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Induction of Ion Transport in Rat Heart Mitochondria by Fluorescamine

Edmundo Chávez[1] and Concepción Bravo[1]

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Abstract

This report describes experimental results which show that the fluorescent reagent fluorescamine induces mitochondrial energy-independent swelling when the incubation media contain the chloride salts of the cations Li^+, Na^+, K^+, Rb^+, and Cs^+. The reaction depends on the concentration of the dye and is inhibited by Mg^{2+}, and its extension is closely related to the amount of the primary amino groups titrated by fluorescamine. Analysis of the labeled inner membrane in polyacrylamide gel shows that the amount of aminofluorescamine complex is lower when mitochondria are in the presence of Mg^{2+}.

Key Words: Heart, biomembranes; mitochondria; ions; transport; diffusion; channels; swelling; fluorescamine; fluorescence.

Introduction

Recently, it has been shown that reagents which react with functional groups of biological membranes induce changes in their permeability to cations as well to anions. It was shown by Sutherland *et al.* (1967) that *p*-chloromercuriphenyl sulfonate, which reacts with thiol groups, inhibits the transport of phosphate across the red cell membrane; also Cabantchik and Rothstein (1972) observed that reagents that interact with membrane amino groups of the red blood cells inhibit anion transport.

In mitochondria, Fonyo and Bessman (1966) and Tyler (1968) showed that the blockage of sulfhydryl groups located in the inner mitochondrial membrane results in inhibition of phosphate transport. Moreover, Moore (1971) showed that the interaction of ruthenium red with the inner mitochondrial membrane results in the inhibition of calcium transport.

In contrast, Brierley *et al.* (1967, 1968) showed that mercurial reagents

[1]Departamento de Bioquímica, Instituto Nacional de Cardiología, Ignacio Chávez, México, 014080, D.F. México.

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that react with membrane thiol groups markedly stimulate the energy-linked transport of K^+ and Mg^{2+} ions in heart mitochondria. These experiments indicate that mercurial reagents increase the affinity for potassium of the mitochondrial potassium transport system. In addition, it was found that dicyclohexylcarbodiimide induces inhibition of potassium uptake in rat liver mitochondria (Gauthier and Diwan, 1979).

It was reported also that the fluorescent dye fluorescamine that reacts with $-NH_2$ groups affects the ATPase activity of rat liver mitochondria by modification of the proton permeability of the inner membrane (Tu, 1979). Thus it appears that primary amino groups may be involved in the maintenance of the permeability of the mitochondrial membrane.

The present report shows that fluorescamine induces changes in the permeability of the mitochondria to cations by interacting with the amino groups of the inner mitochondrial membrane. In addition, the results indicate that the binding of fluorescamine to the membrane is directly related to diffusion of the chloride salts of Li^+, Na^+, K^+, Rb^+, and Cs^+. The uptake of cations, as determined by measurements of mitochondrial swelling, was inhibited by the addition of magnesium.

