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Response of Isolated Rat Liver Mitochondria to Variation of External Osmolarity in KCl Medium: Regulation of Matrix Volume and Oxidative Phosphorylation

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When isolated rat liver mitochondria are incubated in KCl medium, matrix volume, flux, and forces in both hypo- and hyperosmolarity are time-dependent. In hypoosmotic KCl medium, matrix volume is regulated via the K^+/H^+ exchanger. In hyperosmotic medium, the volume is regulated in such a manner that at steady state, which is reached within 4 min, it is maintained whatever the hyperosmolarity. This regulation is Pi- and $\Delta\tilde{\mu}H^+$-dependent, indicating Pi-K salt entry into the matrix. Under steady state, hyperosmolarity has no effect on isolated rat liver mitochondria energetic parameters such as respiratory rate, proton electrochemical potential difference, and oxidative phosphorylation yield. Hypoosmolarity decreases the $NADH/NAD^+$ ratio, state 3 respiratory rate, and $\Delta\tilde{\mu}H^+$, while oxidative phosphorylation yield is not significantly modified. This indicates kinetic control upstream the respiratory chain. This study points out the key role of potassium on the regulation of matrix volume, flux, and forces. Indeed, while matrix volume is regulated in NaCl hyperosmotic medium, flux and force restoration in hyperosmotic medium occurs only in the presence of external potassium.

KEY WORDS: Oxidative phosphorylation yield; external osmolarity; matrix volume; swelling; ionic medium; rat liver mitochondria.

INTRODUCTION

To achieve their functions, cells have to accumulate or lose a number of osmotically active substances which can induce cell swelling or shrinkage, in spite of the activity of numerous systems involved in the regulation of cell volume (Häussinger and Lang, 1991a). Furthermore, cell volume variations have extensively been described in liver as being a new principle of metabolic control (Graf *et al.*, 1988; Lang *et al.*, 1989; Häussinger *et al.*, 1990; Bacquet *et al.*, 1990; Meijer *et al.*, 1992; Bacquet *et al.*, 1993). Cell swelling induces stimulation of anabolic pathways (Häussinger and Lang, 1991b; Watford, 1990) whereas cell shrinkage induces stimulation of catabolic pathways (Häussinger and Lang, 1991b). Cell volume variations, *per se*, are linked to modifications in cytosolic osmolarity which could induce modifications in mitochondrial matrix volume, because isolated rat liver mitochondria are known to behave like an osmometer under particular conditions. The influence of matrix volume variations on mitochondrial metabolism has already been studied on isolated rat liver mitochondria (Halestrap, 1989; Halestrap *et al.*, 1990; Armston *et al.*, 1982; Otto and Ontko, 1982; Halestrap *et al.*, 1986; Halestrap and Dunlop, 1986), and it appears that in various conditions of increasing matrix volume, mitochondrial metabolism is strongly affected. Mitochondrial respiratory rate, pyruvate metabolism, citrulline synthesis, and fatty acid oxidation were stimulated while cytochromes were more reduced (Otto and Ontko, 1982). Moreover, on mitochondria from Hamster brown-adipose tissue, it has been shown that the oxidation of palmitoyl-L-carnitine by mitochondria is highly dependent on the nature of the incubation medium (Nicholls *et al.*, 1972). In conditions of matrix volume condensa-

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tion, an inhibition of mitochondrial substrate oxidation has been observed and interpreted as a consequence of an osmotically sensitive diffusion of quinones through the mitochondrial membrane (Mathai *et al.*, 1993). It has also been shown that under conditions of matrix condensation, state 3 respiratory rate was inhibited, as was ATPase activity (Nicholls and Lindberg, 1972), and ADP/O ratio was slightly decreased (Sitaramam and Rao, 1991).

Furthermore, previous work in our laboratory showed that on isolated hepatocytes, cytosolic hypoosmolarity induces a large increase in mitochondrial volume which is not directly involved in the activation of respiration (Espié *et al.*, 1995). Indeed, the lower the external osmolarity, the higher the overall thermodynamic driving force (i.e., $2\Delta E'h - 10\Delta p$, where $\Delta E'h$ is the difference in redox potential across the electron transport chain and 10 is its H^+/O stoichiometry) for the same respiratory rate. These results point more to an inhibition of respiration at the level of the electron transport chain rather than to a stimulation, as previously proposed for isolated rat liver mitochondria. Moreover, when isolated hepatocytes are incubated in the presence of nonmetabolizable sodium-cotransported amino acids (cytosolic hyperosmolarity), neither matrix volume, respiratory rate, nor thermodynamic parameters are affected (Espié, 1994). So it appears that: (i) when the cytosol is hypoosmotic, matrix volume increases but respiratory rate is not stimulated, and (ii) when the cytosol is hyperosmotic, matrix volume is not affected, which is not in accordance with previous published results concerning the influence of external osmolarity on isolated rat liver mitochondria (Halestrap, 1989; Halestrap *et al.*, 1990; Armston *et al.*, 1982; Otto and Ontko, 1982; Halestrap *et al.*, 1986; Halestrap and Dunlop, 1986, Nicholls and Lindberg, 1972, Devin *et al.*, 1996). In a previous study, we showed that in sucrose medium, the decrease in oxidative phosphorylation yield when external osmolarity increases was linked to adenine nucleotide carrier activity: when external osmolarity increases, its inhibition lowers the state 3 respiration rate close to the state 4 level and consequently leads to a decrease in oxidative phosphorylation yield (Devin *et al.*, 1996). As previously observed by others (Halestrap, 1989; Halestrap *et al.*, 1990), we confirm that on isolated rat liver mitochondria, matrix volume and state 3 respiratory rate both increase in hypoosmotic sucrose medium. Moreover, we observed that this increase in state 3 respiratory rate is associated with a large decrease in protonmotive force. The relationship between JO_2 and Δp is left-shifted in hypoosmotic sucrose medium, in such a manner that for a given respiration rate, the protonmotive force maintained is lesser in hypo- than in isoosmotic medium (Espié *et al.*, 1995). These results are not in favor of an activation of the respiratory chain, for which one might expect an increase in protonmotive force.

