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Factors Limiting Mitochondrial Respiration in Media of High Solute Content

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Abstract

The state-3 rate of respiration of rat-liver mitochondria was depressed in media containing KCl, sucrose, or mannitol at concentrations in excess of 125 mM. At equivalent concentrations, glucose caused less inhibition than sucrose or mannitol, and no inhibition was observed with glycine. These observations establish that solute inhibition of respiration is not a consequence of the reduced chemical potential of water in the system. The accumulation of succinate by mitochondria was not reduced by high sucrose concentrations. Sonication only partially relieved inhibition by sucrose or mannitol, and not at all that by KCl, and the evidence indicates that solute inhibition is not primarily an inhibition of substrate entry into mitochondria. Sucrose in the assay media inhibited succinate dehydrogenase [succinate: PMS oxidoreductase (EC.1.3.91)] and malate dehydrogenase [L-malate: NAD oxidoreductase (EC.1.1.1.37)] activities, but these inhibitions were less than those of succinate- and malate-dependent oxygen uptake by mitochondria. Disruption of the mitochondrial membrane by detergent abolished the inhibition of respiration by sucrose, and the evidence indicates that solute inhibits the functional capacity of the membrane-associated respiratory system.

Introduction

Mitochondria from animal as well as from plant sources may show a decrease in respiratory activity when suspended in media in which the

chemical potential of water is much below that of the mitochondria themselves. Such inhibition has been demonstrated when the chemical potential of water in the medium was adjusted by addition of sucrose, mannitol, potassium chloride, or sodium chloride for animal mitochondria [1], and sucrose and potassium chloride for plant mitochondria [2, 3].

Johnson and Lardy [4] found that inhibition of rat-liver mitochondria in a medium of high sucrose concentration (480 mM) was greater with some substrates than with others, and was not alleviated by uncouplers of oxidative phosphorylation. Atsmon and Davis [1] observed that the oxidation of different substrates was inhibited to the same extent. They found that inhibition was relieved when the mitochondria were disrupted by freezing and thawing, and also that there was a lower level of reduced NAD in mitochondria having an inhibited respiration rate. These results were interpreted as evidence that in inhibited mitochondria, the respiration rate is limited by substrate permeation.

Atsmon and Davis [1] concluded that since different solutes at the same concentration in the medium gave similar depressions of the respiratory rate, the inhibition of respiration was due to the osmolarity of the medium. In other words, the chemical potential of water in the system affected the respiratory rate.

In this study, we have examined the effect of the water potential of the medium on respiration and attempted to define if substrate permeation or enzyme function limits oxygen uptake at high solute concentrations.

Materials and Methods

Rat-liver mitochondria (Wistar or Carnsworth Farm strain) were isolated as previously described [5] and finally suspended at a concentration of 30–35 mg protein ml^{-1} in a medium containing 250 mM sucrose, 1 mM EDTA, and 24 mM Tris buffer, pH 7.6. Before measuring the respiration, each suspension of mitochondria was incubated for 3–5 min in 10 mM succinate with three successive additions of ADP. This operation eliminated the inhibition of the initial state-3 rate of respiration usually observed with plant mitochondria [6]. The preincubated mitochondria were subsequently held at 0° C for at least 15 min before treatments were imposed.

Oxygen uptake was measured polarographically. The standard reaction medium contained 250 mM sucrose, 10 mM KH_2PO_4, 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mg ml^{-1} of ether-washed bovine serum albumin in a volume of 5 ml. The amount of mitochondrial protein was maintained above the critical concentration defined by Raison and Lyons [7]. The usual order of addition of components was:

reaction medium, mitochondria, and succinate. The temperature of the reaction was maintained at 26° C. Mitochondria were given several cycles of state-3–state-4 transitions by successive additions of 200 nmole of ADP per cycle.

Mitochondria were disrupted by sonication (Branson Sonifier B-12) using maximum power in several bursts for a total of 36 sec with the temperature maintained at 0–5° C. Succinate oxidase activity in the sonicated mitochondria was assayed polarographically in the standard reaction medium described above. Succinate dehydrogenase [succinate: PMS oxidoreductase (EC.1.3.99.1)] activity was estimated by the spectrophotometric method described by King [8], but with a constant concentration of phenazine methosulphate of 3 mM. L-Malate:NAD oxidoreductase (EC.1.1.1.37) was assayed by the procedure of Mukerji and Ting [9] (forward reaction) using the supernatant fraction obtained by centrifuging a suspension of sonicated mitochondria at 100,000*g* for 60 min or by the procedure of Ochoa [10].