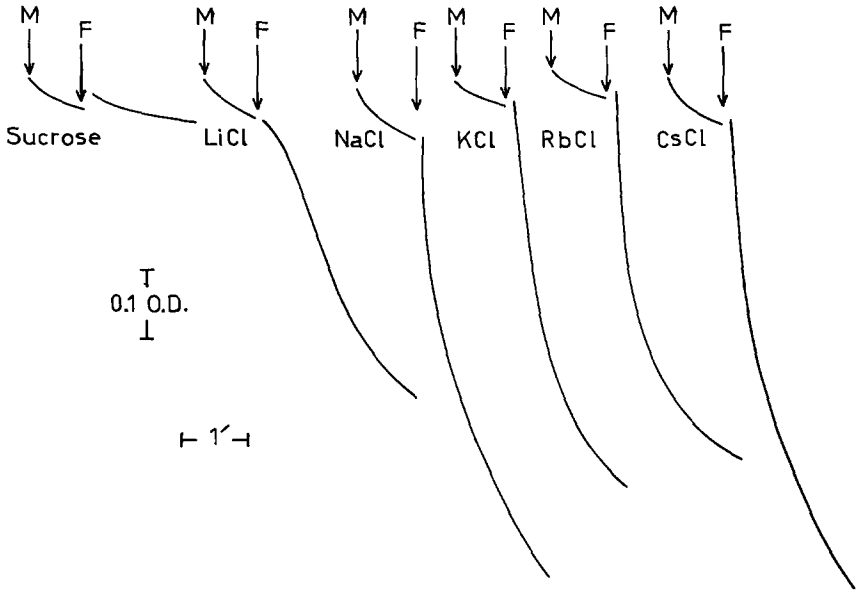

Fig. 1. Mitochondrial swelling induced by fluorescamine. Rat heart mitochondria (2 mg protein) were added to a medium which contained 250 mM sucrose or 120 mM of the indicated salts, 2 mM potassium borate, pH 8.3, and 10 μg rotenone; where indicated, 98 μM of fluorescamine (F) was added, except for the sucrose medium in which 200 μM of fluorescamine was added. Final volume 3 ml, temperature 25°C.

Materials and Methods

Mitochondria from the heart of rats were prepared using the Polytron technique for tissue disruption (Jurkowitz *et al.*, 1974). The preparation media were 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 7.3. Mitoplasts were prepared using the digitonin method reported by Greenawalt (1974). Mitochondrial swelling was monitored following the changes in optical density at 546 nm as a function of time in the mixtures described in the Results section. Determination of primary amino groups that reacted with fluorescamine was made by measurements of fluorescence intensity of the aminofluorescamine complex in an Aminco spectrofluorometer with an excitation wavelength of 390 nm and an emission wavelength of 490 nm; the experimental conditions are described in the Results section. A calibration curve was made with arginine as standard.

Polyacrylamide gel electrophoresis was carried out in 7.5% acrylamide and 0.1% dodecyl sulfate as described elsewhere (Weber and Osborn, 1969); cross-linked hemoglobin obtained from Sigma Chemical Company was used

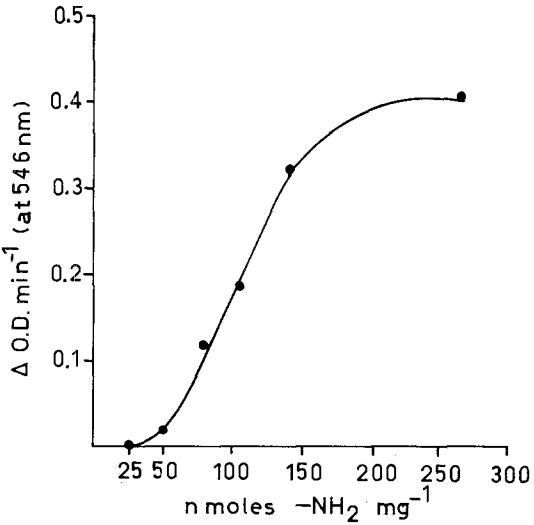

Fig. 2. Relationship between the titrable amino groups and the extent of mitochondrial swelling. Mitochondrial protein (2 mg) was added to 3 ml of a medium of 120 mM KCl containing 2 mM potassium borate, pH 8.3, and 10 μg rotenone. Fluorescamine at the concentrations of 19.6, 39.2, 58.8, 78.4, and 98 μM was added for the titration. The extent of swelling obtained after 1 min of incubation was recorded, and the cuvette was immediately transferred to the spectrofluorometer for assay of fluorescence intensity. Temperature 25°C.

as a molecular-weight marker. Protein was determined by the Lowry method (Lowry *et al.*, 1951). Fluorescamine (Fluram) was obtained from Hoffman-La Roche, Nutley, New Jersey.

