochondrial outer membrane disruption was carried out in the oxygraph medium in the presence of 125 mM KCl, which dissociates endogenous cytochrome *c* from the membrane [20] and in the case of destroyed outer membrane the respiration is decreased due to wash-out of cytochrome *c* and can be stimulated again by adding saturating amount of exogenous cytochrome *c*.

The cytochrome *c* test was carried out by determinations of the respiration rates in the standard oxygraph medium, containing 125 mM KCl. After addition of 1 mM ADP the maximal respiration was recorded, then 8  $\mu$ M cytochrome *c* was added and the respiration rate recorded again. For determination of the intactness of the inner mitochondrial membrane, 35  $\mu$ M carboxyatractyloside (CAT) was added.

### *Solutions*

All solutions which were used in studies with skinned cardiac fibers contained 10 mM EGTA-Ca EGTA buffer (free  $\text{Ca}^{2+}$  concentration 0.1  $\mu$ M), 4 mM  $\text{Mg}^{2+}\text{Cl}_2$ , 20 mM taurine, 0.5 mM dithiothreitol, 20 mM imidazole (pH 7.0). Ionic strength (around 0.16 M) was adjusted by adding potassium 2-(*N*-morpholino)-ethanesulfonate. Free  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations were calculated on the basis of equations described by Fabiato and Fabiato [21] by using known values of dissociation constants. Relaxing solution A, in which fibers were skinned by saponin contained also 5 mM MgATP and 15 mM phosphocreatine. Solution B in which respiration rates were determined contained besides main components 5 mM glutamate, 2 mM malate, 3 mM phosphate and 2 mg/ml of bovine serum albumin (fatty acid free) instead of high energy phosphates. The solutions A and B were composed on the basis of information of composition of muscle cell cytoplasm [22].

### Determinations

Respiration rates of isolated mitochondria and skinned fibers were determined using a Yellow Spring Instruments oxygraph and Clark electrode.

Respiration of skinned fibers and mitochondria was determined in a medium 'B' described above. Some respiratory measurements were done in standard medium contained 125 mM KCl, 20 mM Hepes, 4 mM glutamate, 2 mM malate, 3 mM magnesium acetate, 5 mM  $\text{KH}_2\text{PO}_4$ , 0.4 mM EGTA, 0.3 mM DTT and 2 mg/ml BSA. The solubility of oxygen was taken to be 460 ng atoms/ml at 21°C and 400 ng atoms/ml at 30°C [23].

The medium for respiration rate determination for liver mitochondria contained 0.25 M sucrose, 20 mM potassium-Hepes (pH 7.4), 3 mM magnesium acetate, 10 mM succinate, 0.37 mM dithiothreitol, 4 mM  $\text{KH}_2\text{PO}_4$ , 0.3 mM EGTA and 2 mg/ml bovine serum albumin. In special experiments 100  $\mu\text{M}$  diadenosine pentaphosphate ( $\text{AP}_5\text{A}$ ) for complete inhibition of myokinase was added. ADP solutions used were calibrated by an enzymatic methods [24].

Activities of lactate dehydrogenase were determined in a medium containing 20 mM potassium phosphate (pH 7.4), 120 mM KCl, 0.5 mM dithiothreitol, 0.2 mM NADH, 10 mM pyruvate, 0.2% Triton X-100. After pyruvate addition the changes in absorbance at 340  $\mu\text{m}$  were determined.

For electron microscopy, the skinned fibers were placed into 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.2–7.4 for fixation during 1 h, and then washed 3-times for 5 min in the phosphate buffer. After that they were fixed in 1% solution of  $\text{OsO}_4$  in the same buffer during 1 h at 4°C, washed for

5 min in phosphate buffer and then with 30% and 50% alcohol for 5 min each time. The fibers were kept overnight in 3–5% uranylacetate solution in 70% alcohol at 4°C, and washed with 70% alcohol solution for 20 min. The fibers were then dehydrated in the ascending alcohol solutions, 20 min at each step, and finally dehydrated in acetone. The fixed fibers were embedded in Epon-Araldite at 30°C and 60°C for 48 h. Ultrathin slices were obtained by LKB-5 ultratom, stained with  $\text{Pb}(\text{NO}_3)_2$  and studied with a Jeol 100-CX microscope.

Protein concentration was determined by the biuret or Coomassie method [25,26]. Statistic analyses of data in double-reciprocal plots were made by using methods of linear regression.  $K_m$  mean values and their standard errors are given.

### Reagents

Coupled enzyme system, creatine, nucleotides, Hepes, bovine serum albumin, sucrose, dithiothreitol, malate, glutamate, EGTA, trypsin, trypsin inhibitor, magnesium acetate, saponin (Sigma, were used in this study.

### Results

*The dependence of cellular respiration on the extracellular ADP concentration: effect of creatine and its elimination by creatine kinase detachment from the inner membrane*

Fig. 1 shows the experimental results with saponin-skinned cardiac fibers when the respiration rate was measured in the physiological salt solution at different ADP concentrations. Curve 1 in this figure shows that

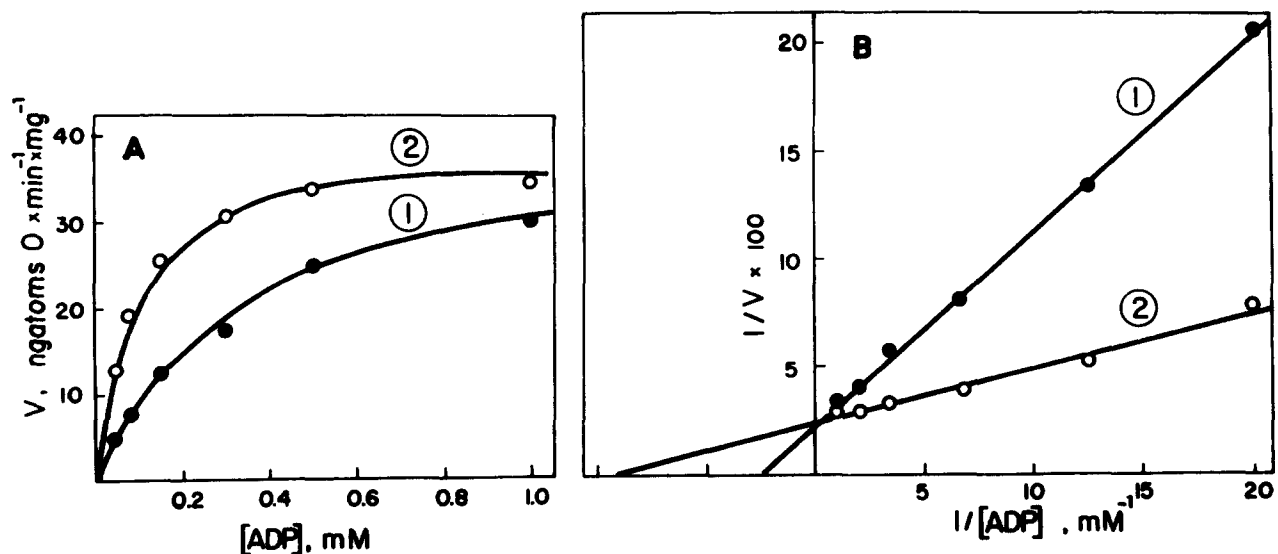


Fig. 1. (A) The dependences of the rate of respiration of the skinned cardiac fibers in medium 'B' on the external ADP concentration in the absence (curve 1) and in the presence of 25 mM creatine (curve 2). The hearts were perfused for 10 min with Krebs solution at 37°C, then the bundles of fibers were skinned by standard method. (B) Linearization of the dependences shown in Fig. 1A in double-reciprocal plots. Note the change in the value of apparent  $K_m$  for ADP by creatine at similar  $V_{\text{max}}$ .