In fact, no thoroughly thermodynamic and kinetic studies of oxidative phosphorylation on isolated rat liver mitochondria under conditions of varying both external osmolarity and ionic force have been done. The evolution of respiratory fluxes and ADP/O ratio as a function of external NaCl osmolarity has previously been measured, and it appears that state 3 respiratory rate increases from hypo- to isoosmolarity and decreases from iso- to hyperosmolarity, while ADP/O ratio is stationary (Sitaramam and Rao, 1991). However, the mitochondria in mammalian cells are exposed to a cytosol that is high in potassium and low in sodium (Garlid, 1994), and it is now well established that potassium, in addition to its osmotic function, is fully involved in matrix volume regulation by the intermediary of K^+ influx and/or efflux by way of K^+/H^+ exchanger (which extrudes an excess of K^+), K^+-channel, and K^+-leak (see Scheme 1 for the mitochondrial K^+-cycle) (Garlid, 1994). Thus, potassium is involved in both mitochondrial enzymatic matrix reactions and control of mitochondrial volume regulation (Garlid, 1980; Gunter, 1994). So, to be closer to *in situ* conditions and to have a better understanding of the mechanism of both respiratory rate changes when varying external osmolarity and discrepancies between *in situ* and *in vitro* results, we studied the influence of external osmolarity in KCl medium on essential thermodynamic parameters in isolated rat liver mitochondria energetics.

We show that state 3 respiratory rate, matrix volume, and membrane electrical potential difference are time-dependent in KCl medium. In iso- and hyperosmotic media, matrix volume is regulated in such a manner that this regulation is dependent on K^+, electroneutral Pi entry, and $\Delta\tilde{\mu}H^+$ maintenance.[2] During the first minutes of isolated rat liver mitochondria incubation in hyperosmotic KCl medium, there is a

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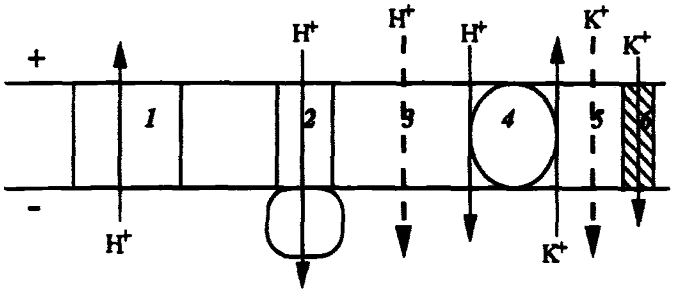
[2] Abbreviations: DMO: 5,5-dimethyloxazolidine-dione; ΔpH: pH difference across the mitochondrial inner membrane; ΔΨ: electrical potential difference across the mitochondrial inner membrane; $\Delta\tilde{\mu}H^+$: electrochemical potential difference across the mitochondrial inner membrane; TMPD: *N,N,N′,N′*-tetramethyl-1, 4-phenylenediamine; TPMP: tetraphenylmethylphosphonium.



Scheme 1 : The mitochondrial K^+ cycle (according to Garlid K.D. (1994) *Journal of Bioenergetics and Biomembranes* **26** 537-542)

(1) : respiratory chain
(2) : ATPsynthase
(3): proton leak
(4) : potassium/proton exchanger
(5) : potassium leak
(6) : potassium channels

mitochondrial matrix shrinkage associated with a decrease in state 3 respiratory rate, which is in accordance with previously published results (Nicholls and Lindberg, 1972). Matrix volume recovery in hyperosmotic KCl medium is related to flux recovery. Furthermore, when a steady state is reached between iso- and hyperosmolarity, matrix volume, respiratory rates, $\Delta\tilde{\mu}H^+$, and NAD(P)H level are unchanged as in isolated hepatocytes in conditions of cytosolic hyperosmolarity (Na^+-cotransported amino acids). This indicates that under our experimental conditions, isolated rat liver mitochondria fully regulate these parameters via the potassium-salt entry. From hypo- to isoosmolarity, state 3 respiratory rate increases, as does ΔpH and consequently $\Delta\tilde{\mu}H^+$. This indicates an activation of K^+/H^+ carrier according to the degree of hypoosmolarity.

EXPERIMENTAL PROCEDURES

Mitochondrial Preparation

Liver mitochondria were prepared from male Wistar rats (250–300 g body weight) starved overnight. Rats were killed by cervical dislocation and liver was rapidly removed and put into an ice-cold isolation medium containing 225 mM sucrose, 20 mM Tris-HCl (pH 7.2), and 1 mM EGTA. Mitochondria were isolated according to Cooper and Lehninger (1956) in the same medium. The mitochondrial pellet was finally resuspended in the isolation medium. Protein concentration was estimated by the biuret method using bovine serum albumin as standard (Gornall *et al.*, 1948).

Mitochondrial Respiration and ATP/O Measurements

The rate of oxygen uptake at various osmolarities was measured polarographically at 26°C using a Clark-type oxygen electrode connected to a microcomputer giving an online display of rate values. Respiration buffer contained 5 µM $TPMP^+$ (triphenylmethylphosphonium), 5 µM DMO (5,5-dimethyloxazolidine-2,4-dione), 5 µM mannitol, 20 mM Tris-HCl (pH 7.2), 1 mM EGTA, 6 mM Tris-respiratory substrates, 5 mM Tris-Pi, and an amount of KCl to adjust the osmolarity to the required value. Depending on experimental conditions, 1–4 mg of mitochondrial protein were used after checking that the respiratory rate was always directly proportional to the milligrams of protein, whatever the osmolarity.

Oxygen concentrations in the different osmolarity media were determined with NADH quantitated spectrophotometrically and yeast mitochondria (Rigoulet *et al.*, 1996).

Phosphorylation rate was measured by ^{32}[P]Pi incorporation in adenine nucleotides as previously described (Rigoulet and Guérin, 1979). These measurements were done in respiratory buffer supplemented with 1 mM Tris-ADP, at various KCl osmolarities. The ATP/O ratio stoichiometries were determined from the yield of oligomycin-sensitive phosphorylation rates versus respiratory rates.