Results

Figure 1 shows that the addition of 98 μM fluorescamine to mitochondria incubated with the chloride salts of Li^+, Na^+, K^+, Rb^+ and Cs^+ resulted in large mitochondrial passive swelling. The rate of fluorescamine-induced swelling was more or less the same with all the cations tested except with Li^+ where the rate was slightly lower. Fluorescamine was dissolved in acetone (10 μl), which had no effect on mitochondrial swelling. The addition of 200 μM of fluorescamine to mitochondria suspended in 0.25 M sucrose did not induce changes in the optical density.

In order to determine whether there is a relation between modification of primary amino groups and mitochondrial swelling, the experiment shown in

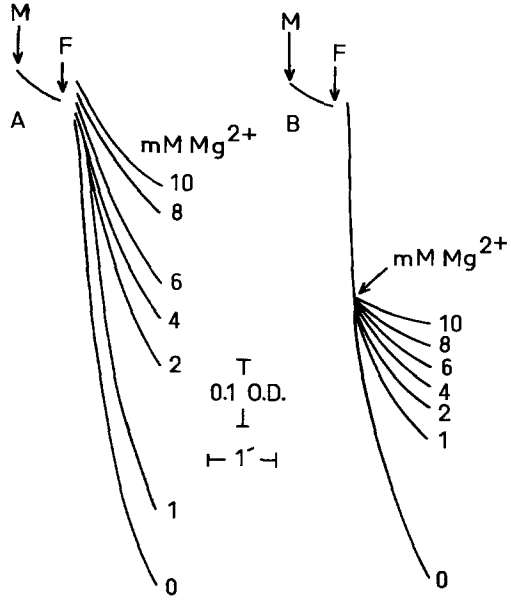

Fig. 3. The effect of magnesium on the mitochondrial swelling induced by fluorescamine. Rat heart mitochondria (2 mg of protein) were added to a medium which contained 120 mM KCl, 2 mM potassium borate, pH 8.3, and 10 μg rotenone. Where indicated, 98 μM fluorescamine and the indicated concentrations of magnesium chloride were added. Final volume 3 ml. Temperature 25°.

Fig. 2 was carried out. The swelling was allowed to proceed for 1 min in the presence of KCl, and the fluorescence of the mixture was determined in order to know the amount of $-NH_2$ titrated for the dye; when the latter was plotted against the rate of swelling, a sigmoidal curve was obtained. Similar results were obtained with the chloride salts of the other monovalent cations used as Fig. 1.

Several studies have established that the addition of magnesium inhibits potassium exchange and can protect mitochondria against swelling in different media (Wehrle *et al.*, 1976; Brierley *et al.*, 1977; Chávez *et al.*, 1977). The results of Fig. 3A showed that the addition of different concentrations of magnesium to a potassium medium induces an inhibition of the fluorescamine-promoted swelling; such inhibition is higher up to a concentration of 10 mM magnesium; however, when magnesium is added after fluorescamine, lesser concentrations of this cation are required in order to reach a similar inhibition of swelling, as is shown in Fig. 3B. The marked differences in the inhibitory effect of magnesium are more evident when the rate of swelling is plotted against the different concentrations of magnesium chloride, as is observed in Fig. 4. Similar results were obtained when calcium at the same concentrations was the divalent cation added (not shown).

The study shown in Fig. 3A allowed us to determine whether the effect of Mg^{2+} could be due to a modification of the proteolipid bulk of the membrane in such a way that under the influence of magnesium certain primary amino groups involved in the control of membrane permeability become inaccessible to fluorescamine. Indeed the results of Table I would argue in favor of this possibility since prevention of fluorescamine-induced mitochondrial swelling