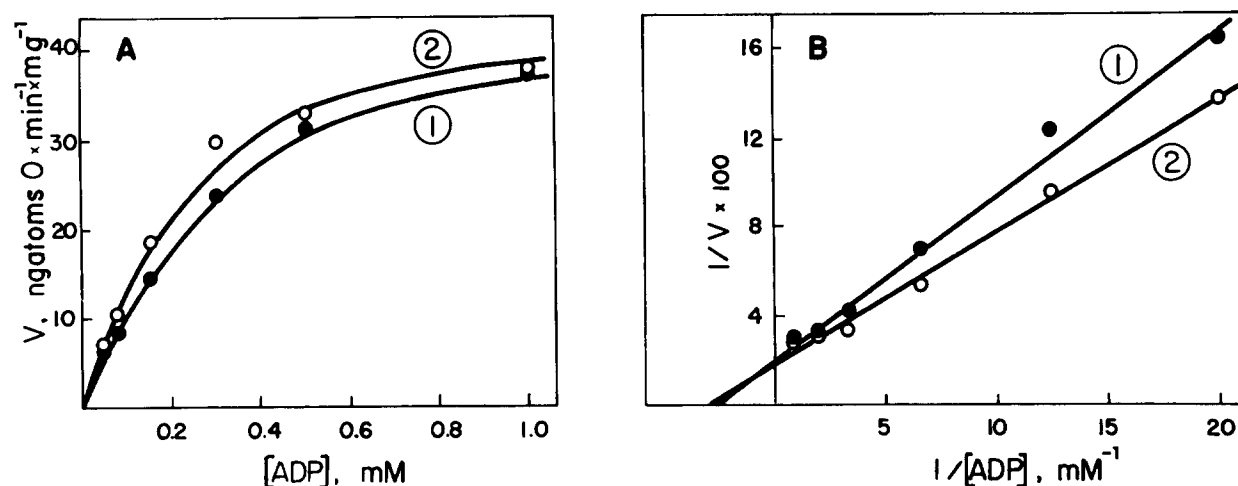


Fig. 2. (A) The dependences of the rate of respiration of the skinned cardiac fibers in the standard oxymetric medium with 125 mM KCl on the external ADP concentration in the absence (curve 1) and in the presence of 25 mM creatine (curve 2). (B) Linearization of the data from Fig. 2A in double-reciprocal plots.

the maximal respiration rate is achieved only at millimolar external ADP concentrations, but the rate of respiration at any ADP concentration is significantly accelerated in the presence of creatine due to activation of mitochondrial creatine kinase reaction. This is in complete accordance with our earlier results [14,15]. Fig. 1B shows that this is due to very significant decrease of the apparent  $K_m$  value for ADP at constant values of  $V_{\max}$ . However, when these experiments were repeated in the medium containing 125 mM KCl, which is known to release mitochondrial creatine kinase from the inner membrane of mitochondria [27], the stimulating effect of creatine on the cellular respiration was completely absent (Fig. 2A and B). We have shown earlier that the detachment of mitochondrial creatine kinase from the surface of the inner mitochondrial membrane by KCl treatment destroys the functional coupling between mitochondrial creatine kinase and

adenine nucleotide translocase [27,28]. Thus, the stimulating effect of creatine on the respiration of saponin-skinned cardiac fibers is directly related to the tight functional coupling between these two proteins mentioned.

Fig. 3A and B show that very similar effect is observed also for saponin-skinned skeletal muscle fibers. The value of apparent  $K_m$  for ADP in the presence of creatine is, however, to some extent higher than that for skinned cardiac fibers (see Table I).

#### *Non-significance of the myofibrillar structures for retarded diffusion of ADP in muscle cells*

To understand which cellular structures might be responsible for the retarded diffusion of ADP we first paid attention to the hypothesis that this may be because of the intermediate binding of ADP to the myosin ATPase [14,15]. To study this question, we treated the

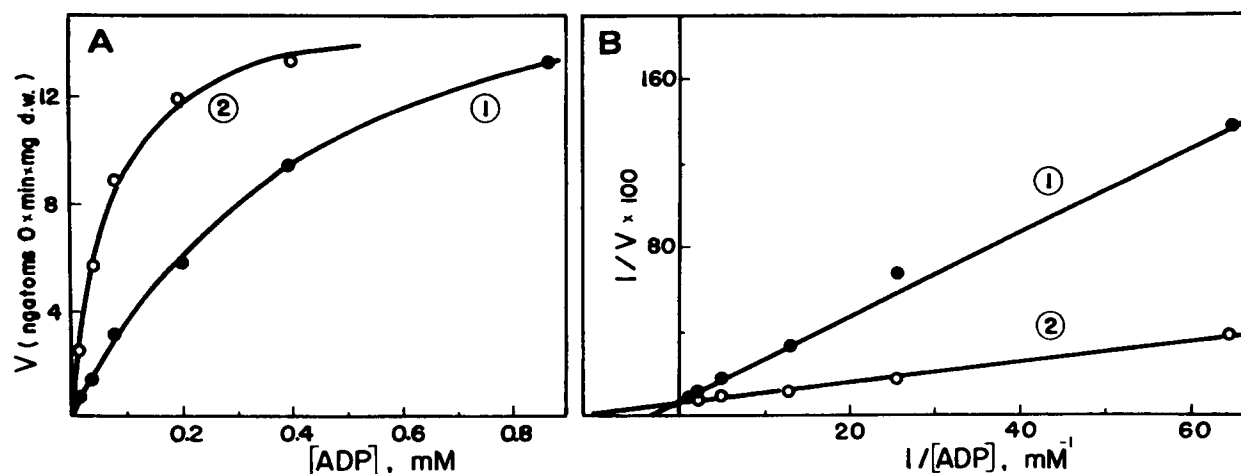


Fig. 3. (A) The dependences of the rate of respiration of the skinned skeletal muscle fibers in medium 'B' on the external ADP concentration in the absence (curve 1) and in the presence of 25 mM creatine (curve 2). (B) Linearization of the data from Fig. 3A in double-reciprocal plots.