The respiration rates reported are the average of at least three state-3 and three state-4 rates. Where mitochondria did not exhibit respiratory control, the respiration rate was determined after the addition of ADP when the rate remained constant. Respiratory control ratio (RCR = state-3/subsequent state-4) and P/O ratios were calculated according to Estabrook [11]. ADP was estimated enzymatically [12] and protein determined by the method of Lowry et al. [13].

The nonionic detergent Terex-X-8 (a gift from I.C.I. Australia) at a final concentration of 0.03% was used to solubilize the mitochondria. Silicone oil, DC 560, was a gift from Dow Corning Australia.

Results

Response of whole mitochondria to changes in concentration

The rate of state-3 respiration of mitochondria from rat liver is severely depressed when sucrose is increased above a certain concentration (Fig. 1A). The rate of state-4 respiration is essentially unaffected by increasing concentrations of sucrose; thus high sucrose concentrations effectively reduce the respiratory-control ratio. This degree of inhibition of state-3 respiration by sucrose is similar to that previously reported [1].

Equivalent concentrations of potassium chloride and sucrose cause similar decreases in respiration (Fig. 1B). Mannitol inhibits respiration about equally to sucrose (Fig. 1B), but glucose is much less inhibitory (Fig. 1A). Respiration of rat-liver mitochondria is essentially unaffected by concentrations of glycine up to 1000 mM (Fig. 1B); a similar result is observed when increasing amounts of proline are added to the medium.

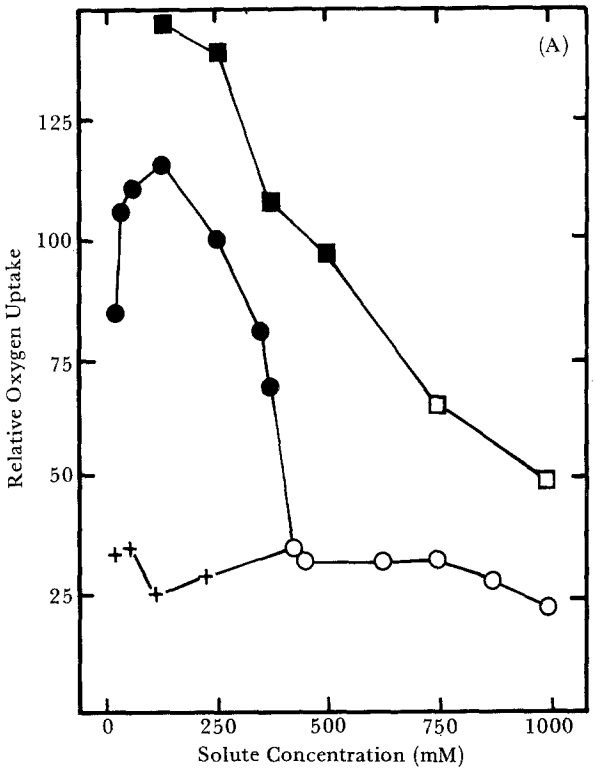
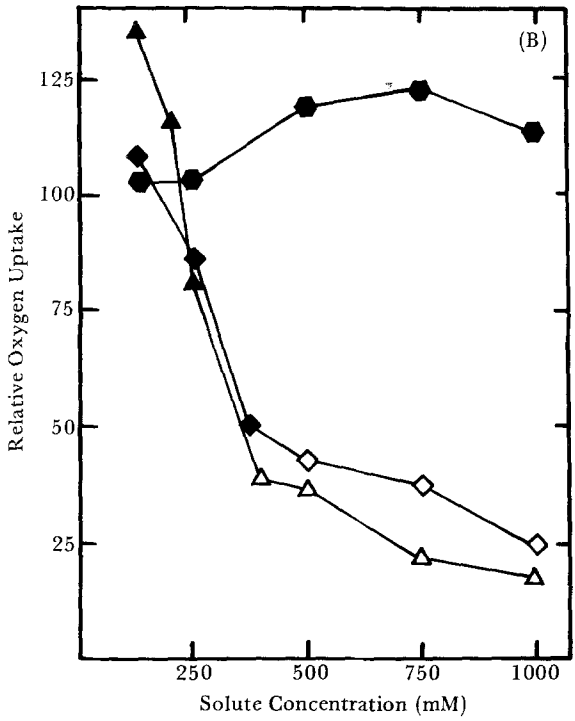