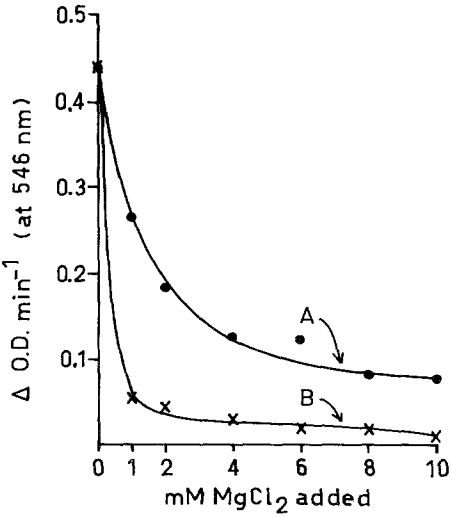

Fig. 4. The inhibitory effect of magnesium on mitochondrial swelling. For experimental details see Fig. 3. (A) Magnesium at the indicated concentrations was added before fluorescamine; (B) magnesium was added after the dye.

Table I. Primary Amino Groups Titrable by Fluorescamine in Sodium and Potassium Chloride Medium in the Absence and in the Presence of Mg^{2+}[a]

Additions	*n* moles–NH_2 mg^{-1}
NaCl	119
NaCl + 10 mM $MgCl_2$	68
KCl	116
KCl + 10 mM $MgCl_2$	76

[a]Mitochondrial protein (2 mg) was added to a medium containing 120 mM NaCl or 120 mM KCl, 2 mM potassium borate, pH 8.3, and 10 μg rotenone. Fluorescamine (98 μM) was added and the fluorescence intensity determined. Final volume 3 ml., temperature 25°C.

is accompanied by a reduction in the number of amino groups that react with fluorescamine.

In order to determine whether reagents other than fluorescamine that react with primary amino groups can induce mitochondrial osmotic swelling, phenylglyoxal at different concentrations was added to a medium containing potassium chloride. As can be observed in Fig. 5, the effect of fluorescamine on mitochondrial swelling is duplicated by phenylglyoxal. However, higher concentrations of this reagent were required in order to attain rates of swelling similar to those induced by fluorescamine. This observation indicates that fluorescamine reacts more easily with some $-NH_2$ groups than phenylglyoxal; a quite satisfactory explanation for these results can be provided if differences in hydrophobicity of these two reagents are taken into account.

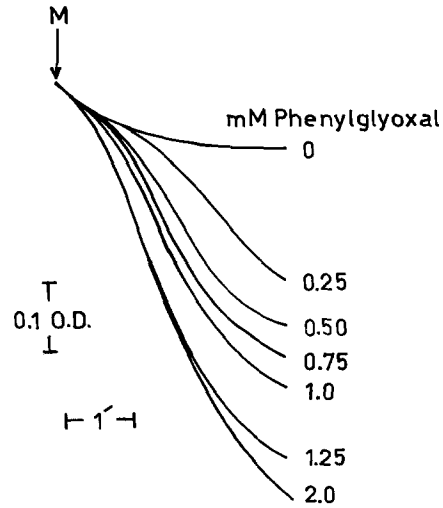

Fig. 5. Mitochondrial swelling induced by phenylglyoxal. Mitochondrial protein (2 mg) was added to a medium containing 120 mM KCl, 2 mM potassium borate, pH 8.3, 10 μg rotenone, and the indicated concentrations of phenylglyoxal.

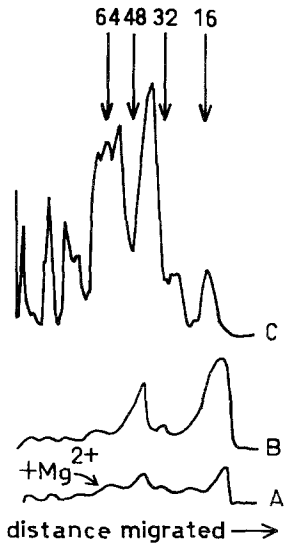

Fig. 6. Fluorescence profile of mitoplasts treated with fluorescamine and fluorescamine plus $MgCl_2$. (A) Protein from mitoplasts (2 mg) was added to a medium (3 ml) which contained 120 mM KCl, 2 mM potassium borate, pH 8.3, 10 μg rotenone, and 10 mM $MgCl_2$. (B) Protein from mitoplasts (2 mg) was added to a similar medium as above minus magnesium. Fluorescamine (98 μM) was added to each experimental medium and, after centrifugation, aliquots containing 150 μg of protein were electrophoresed as indicated in Materials and Methods. (C) Photometric trace at 546 nm of the stained gel.