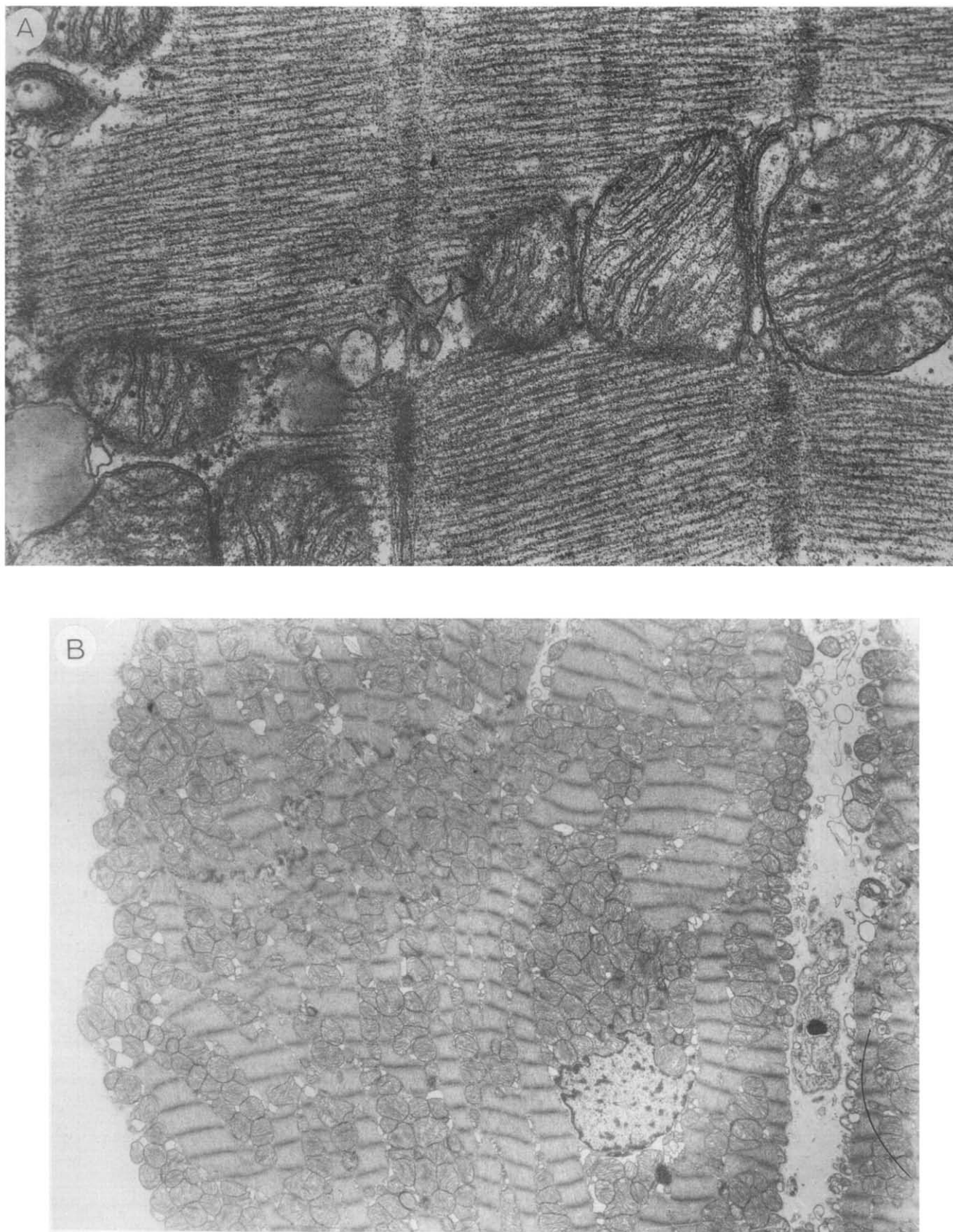


Fig. 4. Electron micrographs of the cardiac fibers. (A) Control, magnification  $40\,000\times$ . (B) After saponin treatment. The phospholipid bilayer of the sarcolemma is destroyed and vesicularized, but intracellular structures (mitochondria and myofibrils) are normal and have an appearance characteristic for these structures in the hyperosmotic physiological salt solution [22] (magnification  $5\,280\times$ ).

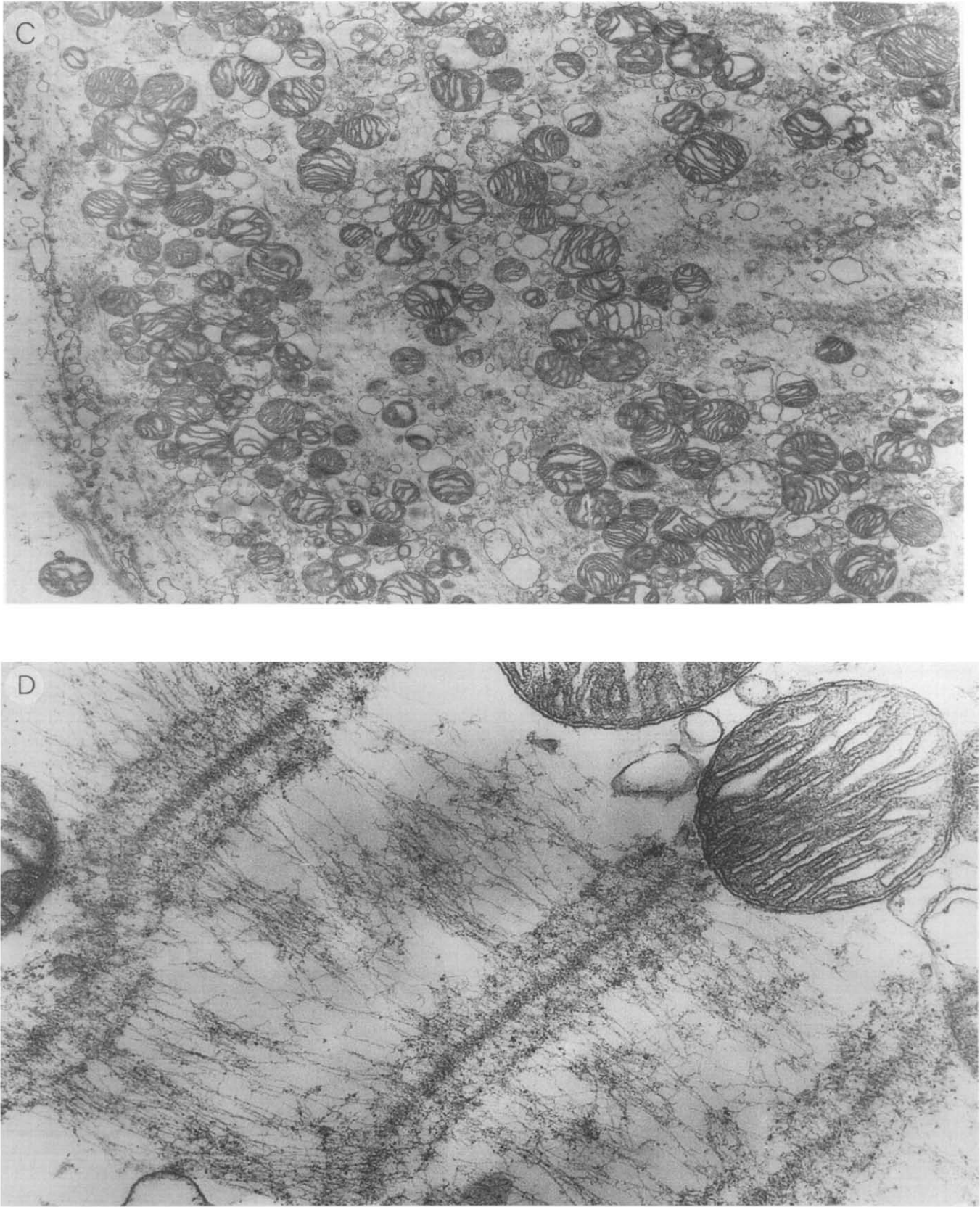


Fig. 4. (C) The saponin-skinned fibers after treatment with 800 mM KCl solution. Note the complete dissolution of thick filaments (magnification  $10\,000\times$ ). (D) The same as C at higher magnification,  $43\,200\times$ . Note the completely intact structure of mitochondria.

TABLE I

$K_m$  values for ADP in regulation of respiration in different preparations at pH 7.2

The parameters for skinned fibers were measured at 21°C according to Veksler et al. [16]. For isolated mitochondria, determinations were performed at 30°C.