Measurement of Matrix Space, ΔpH, and ΔΨ Using Radiolabeled Elements

Routinely, the protonmotive force under different steady states was determined as follows: matrix space was determined using ^{3}[H] water and ^{14}[C]mannitol, an inner membrane impermeable sugar, in the respiratory buffer. After equilibration (3 min), 0.5 mM Tris-ADP was added in order to reach state 3 respiration. After 15 sec, mitochondria were rapidly centrifuged (20 sec) and then treated as described previously (Rigoulet and Guérin, 1979). For state 4 measurements, the rapid centrifugation was done when the net ATP synthesis flux was zero (state 4). The delay after ADP addition necessary to obtain state 4 was checked by oxygen consumption determinations. ΔpH was measured by the distribution of ^{14}[C]DMO according to Rottenberg (1979) and ΔΨ by ^{3}[H]TPMP^{+} distribution.

TPMP^{+} is a lipophilic cation which is partially linked to membranes. For its binding, we applied a correction coefficient of 0.38 to our measurements, determined in our laboratory (Espié *et al.*, 1995).

Mitochondrial NAD(P)H Fluorescence Measurements

NAD(P)H measurements make it possible to evaluate qualitatively the reducing equivalent supply to the respiratory chain. NADH+NADPH fluorescence was monitored at 26°C with a Kontron fluorimeter as previously described (Koretsky and Balaban, 1987). Excitation wavelength was 340 nm and fluorescence emission wavelength was 465 nm. Mitochondria (0.5 mg protein) were suspended in the respiratory buffer at various KCl osmolarities to a final volume of 3 ml. Results were expressed as a percentage of NAD(P)H fluorescence: 0% was an endogenous signal corresponding to a steady state of mitochondria placed in respiratory buffer without added substrate. State 3 fluorescence corresponds to mitochondria in the presence of 6 mM respiratory substrates and 50 μM ADP. State 4 fluorescence was measured when the net flux of ATP synthesis was zero. Maximal NAD(P)H fluorescence (100%) was measured in the presence of 1 μg/ml antimycin. This technique does not allow quantitative determination of the NADH concentration because free and bound NAD(P)H are simultaneously measured. Therefore, it is not possible to evaluate the proportion of either. Furthermore, maximal NAD(P)H fluorescence depends on the enzyme involved and we could not quantitate the NAD^{+} content.

Measurements of ΔΨ Time Dependence

ΔΨ measurements as a function of time were done using Rhodamine 123 as a probe of inner mitochondrial membrane ΔΨ (as described in Emaus *et al.*, 1986); excitation wavelength was 485 nm and emission wavelength was 525 nm. These measurements were done with a Kontron fluorimeter, in 2 ml respiratory buffer supplemented with 1 μM Rhodamine 123, 1 mg of mitochondrial protein, and 0.5 mM ADP for state 3 determination.

Time Dependence of Matrix Volume

As matrix volume measurements using radiolabeled elements required 3 min to reach the equilibrium of distribution of these elements, time and respiration state dependence of matrix volume were measured using a Kontron fluorimeter. Indeed, it is now well known that an increase in mitochondrial matrix volume is accompanied by a decrease in light scattering at 540 nm. This phenomenon was monitored when excitation and emission wavelength were 540 nm. Relative matrix volume measurements were done in 2 ml respiratory buffer and 0.5–1 mg of mitochondrial protein supplemented with 0.5 mM ADP if required, in the same conditions as those used for measurements of respiratory flux time dependence.

Measurements of Medium Osmolarity

Medium osmolarity measurements were done for each osmolarity. Medium osmolarity was measured by using a Roebling osmometer. Osmolarity was determined by the freezing point of an aliquot of the medium under study, since the decrease in the freezing point compared to that of distilled water is directly related to the osmolarity of the medium. Osmolarities indicated in the figures are those measured in the absence of Pi, substrates, ADP, and uncoupler.

RESULTS

Time Dependence

To assess the actual steady state of oxygen consumption under the different osmotic conditions in KCl medium, we checked the time dependence of respiratory rates. Figure 1 shows that in KCl medium,

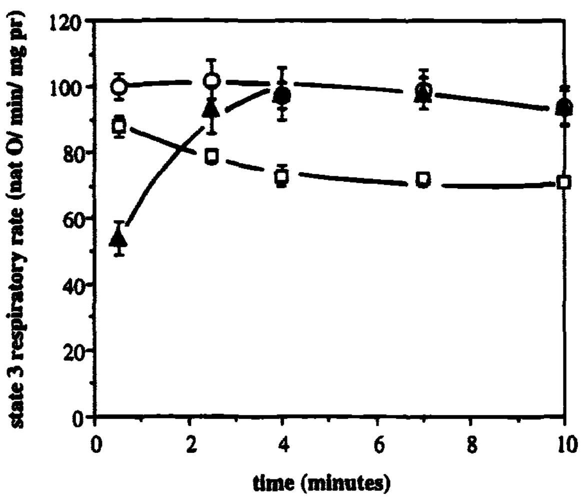


Fig. 1. Time dependence of state 3 respiratory rate. Mitochondria (1 mg/ml) were suspended in respiratory buffer as described in Materials and Methods with glutamate and malate as substrates. State 3 respiratory rate measurements were done 1 min after 1 mM ADP addition. (○) isoosmolarity, i.e., 225 mOsm KCl; (□) hypoosmolarity, i.e., 100 mOsm KCl; (▲) hyperosmolarity, i.e., 400 mOsm KCl. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

when glutamate and malate were the substrates: (i) in isoosmolarity, state 3 respiratory rate did not significantly vary during 10 min of mitochondrial incubation; (ii) in hypoosmolarity, state 3 respiratory rate decreased for 4 min of incubation and was lower than in isoosmolarity. This decrease in state 3 respiratory rate could be linked to a loss in cytochrome *c*, itself linked to a decrease in osmolarity associated with ionic strength. We checked that state 3 respiratory rate in hypoosmolarity was not significantly increased with externally added cytochrome *c*, which shows that the decrease in state 3 respiratory rate was not linked to a loss in cytochrome *c* (not shown); (iii) in hyperosmolarity, state 3 respiratory rate increased for 4 min of mitochondrial incubation and then was stationary and equal to state 3 respiratory rate in isoosmolarity. In the same osmotic conditions, the uncoupled state behaved like state 3 and remained greater while state 4 was stationary whatever the time considered (not shown). This time dependence of respiratory fluxes was not noted in sucrose medium (not shown).