At this stage of the experimental work it was considered interesting to explore which membrane components are modified by the addition of fluorescamine. Figure 6 shows the fluorescence profile of mitoplasts obtained from polyacrylamide gels and, as can be observed, mitoplasts after incubation with 10 mM Mg^{2+} bind less fluorescamine (Fig. 6A) than mitoplasts incubated without Mg^{2+} (Fig. 6B). The control gels (Fig. 6C), stained with Coomasie blue for proteins, showed that the main modification is localized to a polypeptide with an apparent molecular weight of 40,000 daltons; nevertheless there are also important modifications at the front of the gel where presumably the phospholipids are located.

It is worthwhile to point out that Shiuan and Tu (1978) reported on the gel electrophoresis pattern of proteins labeled with fluorescamine. It is interesting that their pattern differs from that shown in Fig. 6. Most likely this is due to several experimental differences, i.e., we used mitoplasts incubated in KCl media, while Shiuan and Tu used mannitol–sucrose media; furthermore the pH of the mixture was drastically different (pH 7.4 in the Shiuan and Tu experiment); finally we are using mitochondria from heart, while the other authors used mitochondria from liver.

Discussion

The present study establishes that the fluorescent dye fluorescamine, used as a labeling reagent for mitochondrial membrane (Shiuan and Tu,

1978), induces a passive osmotic swelling when mitochondria are incubated in the chloride salts of Li^+, Na^+, K^+, Rb^+, and Cs^+. The swelling does not take place when mitochondria are incubated in a medium containing sucrose; therefore this increased swelling resembles the corresponding reaction reported to occur after the addition of mercurial reagents (Brierley *et al.*, 1968) or macrocyclic antibiotics (Lardy *et al.*, 1967; Pressman *et al.*, 1968) since it is closely related to ion uptake.

This passive permeability to ions induced by fluorescamine is related to the number of primary amino groups of the mitochondrial membrane which are titrable by the dye. It should also be noted that fluorescamine behaves as a cooperative effector of ionic permeability, since when fewer $-NH_2$ groups are blocked, there is little swelling. It appears to be necessary to titrate around 75 nmol of presumably important primary amino groups per milligram protein in order to trigger the swelling.

On the other hand, Wehrle *et al.* (1976) have shown that endogenous magnesium can control the permeability of the mitochondria to monovalent cations; also it has long been known that exogenous magnesium protects mitochondria against swelling; this role of Mg^{2+}, attributable to a blocking of specific ionic channels by its binding to the membrane, is also observed in fluorescamine-induced swelling; however, from the results shown in Table I in which Mg^{2+} diminished the amount of primary amino groups titrated by fluorescamine, it is possible to propose that addition of magnesium prior to the dye results in lower reactivity of the primary amino groups. The latter is not the case when Mg^{2+} is added after reaction of the dye with the membrane. Since the total inhibition requires lower concentrations of the bivalent cation, it would appear that under these conditions a smaller number of sites are available for fluorescamine reaction and are involved in the permeability to monovalent cations; *however, the possibility that both magnesium and fluorescamine affect the proteolipid bulk of the membrane but in opposite directions must not be ruled out.*

Finally, with the results reported here, it appears necessary to postulate the participation of primary amino groups in the regulation of the permeability functions of the mitochondrial membrane to monovalent cations, and from the results obtained from the polyacrylamide gels these primary amino groups would be located in phospholipid molecules or in a protein component with a molecular weight around 40,000 daltons; current experiments are directed to determine in which particular component of the mitochondrial membrane they are located.

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