Preparation	$K_m^{\text{app}}$ for ADP ( $\mu\text{M}$ )	
	creatine	+ 25 mM creatine
Skinned cardiac fibers	297 $\pm$ 35	85 $\pm$ 5
Skinned cardiac fibers in KCl, 125 mM	292 $\pm$ 49	369 $\pm$ 11
Skinned skeletal muscle fibers	334 $\pm$ 54	105 $\pm$ 15
Ghost cardiac fibers (without myosin)	349 $\pm$ 24	—
Skinned cardiac fibers with swollen mitochondria	I	II
	315 $\pm$ 23; 32.3 $\pm$ 5	—
Isolated mitochondria		
Heart	17.6 $\pm$ 1.0	13.6 $\pm$ 4.4
Liver	18.4	—

saponin-skinned fibers with 0.8 M KCl solution to dissolve thick myosin filaments and to obtain so-called 'ghost fibers' [19].

Fig. 4 shows the electron micrographs of the cardiac fibers before and after treatment with saponin and then with KCl. In contrast to control, normal fibers (Fig. 4A), the sarcolemmal membrane is destroyed by saponin: the phospholipid bilayer is dissolved and forms vesicles under the layer of glycocalyx which is significantly removed from sarcomers and is in some places disrupted (Fig. 4B). However, both sarcomers and mitochondria are normal (Fig. 4B). After 0.8 M KCl

treatment the thick filaments of the sarcomere are completely dissolved but the structures of Z-line and thin filaments are more or less preserved (Fig. 4C, D). This very hyperosmotic treatment, however, does not change the structure of mitochondria which preserve their normal appearance and perfectly intact outer membrane (Fig. 4C, D).

Fig. 5 shows the kinetic analysis of the ADP-dependence of the respiration of these 'ghost fibers'. There is no difference between the experimental dependences before (curve and line 1) and after (curve and line 2) myosin thick filament dissolution. In both cases the value of the apparent  $K_m$  for ADP is high and equal to  $349 \pm 24 \mu\text{M}$ . Maximal rates of respiration, calculated per mg of dry weight, increase in comparison to control fibers due to extraction of proteins from fibers in 0.8 M KCl. Thus, the binding of ADP to the myosin ATPase active centers is not the reason for the retarded diffusion of ADP in these cells.

#### Outer mitochondrial membrane as a possible barrier for ADP diffusion in the muscle cells

In recent years, new hypotheses have been very actively discussed regarding the possible selective barrier function of the outer mitochondrial membrane for metabolites [29–42]. After ruling out the possible role of myosin, this membrane may be one of the real structures which may be suspected to retard ADP diffusion from external medium and from the cytoplasm into the mitochondrial intermembrane space and to the adenine nucleotide translocase. Using the well-controlled conditions for heart mitochondrial swelling in the hypoosmotic medium worked out by

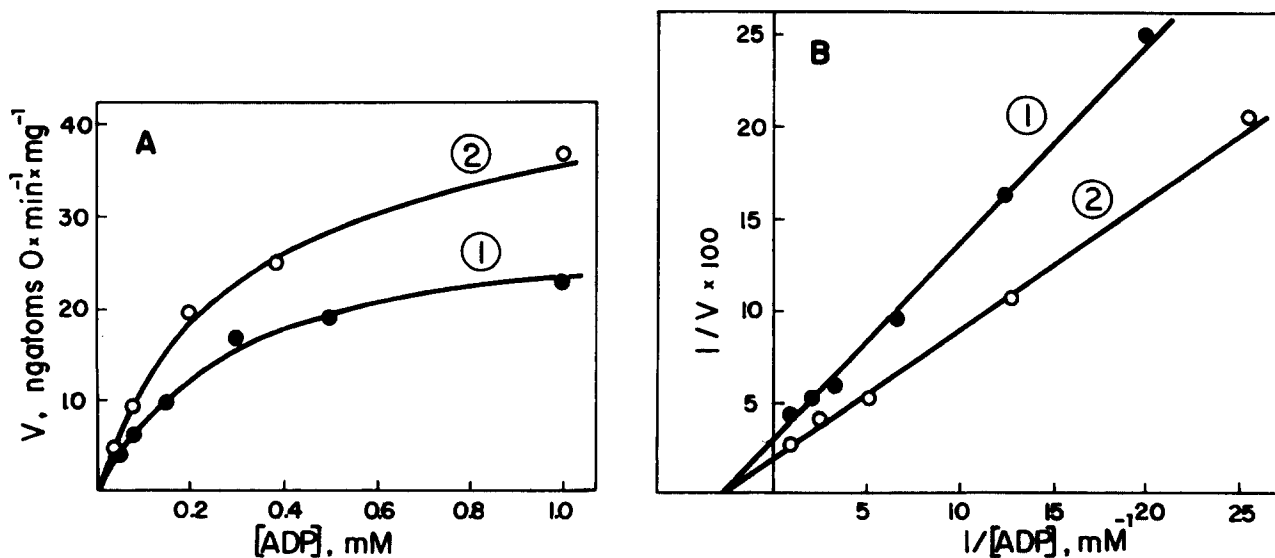


Fig. 5. (A) The dependence of the respiration rate of the skinned and 'ghost' fibers on the external ADP concentration. 1, control, skinned fibers; 2, ghost fibers, after treatment of skinned fibers with 800 mM KCl solution. Addition of 4 IU/ml hexokinase and 20 mM glucose to oxymetric medium (as ADP-regenerating system) did not change kinetic parameters obtained for extracted fibers. (B) Linearization of the dependences shown in Fig. 5A, in double-reciprocal plots.