To investigate the segmental response of the respiratory chain to osmotic and ionic changes, we also used succinate (in the presence of rotenone) and TMPD/ascorbate (in the presence of antimycin) as substrates. It appeared that respiratory rates using succinate as substrate behaved like respiratory rates with glutamate and malate, while respiratory rates with TMPD/ascorbate were time-independent and osmolarity-insensitive (not shown). This last result is in accordance with previous studies showing that cytochrome-*c*-oxidase is osmolarity-insensitive (Mathai *et al.*, 1993).

As we observed the same behavior using glutamate and malate or succinate as substrates, all the following experiments were done using the more physiological substrates, i.e., glutamate and malate.

To check if time dependence was linked to the salt used, and since the respiratory rate is stationary in sucrose medium, we measured respiratory rates in both sodium chloride and potassium glucuronate medium. Glucuronate is a nonpermeant anion and sodium movements across the inner mitochondrial membrane occur differently from those of potassium (Brierley *et al.*, 1994). Comparison between Fig. 1 and Fig. 2A shows that, in potassium medium, whatever the anion considered, state 3 respiratory rate (i) was time-dependent in both hypo- and hyperosmotic medium, and (ii) it had almost the same value whatever the osmolarity considered. Figure 2B shows that in sodium chloride medium, (i) the respiratory rate in phosphorylating conditions was slightly time-dependent whatever the osmolarity considered; (ii) iso- and hypoosmotic state 3 respiratory rates varied similarly as a function of time, and the difference in their values was comparable to that found in KCl medium at the same osmolarity; (iii) the amplitude of respiratory rate restoration in hyperosmotic medium was less than in potassium medium. In view of these behaviors in both potassium and sodium media, after 4 min of incubation (which is necessary to obtain an actual steady state), we observed (Fig. 3) that (i) in hypoosmotic medium (100 mOsm) the state 3 respiratory rate was nearly the same whatever the considered medium; (ii) from 100 to 225 mOsm, the respiratory rate as a function of external osmolarity was comparable between NaCl and KCl media; (iii) in hyperosmolarity, this rate was strongly inhibited in sucrose medium, restored in potassium medium, and partly restored in NaCl medium. So in sucrose medium in which respiratory rates are not time-dependent, there is a simple and instantaneous osmotic response of flux to external osmolarity, as previously shown (Nicholls and Lindberg, 1972). In hyperosmotic ionic medium, flux response to external osmolarity is dependent on the medium considered; its restoration is complete in K^+-salts media while in NaCl medium, flux response to external osmolarity seems to be more swift.

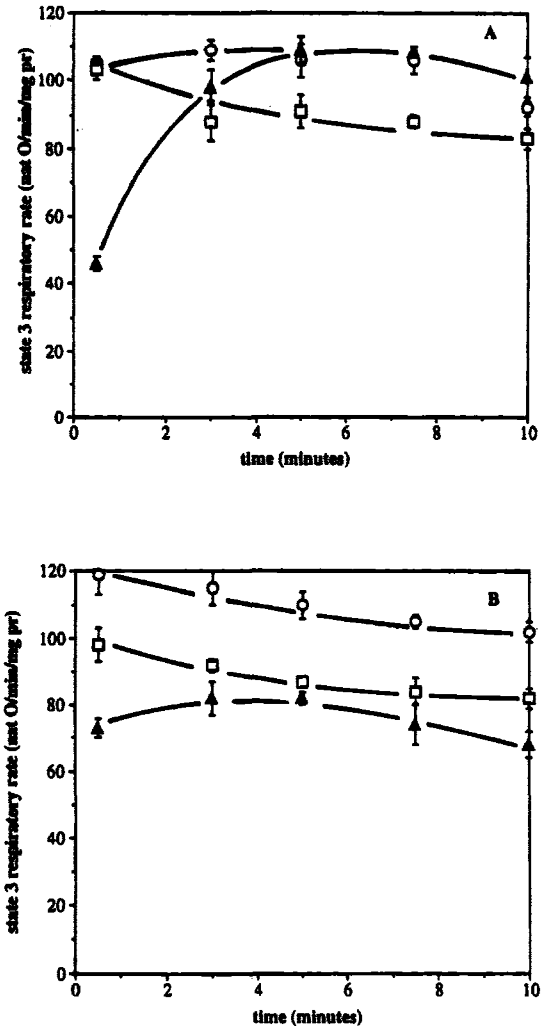


Fig. 2. Time dependence of state 3 respiratory rate in (A) potassium glucuronate medium and (B) sodium chloride medium. Mitochondria (1 mg/ml) were suspended in respiratory buffer as described in Materials and Methods with glutamate and malate as substrates. State 3 respiratory rate measurements were done 1 min after 1 mM ADP addition. (□) hypoosmotic medium, i.e., 100 mOsm (○) isoosmotic medium, i.e., 225 mOsm; (▲) hyperosmotic medium, i.e., 400 mOsm. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

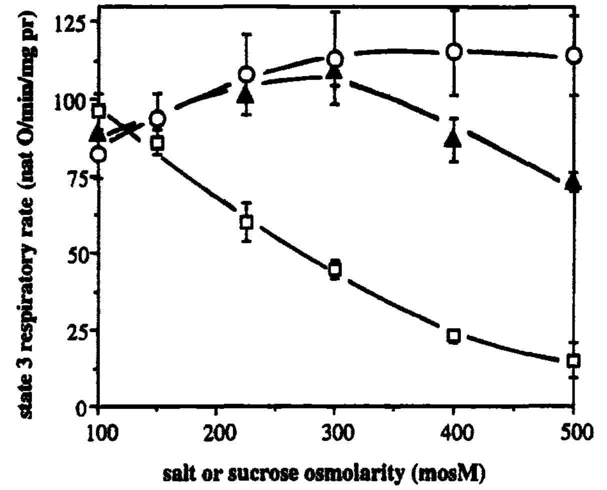


Fig. 3. State 3 respiratory rate evolution as a function of external osmolarity in different media. (○) KCl medium; (□) sucrose medium; (▲) NaCl medium. Mitochondria (1 mg/ml) were suspended in the considered salt medium supplemented with 5 μM TPMP$^+$, 5 μM DMO, 5 μM mannitol, 20 mM Tris-HCl (pH 7.2), 1 mM EGTA, 6 mM glutamate and malate, 5 mM Tris-Pi, and an amount of either KCl, NaCl, or sucrose to bring the osmolarity to the required value indicated in the figure. Phosphorylations were induced by the addition of 1 mM ADP. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