Figure 1. Effect of solute concentration in the medium on succinate oxidase activity of rat-liver mitochondria. State-3 respiration rate in (A, above) sucrose (○) and glucose (□) media, and (B, facing page) in KCl (△), glycine (⇨), and mannitol (◇) media all expressed relative to the state-3 respiration rate in 250 mM sucrose (100). State-4 respiration rate in sucrose (+) is also expressed relative to the state-3 rate in 250 mM sucrose. The state-3 rates in 250 mM sucrose media for the various preparations of mitochondria ranged from 82 to 114 nmole O_2 min^{-1} mg^{-1} protein. Closed symbols refer to samples with, and open symbols to those without respiratory control.

These results clearly show that inhibition of respiration varies with the solute added to the medium, and that factors other than the water potential of the medium regulate the inhibition.

Substrate availability as a rate-limiting factor in succinate oxidation

If mitochondrial respiration at high solute concentrations was depressed because of restricted permeability of the membrane to substrate, then



the inhibition should not be apparent in sonicated mitochondria. As shown in Fig. 2, disruption of rat-liver mitochondria by sonication substantially relieves the inhibition of succinate oxidation by high concentrations of sucrose and mannitol. Glucose also causes little inhibition of succinate oxidation by sonicated mitochondria. Prolonging the sonication treatment does not further relieve the inhibition by sucrose or mannitol. Inhibition by potassium chloride increases following sonication.

If the rate of permeation of substrate into mitochondria under conditions of high solute concentration limits respiration, rapidly changing the solute concentration from 250 mM to 1000 mM should induce no depression in the rate of respiration until the endogenous succinate concentration becomes rate limiting. But, when mitochondria respiring in state-3 in 250 mM sucrose with sufficient ADP and succinate to maintain a constant rate of respiration are rapidly subjected to an increase in sucrose concentration to 1000 mM, the respiration rate is depressed within 30 sec (Fig. 3A) to about the rate of mitochondria in 1000 mM sucrose. Dilution to 250 mM sucrose of mitochondria respiring

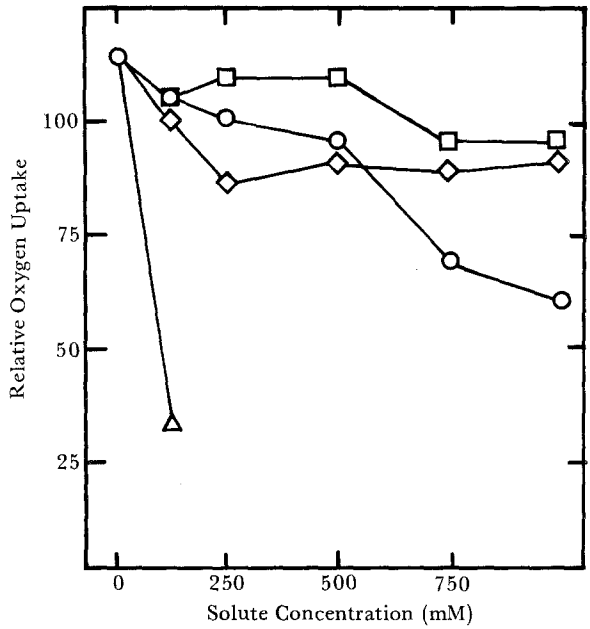


Figure 2. Effect of solute concentration in the medium on succinate oxidase activity by sonicated rat-liver mitochondria. Oxygen uptake by sonicated mitochondria in media containing succinate (4 mM) and ADP (2 mM), and either sucrose (○), glucose (□), KCl (△), or mannitol (◇) is expressed relative to the oxygen uptake in 250 mM sucrose (100), which was 71 nmole min^{-1} mg^{-1} protein.

in a medium containing 1000 mM sucrose, with succinate and ADP present, results in a state-3 rate which is initially greater than the control (250 mM sucrose) rate (Fig. 3B). This stimulated rate declines to the control rate after about 5 min. Thus changes in the solute concentration of the medium are rapidly reflected in changes in the rate of respiration.