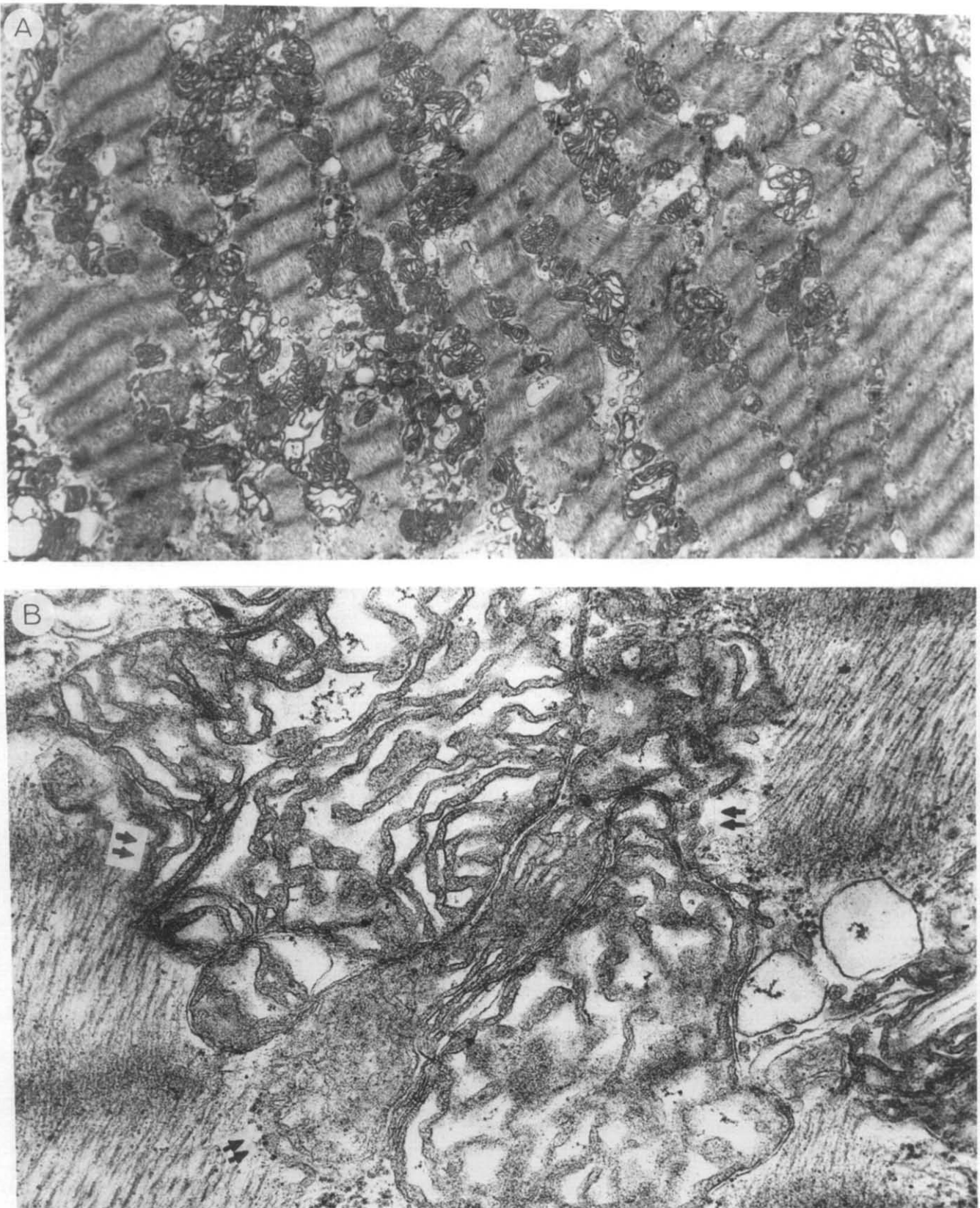


Fig. 6. The effect of the hypoosmotic treatment on the mitochondrial structures in the saponin-skinned cardiac fibers. (A) Fibers after 30 min in the 40 mosM solution (magnification  $9000\times$ ). For control before osmotic shock, see Fig. 4B. (B) The same as A, magnification  $50000\times$ . Mitochondrial outer membrane rupture is obvious (double arrows);



Fig. 6. (C) The same as B, magnification  $54000\times$ . The mitochondrial population with the well-preserved outer membranes (arrows) is seen.

Stoner and Sirak [20], we performed the experiments with disruption of the outer mitochondrial membrane in the saponin-skinned cardiac fibers with careful morphological and biochemical analysis of the extent of disruption.

Fig. 6 shows that incubation of skinned cardiac fibers in the 40 mosM solution in fact results in rather serious disruption of the outer membrane of mitochondria inside the cells. On the electron micrographs the disrupted areas are clearly seen in many places as shown by double arrows (Fig. 6B). However, significant areas of preservation of the outer membrane could also be observed (see arrows in Fig. 6C). Obviously, after hypoosmotic treatment there are several populations of mitochondria in the skinned fibers, with intact and with disrupted membranes. For the rough estimation of the ratio of these populations we used the cytochrome *c* test described in Materials and Methods. Fig. 7 shows that before hypoosmotic treatment (trace 1) addition of the exogenous cytochrome *c* has no effect on the respiration of skinned fibers at the maximal ADP concentrations: due to the intact outer membrane, endogenous cytochrome *c* is not released in spite of the presence of KCl and also the exogenous one cannot cross the membrane and is not necessary. On the contrary, after the osmotic shock the maximal rate of respiration is significantly decreased (trace 2)

but can be restored by adding exogenous cytochrome *c* to the final concentration of  $8\text{ }\mu\text{M}$ . The effect of exogenous cytochrome *c* on the respiration is a stimulation of about 50%. This very approximate estimation shows that probably about half of mitochondrial population has disrupted outer membranes. As it is shown in Fig. 8, that significantly changes the dependence of

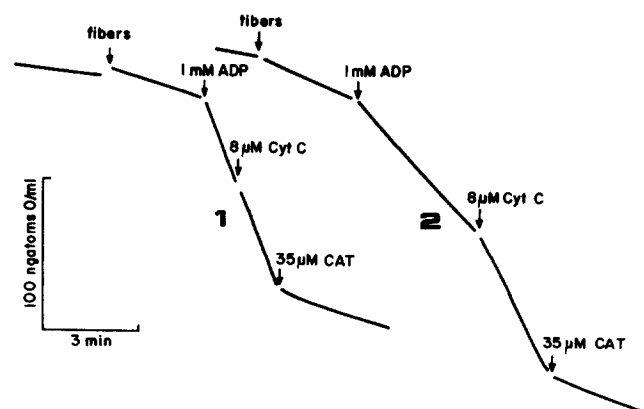


Fig. 7. The oxygraph traces of recording of respiration of skinned cardiac fibers before (1) and after (2) osmotic shock in the 40 mosM solution. The respiration rates were recorded in the medium containing 125 mM KCl to detach the cytochrome *c* from the membrane and in this way to test the intactness of outer mitochondrial membrane.

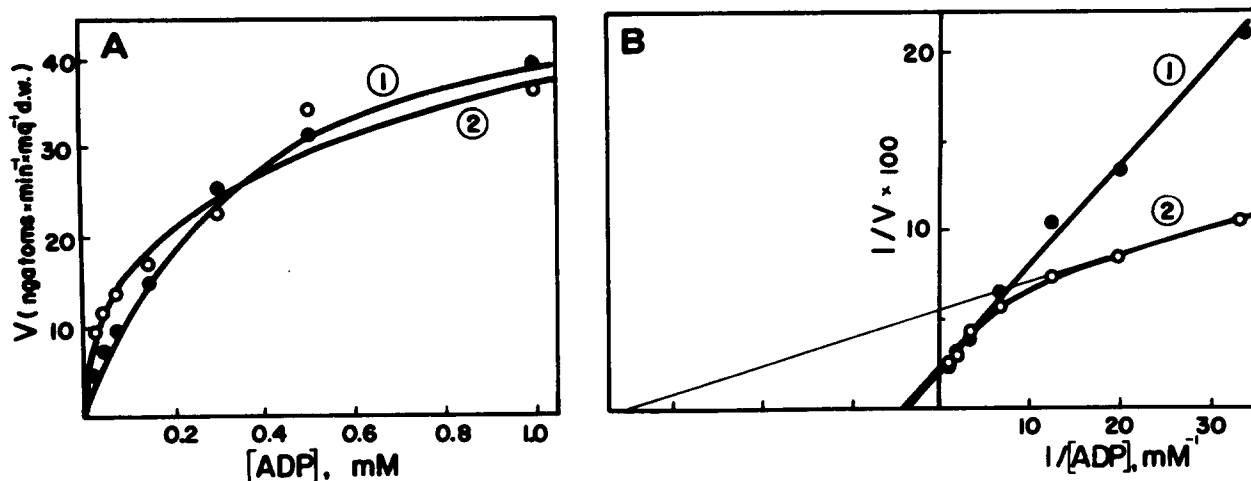


Fig. 8. (A) The dependence of the respiration rates of the skinned cardiac fibers before (curve 1) and after (curve 2) osmotic shock in the 40 mosM solution on the external ADP concentration. (B) Linearization of the dependences shown in Fig. 8A in double-reciprocal plots. 1, control; 2, after the osmotic shock in the 40 mosM solution. In the latter case two different kinetics are seen corresponding to two populations of mitochondria with disrupted and intact outer membranes (Fig. 6B and C, respectively).

the respiration rate on the external ADP concentration: at low ADP concentrations the respiration is higher after osmotic shock, and an analysis of this dependence in the double-reciprocal plots shows the clear existence of two types of processes of respiration regulation in these fibers: one is characterized by low apparent affinity to external ADP equal to that of fibers before osmotic shock, and there is a second

process characterized by apparent  $K_m$  for ADP equal to  $32.3 \pm 5.0 \mu\text{M}$  that is rather close to the parameter for isolated mitochondria, or for mitoplasts deprived of the outer membrane [14,15,28].