Matrix volume time dependence was monitored by light scattering. It is well known that light scattering decreases when matrix volume increases and reciprocally. Firstly, to check this phenomenon on isolated rat liver mitochondria and to quantitate the light scattering signal, we compared the matrix volume measured at different osmolarities and under steady state using radiolabeled elements and light scattering. This was done in sucrose medium because of osmometric behavior and because there is no time dependence of matrix volume of isolated rat liver mitochondria in iso- and hyperosmotic medium. Figure 4 shows an almost linear relationship between this matrix volume and light scattering for matrix volumes from 0.9 to 0.35 μl/mg protein. However, considering the error bars, such a method only assesses matrix volume evolution qualitatively. Figure 5 shows that under isoosmotic conditions (A), matrix volume slightly increased in the presence of respiratory substrates, and that this increase is stimulated by Pi addition. ADP addition induced a slight shrinkage of mitochondrial matrix. Under hyperosmotic conditions (B), matrix volume was less than in isoosmotic medium in the absence of respiratory substrates, indicating a primary shrinkage of mitochondrial matrix. Glutamate/malate addition induced an increase in matrix volume, which was highly stimulated when external Pi was added. Again, ADP addition induced a shrinkage of mitochondrial matrix, which was greater compared to that in isoosmotic medium. Furthermore, final light scattering in the presence of substrates and Pi was the same between iso-and hyperosmolarity. Under hypoosmotic conditions (C), neither substrates nor Pi modified mitochondrial swelling. This was comparable

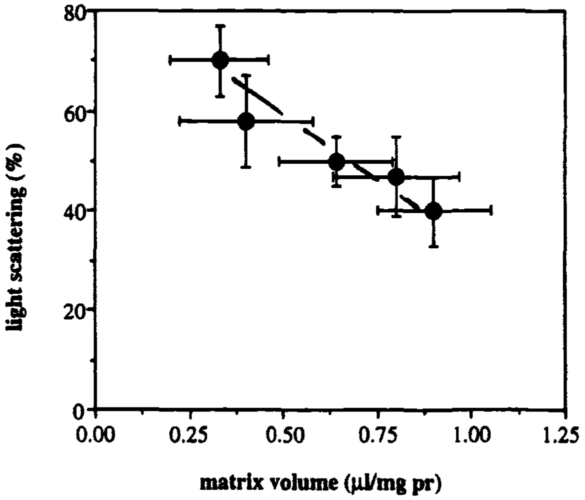


Fig. 4. Light scattering as a function of matrix volume. Light scattering (%) was measured as described in Materials and Methods. 100% corresponds to the signal obtained for mitochondria incubated in 500 mM sucrose medium (maximal shrinkage), and 0% corresponds to the signal obtained for mitochondria incubated in 0 mM sucrose medium (maximal swelling or membrane breaking). Matrix volume was measured as described in Materials and Methods. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

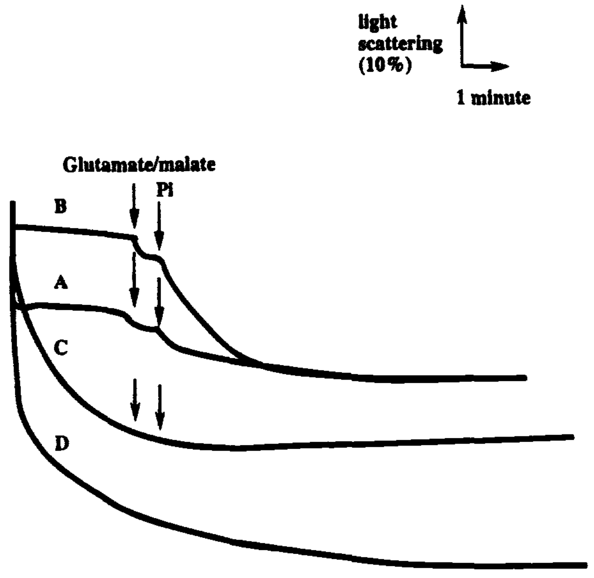


Fig. 5. Mitochondrial swelling and shrinkage measured by light scattering. Light scattering signal was measured as described in Materials and Methods. Substrates, Pi, and ADP were added as indicated in the figure. (A) isoosmotic KCl medium; (B) hyperosmotic KCl medium; (C) hypoosmotic KCl medium; (D) hypoosmotic KCl medium in the presence of antimycin (2.5 µg/mg protein) and oligomycin (1.25 µg/mg protein). This experiment is representative of three such experiments.

to that measured in sucrose medium. Furthermore, we have previously shown that, in this medium, hypoosmotic matrix volume is less than expected by a pure osmometric behavior (see Fig. 10 in Devin *et al.*, 1996), which may indicate that volume regulatory mechanisms such as K^+/H^+ carrier occur, as previously described (Garlid, 1980). This hypothesis is sustained by the fact that in the presence of antimycin A and oligomycin, the matrix volume increase in hypoosmotic medium was much greater than that under energized conditions (D). It should be stressed that whatever the osmolarity, mitochondrial volume varied for 4 min of mitochondrial incubation after substrate addition, whatever the respiratory state.

These experimental results raise the question of how the matrix volume increases in both iso- and hyperosmotic media. Indeed, in both, this swelling is necessarily linked to the entry of osmotic active substances, which could be K-Pi under our experimental conditions. Firstly, this swelling needs respiratory substrates and is strongly inhibited in the presence of uncoupler, so it is an energy-dependent phenomenon (not shown). Moreover, when Pi is replaced by either acetate or thiocyanate, the swelling is partly decreased in acetate medium and drastically decreased in thiocyanate medium (not shown). Therefore, it is greatly dependent on anion entry by an electroneutral mechanism depending on ΔpH.

To discriminate the way K^+ enters the mitochondrial matrix, we studied the influence of known inhibitors of a part of the mitochondrial K^+ cycle on this swelling. Neither $MgCl_2$ [inhibitor of K^+/H^+ exchanger (Garlid, 1980)], Pindolol (inhibitor of K^+/H^+ exchanger), nor glybenclamide [inhibitor of K_{ATP} channels (Sturgess *et al.*, 1985; Inoue *et al.*, 1991)] had any effect on this swelling. Quinine (Garlid *et al.*, 1986) aspecifically inhibits the respiratory chain for concentrations inhibiting K^+/H^+ exchanger, so it could not be used under our experimental conditions.