Van Dam and Tsou [14] have shown that substrates accumulate in rat-liver mitochondria even when conditions prevail such that energy is not generated. They showed that the K_m for succinate oxidation is 1.5 mM and that the internal succinate concentration is higher in mitochondria in 150 mM sucrose than in 50 mM sucrose. In our experiments, the reaction medium contained 4 mM succinate; hence, only a rapid efflux of succinate against a concentration gradient could cause the intramitochondrial succinate level to be rate limiting after the mitochondria were transferred from low to high sucrose concentrations (Fig. 3A).

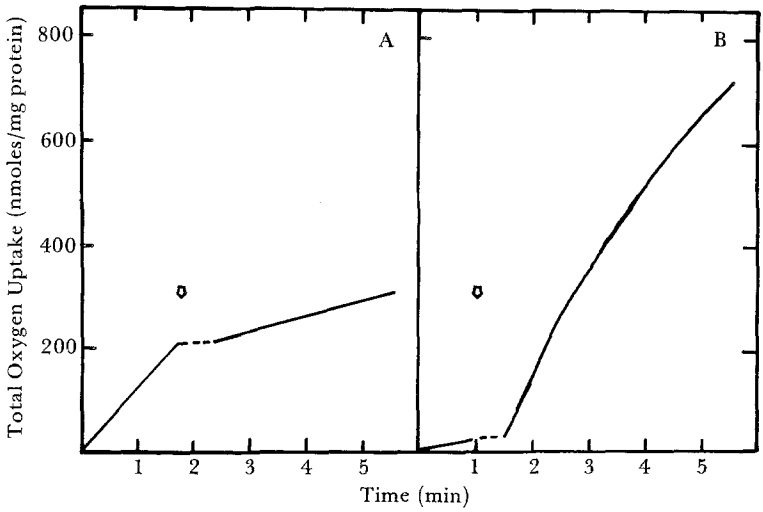


Figure 3. Effect of oxygen uptake on rat-liver mitochondria subjected to rapid changes in solute concentration. (A). Mitochondria respiring in state-3 (118 nmole O_2 min^{-1} mg^{-1} protein) with succinate (4 mM) and ADP (2 mM) in a reaction medium containing 250 mM sucrose, were subjected, at the time indicated by the arrow, to a change in the sucrose concentration to 1000 mM; all other reaction components were maintained at a constant concentration. (B). Mitochondria respiring in 1000 mM sucrose reaction mixture with succinate (4 mM) and ADP (2 mM) were diluted rapidly to 250 mM sucrose keeping other reaction components at a constant concentration. The 30 sec gap in the time scale represents the time required to change the volume and attain a new rate of respiration.

To measure more directly the influence of solute concentration on the uptake of succinate, rat-liver mitochondria were incubated in a reaction medium containing either 250 mM sucrose or 500 mM sucrose, each containing [2,3-$^{14}C_2$] succinate. The mitochondria were centrifuged through a layer of silicone oil into perchloric acid, and radioactivity in the perchloric acid extracts reasured [14]. As shown in Table I, the two groups of mitochondria contained equal amounts of succinate. In a medium containing 500 mM sucrose, respiration of mitochondria is depressed by more than 50% (Fig. 1A). From calculations which assume a mitochondrial volume per unit of mitochondrial protein of 2 [15], an approximately four-fold accumulation of substrate within the mitochondria can be computed. Such an accumulation is in accord with the results of Van Dam and Tsou [14]. These experiments support the conclusion that respiration of mitochondria in media of high solute concentration is not limited by the amount of substrate available to the dehydrogenase.

TABLE I. Effect of sucrose concentration on the succinate content of rat-liver mitochondria[a]

Succinate distribution	Sucrose concentration	
	250 mM	500 mM
cpm μl^{-1} of reaction mixture	19,000	18,000
cpm in mitochondrial pellet	535,000	537,000

[a] Rat-liver mitochondria (1.9 mg) were incubated in 5 mM [2,3-$^{14}C_2$] succinate for 1 min at 0°C, and then centrifuged through a layer of DC 560 silicone oil into a layer of 2 M perchloric acid. Aliquots of the reaction mixture and the final perchloric acid extract were taken for the radioactive determinations.

Effect of solute concentration on substrate dehydrogenase activity

Sucrose, glucose, and potassium chloride depress the activities of malate and succinate dehydrogenases of rat-liver mitochondria, with the salt having the largest effect (Table II). The rate of oxygen uptake of mitochondria oxidizing malate ranged up to 100 nmole min^{-1} mg^{-1} protein, and the activity of malate dehydrogenase was well in excess of this rate even in the presence of 1000 mM potassium chloride.