These results demonstrated in Fig. 8 led us to the conclusion that the outer membrane of mitochondria is a major intracellular structure which retards the ADP diffusion into the intermembrane space. Logically, the

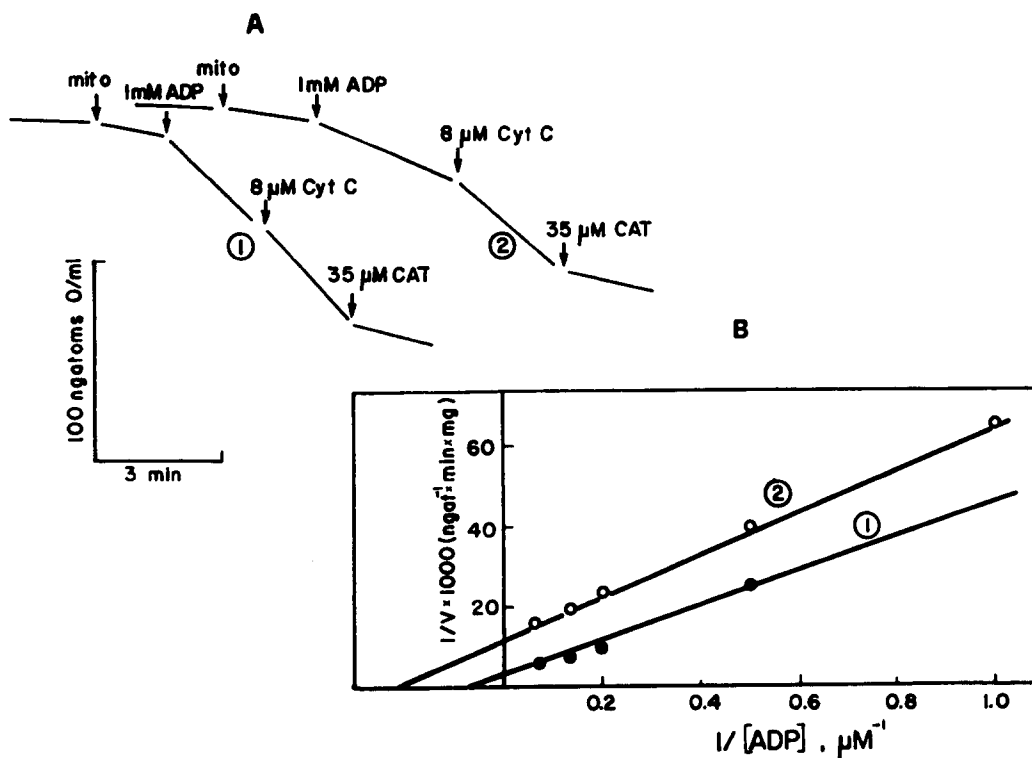


Fig. 9. The experiment with isolated rat heart mitochondria. (A) Oxygraph traces of respiration recordings for intact mitochondria (1) and after osmotic shock in 40 mosM solution (2), cytochrome *c* test. (B) The dependences of the respiration rates of isolated rat heart mitochondria before (1) and after osmotic shock (2) in double-reciprocal plots. Note that in both cases one single straight line is seen.

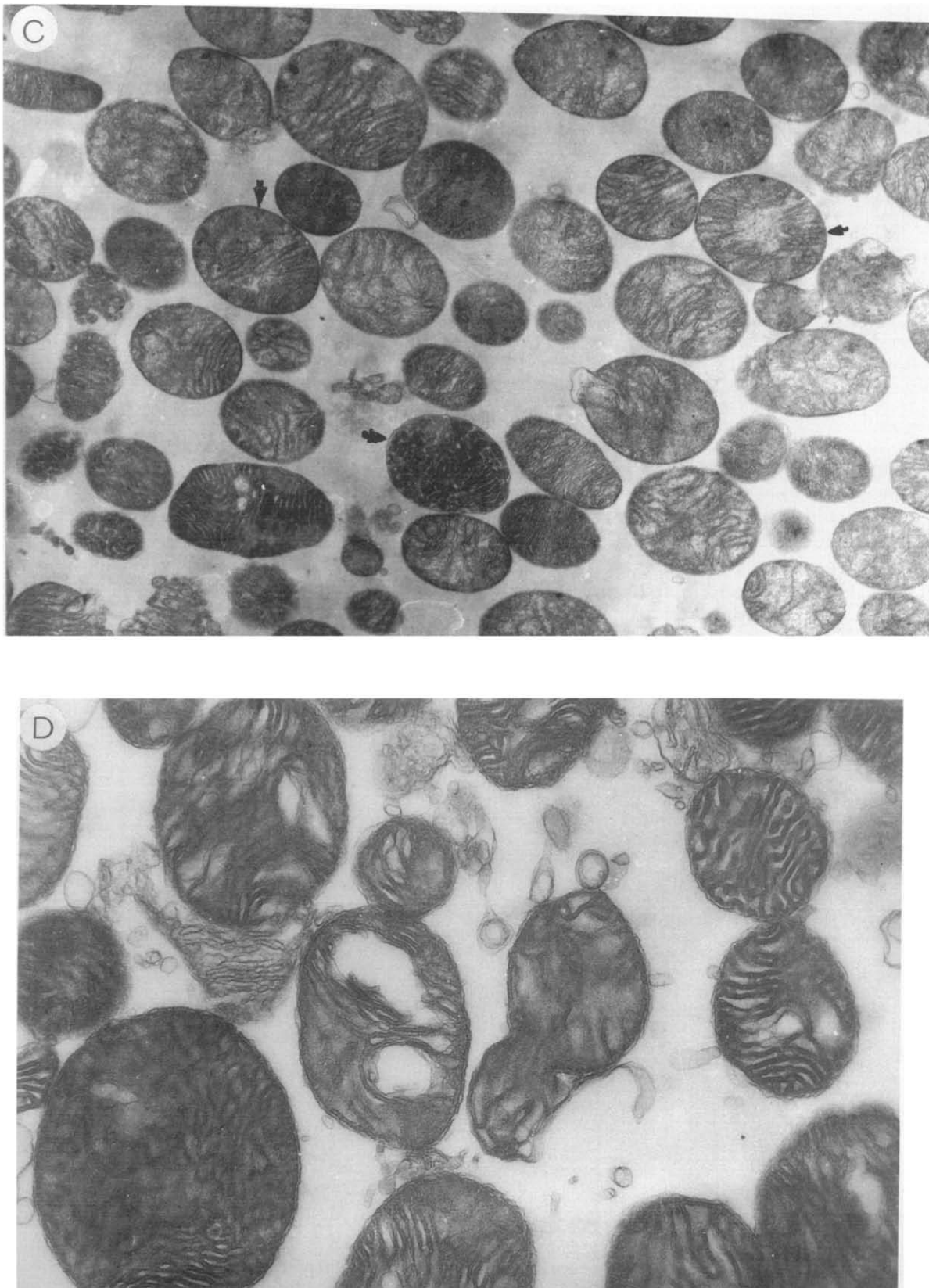


Fig. 9. (C) Electron micrograph of isolated rat heart mitochondrial population in sucrose medium. Note the intact outer membrane, arrows 20000 $\times$ . (D) Electron micrograph of isolated rat heart mitochondria in solution 'B', high resolution 40000 $\times$ .