As there was no one-to-one correlation between matrix volume and respiratory rate evolution as a function of time, it was of interest to estimate inner mitochondrial membrane electrical potential difference as a function of time. Whatever the external osmolarity considered, neither respiratory rate nor $\Delta\Psi$ were time-dependent under state 4 respiration (not shown). Under state 3 respiration, (i) in isoosmotic medium (Fig. 6A), respiratory rate and $\Delta\Psi$ were not significantly time-dependent; (ii) in hyperosmotic medium (Fig. 6B), in the first 4 min, the increase in

JO_2 was related to a decrease in $\Delta\Psi$; (iii) in hypoosmotic medium (Fig. 6C), during the first 4 min, JO_2 decreased for a constant $\Delta\Psi$.

Steady State

The evolution of matrix volume as a function of external osmolarity after 4 min of mitochondrial incubation was different compared to that in sucrose medium, in which matrix volume decreased when external osmolarity increased (Halestrap, 1989, Halestrap and Quinlan, 1983, Devin *et al.*, 1996). Indeed, in KCl medium, the lack of volume variations from iso- to hyperosmolarity indicates osmotic active substance movements across the inner mitochondrial membrane, which make it possible to equilibrate osmolarity changes. In hypoosmotic medium, matrix volume increased when external osmolarity decreased in the same range as that observed in sucrose medium (not shown).

In KCl medium, state 4 respiratory rate slightly decreased from hypo- to isoosmolarity and then was stationary from iso- to hyperosmolarity (not shown). In sucrose medium, when matrix volume increased, state 3 respiratory rate and uncoupled state increased (Halestrap, 1989). Conversely, in KCl medium, with regard to hypoosmolarity, when matrix volume increased from iso to hypoosmolarity, state 3 respiratory rates slightly decreased (Fig. 3), as did the uncoupled state (not shown). It should be stressed that for the same increase in matrix volume, state 3 respiratory rate was comparable in sucrose and KCl hypoosmotic medium. Indeed, the decrease in state 3 respiratory rate in hypoosmotic KCl medium is linked to its stimulation in isoosmotic KCl medium compared to sucrose medium (Devin *et al.*, 1997). From iso- to hyperosmolarity, respiratory rates did not significantly vary (Fig. 3 for state 3 respiratory rate).

The NAD(P)H level is representative of the reduced equivalent supply to the respiratory chain.

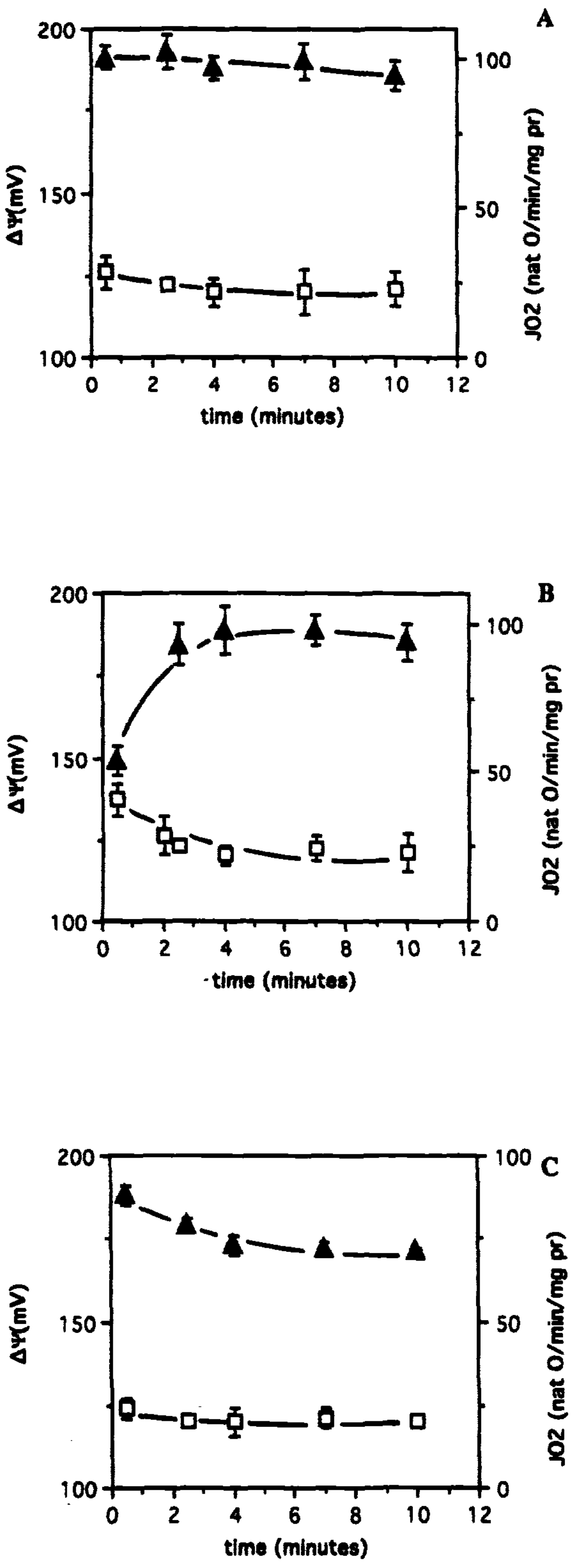


Fig. 6. Time dependence of state 3 respiratory rate and ΔΨ in either (A) isoosmotic, (B) hyperosmotic, or (C) hypoosmotic KCl media. Mitochondria (1 mg/ml) were suspended in respiratory buffer as described in Materials and Methods. Respiratory rate values (▲) are from Fig. 1. Membrane electrical potential (□) was measured using rhodamine 123 as described in Materials and Methods. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

This level slightly increased from hypo- to isoosmolarity and was stationary from iso- to hyperosmolarity (not shown). To determine protonmotive force evolution in KCl medium, we measured ΔpH and ΔΨ respectively as a function of external osmolarity. From hypo- to isoosmolarity, whatever the respiratory state, ΔpH slightly increased (Figure 7A). On the other hand, Fig. 7B shows that Δψ was not significantly modified when osmolarity varied. Consequently, the protonmotive force slightly increased from hypo- to isoosmolarity while it was stationary from iso- to hyperosmolarity (Fig. 7B).