TABLE II. Effect of solute concentration on malate and succinate dehydrogenase activity of rat-liver mitochondria[a]

Enzyme	Solute concentration (mM)	Percentage activity			
		Sucrose	Glucose	Glycine	KCl
Malate dehydrogenase	0	100	100	100	100
	125	92	102	—	—
	250	86	96	119	68
	500	69	69	119	49
	750	65	65	—	—
	1000	59	59	128	42
Succinate dehydrogenase	0	100	—	100	100
	125	83	—	—	—
	250	80	—	75	45
	500	83	—	99	32
	1000	43	—	109	19

[a] Dehydrogenase activity was measured as described in *Methods*. Malate dehydrogenase activity without added solute was 0.33 μmole min^{-1} mg^{-1} protein. Succinate dehydrogenase activity without added solute was 0.09 μmole min^{-1} mg^{-1} protein.

The activity of succinate dehydrogenase did not exceed the rates of succinate-dependent oxygen uptake by mitochondria in optimal sucrose concentration. However, under these assay conditions, succinate dehydrogenase may not have exhibited its full potential. The reversible conversion of succinate dehydrogenase from an active to an inactive form [16,20] could contribute to the control of its activity. Oxygen uptake by mitochondria was more sensitive to increments in the sucrose concentration than was enzyme activity. In 500 mM sucrose, succinate-dependent oxygen uptake was inhibited by 70% and succinate dehydrogenase by 20%.

Detergent relief of inhibition by high solute concentrations

As illustrated by Fig. 4, addition of the nonionic detergent Terex-X-8 to mitochondria abolishes the depression of respiration caused by high

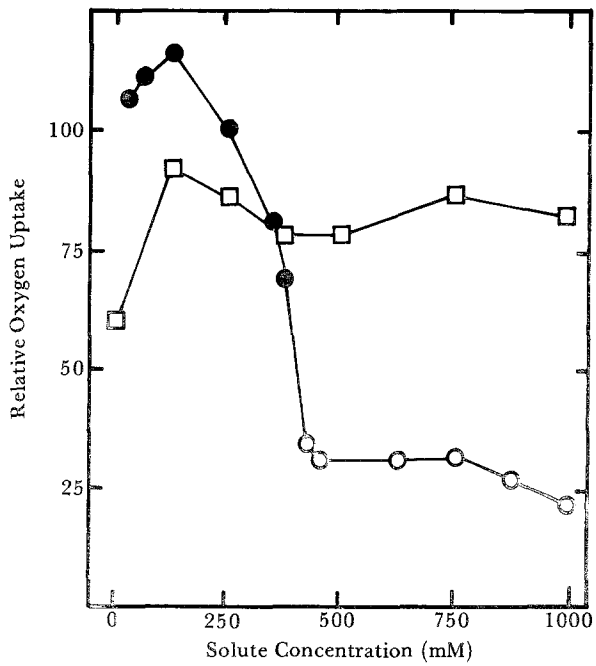


Figure 4. Effect of detergent on oxygen uptake by rat-liver mitochondria at various solute concentrations. Oxygen uptake was determined in media containing succinate (4 mM), excess ADP, and varying sucrose content before (○) and after (□) the addition of Terex-X-8 (0.03% v/v). Oxygen uptake is expressed relative to that in 250 mM sucrose (141 nmole min^{-1} mg^{-1} protein). Closed symbols refer to samples with, and open symbols to samples without respiratory control.

sucrose concentrations. The detergent concentration necessary to alleviate the inhibition is 0.03%, and this produces a clear solution.

Discussion

These experiments clearly show that the respiration of rat-liver mitochondria is not equally affected by different solutes in the medium at a given concentration (Fig. 1). Thus the water potential or water activity of the medium is not the sole factor influencing the rate of oxygen uptake as the solute concentration is increased. Hunter and Brierley [15] found no difference in the extent to which sucrose, mannitol, and KCl permeate beef-heart mitochondria and also found that there existed a compartment impermeable to each of these solutes for at least 30 min. Glucose and sorbitol penetrate the membranes of animal mitochondria very slowly, and ribose somewhat more quickly [17]. There is, then, a general inverse correlation between the inhibitory effects of solutes on respiration and their rates of penetration of the mitochondrial membrane. However, the evidence does not suggest that lack of inhibition is dependent upon osmotic adjustment. In ribose media, osmotic adjustment is not complete within 10 min, and after 2 min it is less than 50% complete. But state-3 respiration rates are constant throughout this period. Similarly, in glucose media, state-3 respiration rates are constant with time during the period of glucose penetration and osmotic adjustment. The studies of solute penetration [17, 18] have been made with mitochondria without added substrate. It is assumed that actively respiring mitochondria respond similarly to solutes. If this is so, inhibition of respiration does not result from a gradient of solute across the mitochondrial membrane.