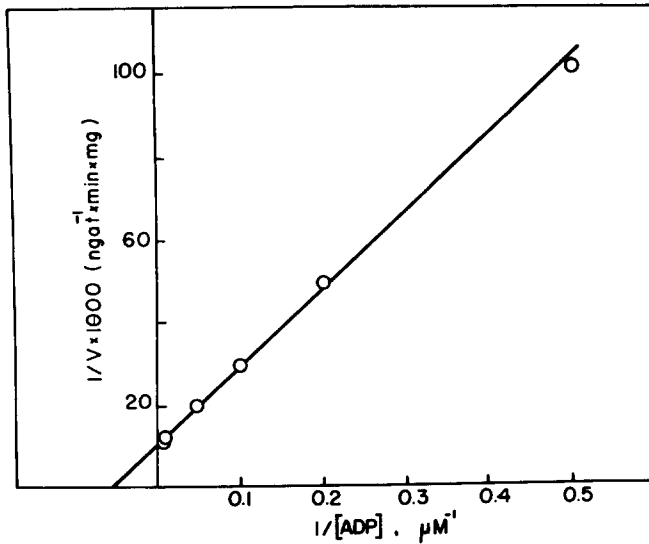


Fig. 10. The dependence of the respiration of rat liver mitochondria on the external ADP concentration in double-reciprocal plots.

following question may be asked: what is the permeability of the outer membrane for ADP in the isolated but intact mitochondria, why it is conventionally accepted that the outer mitochondrial membrane is not a barrier for ADP?

To understand this situation, we repeated the experiments with isolated heart mitochondria in more detail. The results of these experiments are shown in Fig. 9. Fig. 9C, D show that the major part of population of mitochondria possess morphologically intact outer membrane, as it is seen from transmission electron micrographs. This conclusion is confirmed in Fig. 9A which shows only slight (not more than 15–20%) activation of respiration of isolated mitochondria by exogenous cytochrome *c*. In examination of large number of isolated mitochondria approximately the same small percentage mitochondria with morphologically damaged outer membrane due to isolation procedure may be found (Fig. 9C). However, in spite of this, the kinetics of respiration activation by ADP in double-reciprocal plots shows one single straight line giving a very low value for apparent  $K_m$  for ADP ( $17.6 \pm 1 \mu M$ ; Table I). Very similar results were obtained when isolated intact liver mitochondria were used (Fig. 10, Table I). After osmotic shock, the respiration of mitochondria was decreased but significantly activated by addition of  $8 \mu M$  cytochrome *c* (Fig. 9A). Nevertheless, the linearity of respiration-dependence on ADP concentration in double-reciprocal plots was preserved and  $K_m$  for ADP was close to that before osmotic shock (Table I) (some differences may be due to lower  $V_{max}$  value after osmotic shock). Thus, the permeability of the outer membrane for ADP of isolated intact heart and liver mitochondria is very high and the barrier function of this membrane for ADP is lost in the course of mitochondrial isolation.

## Discussion

The results of this study show that the retarded diffusion of ADP in cardiac cells described earlier [14,15] is mainly due to the outer membrane of the mitochondria whose permeability is probably regulated by some unknown intracellular factor, and may also to a smaller extent be related to an unstirred layer effect due to very low physiological concentrations of ADP. These findings give more detailed information of the cellular mechanism of compartmentation of adenine nucleotides and of the important role of the creatine kinase system in the integration of muscle energy metabolism by interconnecting multiple cellular adenine nucleotide compartments. The key role is played by the mitochondrial creatine kinase which significantly amplifies the minor ADP signal which may pass through outer membrane channels and in this way controls mitochondrial oxidative phosphorylation.

Earlier, we have hypothesized that retarded diffusion of ADP may be related to its intermediate binding to the myofibrillar elements. In this work we have completely ruled out the possible importance of ADP binding to myosin thick filaments. Some binding of ADP to the actin filaments is still not excluded (see below), but the main barrier for ADP seems to be the outer mitochondrial membrane.

High permeability of the outer mitochondrial membrane for low-molecular-mass substances has been known for a long time [29–39]. First, it was assumed that this is just a 'leaky' membrane. Very intensive and detailed studies during the last decade from several laboratories have shown that the high permeability is due to the existence of protein pores, or voltage-dependent anion-selective channel (VDAC) with pore sizes in the range of 2 nm [32,33]. Historically, in 1963 Parsons et al. have published electron micrographs with first indications for the pore-like structures in the outer mitochondrial membrane [34]. Similar structures were found using X-ray analysis by Manella and Bonner [35]. Colombini and then many others have extracted this channel-forming protein from rat liver mitochondria and incorporated it into artificial phospholipid membranes [36,37]. The 35-kDa protein forming these pores has been isolated from different sources and characterized by molecular cloning and is called 'mitochondrial porin' [29–37]. The channels formed by mitochondrial porin conduct anions better than cations and switch to low conductance at applied voltages above 10–20 mV [31,36,37]. These channels are easily regulated by several factors which may interact with a special sensor part in the channels or to induce general conformation changes in the channel protein. Besides electric field, these changes may be induced by high-molecular-mass polymers such as dextran sulfate and a synthetic polyanion, a copolymer of methacrylate,

maleate and styrene [29,30,33,38]. In the latter case the oncotic pressure has been supposed to be one of regulatory factors decreasing the channel permeability [33].

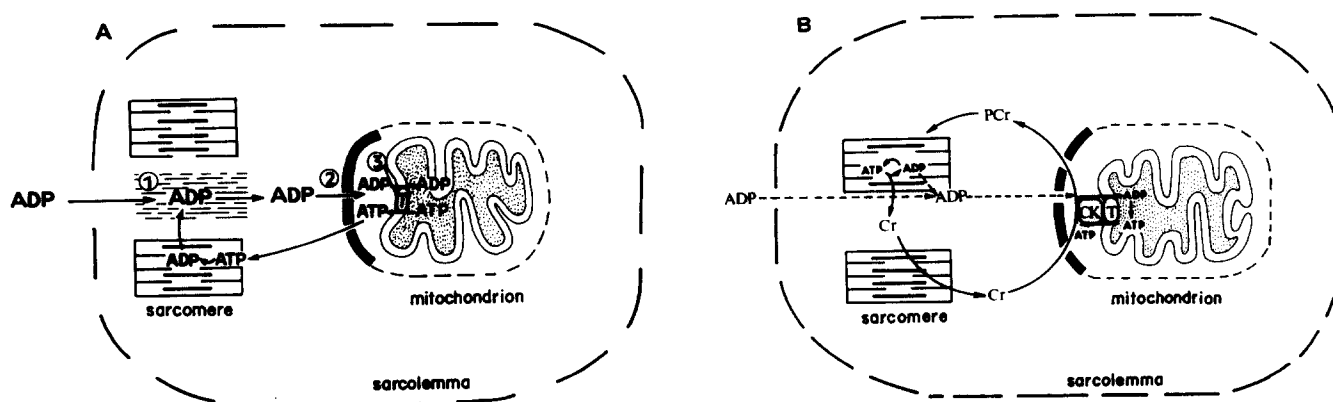
In the series of publications from Brdiczka's and Wallimann's laboratories it has been found that mitochondrial porin channels may be involved in formation of the dynamic contacts between the inner and outer mitochondrial membranes, in particular with participation of the mitochondrial creatine kinase octamers [40–42]. Such a supramolecular complex of porin-creatine kinase-adenine nucleotide translocator assumes direct channeling of substrates – ATP and ADP – and very efficient phosphocreatine production from mitochondrial ATP and cytoplasmic creatine [42]. It may be also assumed that the permeability of the pores may be changed by its attachment into the contact sites [40].