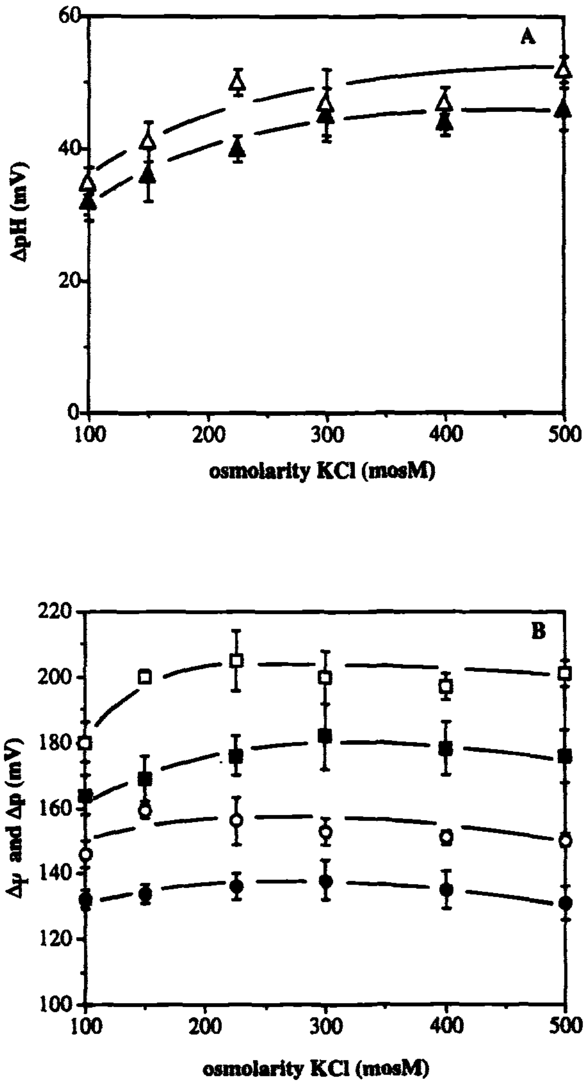


Fig. 7. Influence of KCl external osmolarity on either ΔpH (A) or ΔΨ (B) under steady-state conditions. ΔpH and ΔΨ were measured as described in Materials and Methods after 4 min of mitochondrial incubation. (▲) state 3 ΔpH; (△) state 4 ΔpH; (●) state 3 ΔΨ; (○) state 4 ΔΨ; (■) state 3 Δp; (□) state 4 Δp. State 3 conditions were measured in the presence of 1 mM ADP. The values presented are means ± SD for at least three such experiments carried out with three different mitochondrial preparations.

It has previously been shown that when external osmolarity increases in sodium chloride medium, the ADP/O ratio decreases slightly (Sitaramam and Rao, 1991). In our study, in KCl medium, the ATP/O ratio did not significantly vary whatever the osmolarity with either succinate or glutamate/malate as substrates (not shown).

DISCUSSION

It is generally accepted that isolated rat liver mitochondria behave as an osmometer and that changes in matricial volume can modify mitochondrial metabolism (Halestrap, 1989; Halestrap *et al.*, 1990; Armston *et al.*, 1982; Otto and Ontko, 1982; Halestrap *et al.*, 1986; Halestrap and Dunlop, 1986). However, numerous anionic and cationic channels or exchangers have been described as occurring during matrix volume regulation. Previous studies in isolated rat liver mitochondria in sucrose medium have shown that hyperosmolarity strongly decreases matrix volume in such a way that the relationship between matrix volume and 1/osmolarity is linear (Halestrap and Quinlan, 1983; Devin *et al.*, 1996). Meanwhile, in hypoosmotic medium, the increase in matrix volume is less than expected for an actual osmometer, indicating the activity of the K^+/H^+ exchanger. This points to the importance of cations and anions in the regulation of matrix volume during osmotic changes. In the present work, we show that in KCl medium, matrix volume in hyperosmolarity is efficiently regulated in such a way that under steady state, it is completely recovered compared to its value in isoosmolarity. Furthermore, the mechanisms of matrix volume regulation depend on the energy status of isolated rat liver mitochondria. Indeed, when matrix volume evolution is monitored by light scattering, it appears that (i) substrate addition to isolated rat liver mitochondria in KCl iso- or hyperosmotic medium induces an increase in matrix volume which is strongly increased in hyperosmotic medium by Pi addition, whatever the time considered; (ii) ADP addition induces a slight shrinkage in both iso- and hyperosmotic KCl medium; (iii) replacing Pi by (a) an anion whose diffusion is also ΔpH-dependent but whose accumulation is less so (acetate), decreases the swelling; (b) a charged diffusible anion (thiocyanate) strongly decreases this swelling. So mitochondrial swelling in iso- and hyperosmotic KCl medium seems to be energy- and Pi-dependent. It is therefore probably linked to Pi-K salt accumulation in the mitochondrial matrix. In such a hypothesis, the fact that ADP addition induces a shrinkage is probably linked to a shift in free Pi in phosphorylating conditions. Moreover, the latter induce a slight decrease in ΔpH which could modify the ratio between external and internal Pi. Potassium fluxes under our experimental conditions have never been inhibited by the use of known inhibitors of a part of the mitochondrial potassium cycle. The main interpretation is that potassium enters the mitochondrial matrix via both potassium leak and potassium channels. Taken together, these results indicate that matrix volume regulation is essentially due to an entry of K-Pi. In hypoosmotic medium, the behavior of isolated rat liver mitochondria is comparable to that in sucrose medium (Devin *et al.*, 1996). Indeed, in this medium, the increase in matrix volume is less than expected by the linear relationship obtained between matrix volume and 1/osmolarity in iso- and hyperosmolarity incubation conditions. This may indicate that in hypoosmotic medium, volume regulatory mechanisms occur: as previously proposed, K^+/H^+ carrier in such conditions (i.e., increase in matrix volume inducing a decrease in intramatricial $[Mg^{2+}]$) can induce an extrusion of potassium associated with an intrusion of protons (Garlid, 1980).