Inhibition in media of high solute concentration is more marked in state-3 than state-4 respiration in rat-liver mitochondria; state-4 respiration is reduced only in sucrose concentrations above 750 mM. In certain solutes, the state-3 rate is reduced to the state-4 value, and thus, under these conditions, mitochondria fail to exhibit respiratory control. This observation is similar to that of Flowers and Hanson [3] on soybean mitochondria. However, Atsmon and Davis [1] concluded of rat-liver mitochondria that "hyperosmolarity caused an essentially similar (to state-3) though much less marked, inhibition of state-4 respiration."

If, as Atsmon and Davis [1] concluded, substrate permeation is the rate-limiting parameter in substrate oxidation by rat-liver mitochondria exposed to high solute concentrations, then disruption of the membrane system by sonication should alleviate the inhibition, since under these conditions succinate dehydrogenase would be located on membrane fragments directly exposed to the bulk solution [19]. Sonication reduces but does not abolish the inhibition (Fig. 2). The change to the low

respiration rates in media high in sucrose, yet containing succinate in excess of the K_m for succinate oxidation, is achieved so quickly (Fig. 3) that an active efflux of substrate must be proposed, if the explanation for inhibition is sought in terms of limited substrate within the mitochondria.

Succinate is accumulated to the same concentration in mitochondria in 500 mM as in 250 mM sucrose (Table I), hence the evidence from our experiments fails to support the conclusion that the availability of substrate is limiting oxidation in mitochondria in media of high sucrose content. While the abolition of the respiratory inhibition by detergent treatment is consistent with the view that, in media of high sucrose content, substrate access is rate limiting, other evidence does not support this view, and the detergent effect could indicate that solute inhibition results from modification of a lipid-mediated structure.

Solutes do not influence succinate or malate dehydrogenase in a manner similar to their influence on succinate or malate oxidation by mitochondria (Table II, cf. Fig. 1). The response pattern of the enzymes to different solutes is different to that of the mitochondria, and the concentration dependency also differs. The in vitro assay of a dehydrogenase may be a poor model for its function in intact mitochondria, where it may be subject to reversible deactivation [16, 20] or other forms of control. The evidence of Table II, while establishing that the dehydrogenases are not the sole loci of inhibition by solutes, does not eliminate the possibility of a control of their activity through a solute effect on the interaction of the dehydrogenases with the mitochondrial membrane.

An uncoupler-stimulated ATPase is inhibited by sucrose and by mannitol at concentrations broadly similar to those which inhibit respiration [21, 22]. In addition, adenine-nucleotide exchange in phosphorylating inner-membrane preparations of rat-liver mitochondria is almost completely inhibited by 500 mM sucrose [23]. While neither of these points of evidence may bear directly on coupled respiration in intact mitochondria, an inhibition by solutes of phosphorylation would reduce state-3 respiration in much the manner that we have observed. The partial loss of inhibition by sonication may reflect the release of inhibition of the phosphorylation site, revealing residual inhibition, at higher solute concentrations. The latter may be identical to the slight inhibition we observe of state-4 respiration (Fig. 1).

Our evidence establishes that the inhibition is a direct effect of solutes, not mediated by an effect on the chemical potential of water, and that inhibition is neither on substrate uptake nor peculiarly on dehydrogenase function. Inhibition appears to be of membrane-associated function, and it may be on phosphorylation or on the electron-flow system as an entity. If the phosphorylation site is the first affected, this may be because it is affected directly by the solutes, or

because its function is particularly susceptible to a more general modification of the membrane. Alterations to the physical state of membrane lipids, as a consequence of a phase change or in response to detergent treatment, affect the activity and the Arrhenius activation energy of the succinate oxidase of mitochondria [24, 25]. In addition, direct studies, by spin labeling and electron spin-resonance spectroscopy, show that 1000 mM sucrose decreases the molecular ordering of membrane lipids [26]. A solute effect on membrane lipids is one possible mechanism for the inhibition of membrane function.

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