Thus, sufficient data have been found in the in-vitro experiments with isolated mitochondria and artificial phospholipid membranes to suppose that the VDAC in the outer membrane may be of importance in regulation of metabolic fluxes between cytoplasm and intramitochondrial space [38,42].

The results reported in this work demonstrate, to our knowledge for the first time, the role of the outer membrane of mitochondria in regulation of ADP diffusion from cytoplasm into the intermembrane space in the muscle cells in vivo. In contrast to isolated mitochondria with morphologically well-preserved membrane but with high permeability to ADP, in the muscle cells in vivo the permeability of this membrane is low. Clearly, there is an important regulatory factor decreasing the VDAC permeability to ADP inside the cells. Since we have used the skinned fibers with completely removed cytoplasmic proteins (lactate dehydro-

genase was removed by 90–95%) and have also used a hyperosmotic physiological salt solution which condenses the matrix space and increase the intermembrane space volume (see Fig. 10D) the role of oncotic pressure and the possible influence of contact sites might be minimal in our experiments. Most probably, there is a still unknown intracellular factor associated with the outer membrane in vivo which controls its permeability. This may be similar to the VDAC modulator recently isolated from potato tubers mitochondria by Liu and Colombini [39]. Most probably, this may be a cytoplasmic factor which remained attached to the outer membrane in Colombini's experiments. Since in our experiments in skinned fibers we have seen the saturation kinetics with respect to ADP with high apparent  $K_m$ , it is not excluded that this factor binds ADP before its controlled movement across the membrane. It needs further experimental research to identify the nature of this factor (if we think in line with Marco Colombini who has supposed that the channel sensor which is effected by membrane potential or polyanions may be viewed as a 'door knob' allowing the opening or closing of the channel [30], now we have to look also for a 'doorman' who is using that knob in the cells in vivo to let ADP in). Colombini et al. [38] have also found that in the 'closed', or low-conductivity state the channel diameter is about 0.9 nm and is still permeable for low-molecular-mass compounds. Our data show that this membrane is not a barrier for creatine and probably phosphocreatine (Figs. 1–3) [14,15].

Besides the outer membrane, still some importance for retarded ADP diffusion may be ascribed to the unstirred layers in the cells [43–44] and probably to



Scheme I. (A) Presentation of the intracellular mechanisms of retarded ADP diffusion. Three process are controlling ADP movement in the cells. (1) diffusion through unstirred layers and possible binding to actin filaments is not excluded; (2) ADP movement through the porin channels in the outer membranes which is strictly controlled by some intracellular factors; (3) translocation across the inner mitochondrial membrane by adenine nucleotide translocase into the matrix for rephosphorylation. (B) The coupled creatine kinase systems in mitochondria and in myofibrils control the ADP movement and overcome the problems related to the retarded diffusion of ADP via the mitochondrial outer membrane. In myofibrils, ADP is rephosphorylated at the expense of phosphocreatine and creatine diffuses into mitochondria along the large concentration gradient. In mitochondria the creatine kinase reaction coupled to oxidative phosphorylation via translocase manifold amplifies the weak ADP signal which may come in controlled manner through the outer membrane, and creatine itself exerts acceptor control of oxidative phosphorylation. This seems to be a central cellular mechanism of regulation of respiration in muscle cells.

some binding to the actin filaments, since the  $K_m$  for ADP in the presence of creatine is higher in the cells with larger diameters (skeletal muscle vs. cardiac muscle cells, see Table I). All these possible processes retarding ADP diffusion are described schematically in Scheme IA.

In the living cells the oncotic pressure, due to the high protein concentration in cytoplasm [45] might be an additional factor decreasing the VDAC permeability for ADP. That will mean that at physiological ADP concentration the outer membrane may be almost impermeable for ADP, or only a very limited amount of it may cross the membrane in a controlled manner to activate the oxidative phosphorylation coupled to the creatine kinase reaction. This reaction is a powerful amplification mechanism regulating and controlling the rate of oxidative phosphorylation, as shown in Scheme IB. For efficient control mechanism, the creatine kinase should stay at the membrane to ensure the direct and precise substrate channeling. In a very recent work by Soboll et al. new thermodynamic evidence for such a close connection between phosphocreatine production and oxidative phosphorylation was presented [46]. In very good accordance with several earlier observations [22,27] detachment of creatine kinase from the membrane by phosphate solution (20 mM) eliminated the thermodynamic coupling in Soboll's work [46]. In the present study, KCl solution eliminated the accelerating effect of creatine on respiration (Fig. 1) due to the same effect of enzyme detachment. This shows the necessity of the supramolecular structure of the complex of creatine kinase with translocase and possibly with porin for acceptor control of respiration by creatine. These control mechanisms have very recently been reviewed in detail by Wallimann et al. [47] and Wyss et al. [48]. All these developments leave very little doubt that the phosphocreatine pathway for energy transfer is a central process in muscle energetics.

Very clearly, the in-vivo regulation of oxidative phosphorylation is a multifactoral process, different systems and factors playing their changing role with different control strength under different metabolic and functional conditions [1,3,11,50]. Theories of the control of respiration by  $\text{Ca}^{2+}$ -activated mitochondrial dehydrogenases were very elegantly and precisely elaborated by the Hansford, Denton and MacCormack groups and recently reviewed in several articles [1,8–13]. This important mechanism is, however, mostly intramitochondrial increasing the flux through Krebs cycle and most probably modulatory with respect to the feedback mechanism between energy utilization (contraction) and production (respiration), since inhibition of  $\text{Ca}^{2+}$  entry into mitochondria by Ruthenium red does not change the linear response of respiration to workload elevation but only changes intracellular metabolic situation [2,3,8,51]. Significance of the re-

ported NMR data on the constancy of PCr/ATP ratio at different workloads, however, should probably be re-evaluated, since this technique allows to determine only the overall tissue contents of high-energy phosphates but does not take into account the phenomenon of adenine nucleotide compartmentation [52] until the magnetization transfer is used and carefully analyzed to determine the energy fluxes [52–55]. It becomes more and more possible to assume that in the cells in vivo very small fluctuations in small ADP compartments may be a key signal in the regulation of respiration due to their manifold amplification in the coupled creatine kinase systems in the heart and many other cells [14,15,47,48,56].

This concept will be especially important for our understanding of regulation of cellular energetics, if low permeability of the mitochondrial outer membrane to the ADP in vivo will be verified in further experiments and the cellular factors responsible for this process indentified.

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