In addition to matrix volume regulation, state 3 respiratory flux varied in hypo- and hyperosmotic KCl medium for 4 min of mitochondrial incubation. Firstly, under hyperosmotic KCl incubation conditions, the respiratory rate increased in the first 4 min. This increase was observed only in hyperosmotic potassium medium. Indeed, flux restoration was not complete when mitochondria were incubated in NaCl medium, but the phenomenon was faster. Moreover, it should be pointed out that the variations in matrix volume and state 3 respiratory rate are in accordance with the results of others: (i) in sucrose medium, an increase in matrix volume is associated with an increase in state 3 respiratory rate; (ii) in KCl hyperosmotic medium, before steady state attainment, matrix shrinkage is associated with an inhibition of state 3 respiratory rate; (iii) matrix volume recovery in hyperosmotic KCl medium is associated with state 3 respiratory rate recovery. However, this respiratory rate restoration in KCl medium is not only linked to matrix volume evolution under energized conditions. Indeed, in NaCl medium, matrix volume in hyperosmotic medium is the same as in isoosmotic medium and comparable to that in KCl medium (not shown), while state 3 respiratory rate is not completely recovered (see Fig. 3). So it seems that the respiratory rate in state 3 depends on both matrix volume and matrix potassium concentration. In relation to the increase in respiratory rate, the membrane electrical potential difference decreases in hyperosmotic KCl medium until 4 min of mitochondrial incubation.

Membrane electrical potential difference measurement is done by using rhodamine 123. This method presents some technical limitations, particularly when matrix volume changes during ΔΨ measurement. Indeed, an increase in matrix volume can induce an increase in rhodamine accumulation, without any change in ΔΨ. However, in hyperosmotic KCl medium, during the increase in matrix volume, we observed a decrease in the amount of rhodamine accumulated, which perhaps led to an underestimation of ΔΨ decrease as a function of time. So in hyperosmotic KCl medium and during the first minutes of mitochondrial incubation, it can be hypothesized that there is a potassium energy-dependent entry which decreases ΔΨ and consequently stimulates state 3 respiration. Under hypoosmotic incubation conditions, the state 3 respiratory rate decreased during the first minutes of mitochondrial incubation while the rhodamine signal did not significantly vary. As mentioned above, the fact that the amount of rhodamine accumulated in the matrix space did not vary while matrix volume increased could yield a decrease in ΔΨ.

Previous work in our laboratory showed that on isolated hepatocytes incubated in hypoosmotic media, a large increase in mitochondrial volume was not directly involved in the activation of respiration (Espié *et al.*, 1995). Indeed, the lower the external osmolarity, the higher the overall thermodynamic driving force for the same respiratory rate. These results point more to an inhibition of respiration at the level of the electron transport chain rather than to a stimulation, as previously proposed on isolated rat liver mitochondria. Yet the present results obtained on isolated rat liver mitochondria incubated in KCl medium are in agreement with those obtained on hepatocytes. Indeed, under hypoosmolarity and when steady state is reached, state 3 respiratory rate, NAD(P)H level, and protonmotive force decrease and oxidative phosphorylation yield does not significantly vary. It should be stressed that

NAD(P)H level measurement is not a quantitative one and does not assess the redox span between NADH/ NAD^+ and O_2/H_2O couples as in cells. So the main influence of hypoosmolarity in such an ionic medium on oxidative phosphorylation is a kinetic control upstream the respiratory chain. Furthermore, studies on isolated rat hepatocytes in conditions of cytosolic hyperosmolarity (i.e., sodium-cotransported aminoacid accumulation) have shown that neither JO_2, matrix volume, nor mitochondrial membrane electrical potential vary when amino acids are not metabolized (Espié, 1994). This cannot be related to results obtained on isolated rat liver mitochondria in sucrose medium (Nicholls and Lindberg, 1972; Devin *et al.*, 1996). In the present study, in KCl medium and when steady state is reached, from iso- to hyperosmolarity, and under state 3, neither respiratory rate, NAD(P)H level, oxidative phosphorylation yield, nor protonmotive force varied. This correlates well with the influence of cytosolic hyperosmolarity on isolated hepatocyte energetic parameters.

This study shows that when isolated rat liver mitochondria are studied in KCl medium, matrix volume and oxidative phosphorylation activity tend to be regulated as in cells. It should be stressed that the key role of potassium is also observed in hypoosmotic sucrose medium. Indeed, in these conditions, the regulation of matrix volume depends on the activity of the mitochondrial K^+/H^+ exchanger (Garlid, 1980). Moreover, under hyperosmotic conditions, salt entry allows matrix volume recovery. This is not dependent on the cation under consideration because sodium is also suitable for this restoration of matrix volume in hyperosmotic medium (not shown). When oxidative phosphorylation activity is concerned, it appears that potassium, *per se*, is necessary for both flux and force regulation. Indeed, in NaCl medium, state 3 respiratory rate and ADP/O ratio decrease in hyperosmolarity (Sitaramam and Rao, 1991), while we show in this study that oxidative phosphorylation yield is not affected by KCl hyperosmotic medium. It has been proposed that the respiratory rate in the phosphorylation state strongly depends on matrix volume. Our results are in agreement with this, considering that (i) in both KCl and sucrose hypoosmotic medium, the matrix volume increase is comparable and state 3 respiratory rate is the same (see Fig. 3); (ii) in hyperosmotic KCl medium, matrix volume decrease and recovery are related to a decrease and a recovery of state 3 respiratory rate; (iii) in iso- and hyperosmotic KCl medium, the same flux is obtained for the same volume. Therefore, this paper shows that the intramitochondrial potassium concentration is another important parameter intervening in the control of respiration. Indeed, under isoosmotic conditions, there is a slight increase in matrix volume, while there is a large increase in state 3 respiratory rate in KCl medium compared to sucrose medium (Devin *et al.*, 1997). Under hypoosmotic conditions, the same state 3 respiratory rate is obtained in both sucrose and KCl medium, and the mechanism implicated in matrix volume regulation is K^+/H^+ exchanger. Therefore, one could expect comparable intramatricial concentrations in potassium. In hyperosmotic KCl medium, potassium entry permits both matrix volume and state 3 respiratory flux restoration.

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