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Differential effects of osmotic pressure on mitochondrial respiratory chain and indices of oxidative phosphorylation

V. Sitaramam, D. Sambasivarao and John C. Mathai

Department of Zoology (Biotechnology), University of Poona, Pune (India)

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Oxidative phosphorylation was critically evaluated in terms of activities which are sensitive and insensitive to variations in external osmotic pressure in mitochondria. Integrity of mitochondria was determined in terms of a variety of parameters, including the latency of the occluded enzymes, by careful titrations as a function of external osmotic pressure as well as detergent concentrations. The evidence indicated that the rate-limiting step in respiratory states 2 and 4 would be osmotically insensitive, as opposed to the osmotically sensitive respiration of states 1 and 3 and uncoupler-stimulated respiration with glutamate + malate and succinate. Cytochrome oxidase activity in mitochondria as well as in purified reconstituted systems exhibited osmotic insensitivity but marked sensitivity to ionic strength, offering an interesting model to study the osmotically insensitive respiration. Cytochrome oxidase activity led to permeation of mannitol across the mitochondrial inner membrane. Stimulation of cytochrome oxidase activity by uncouplers did not require an intact membrane.

Introduction

Dynamic perturbations in the structure of the lipid bilayer, as a consequence of energy field–membrane interactions, was shown to be consistent with a number of experimental observations, including those with the mitochondrial oxidative phosphorylation [1–5]. Consensus exists that the bilayer would not be invariantly planar and that fluctuations must accompany the function of biological membranes [1,6–9]. The magnitude and physical description of these lipid fluctuations, in unenergized membranes, however, remains conjectural in view of the cost of exposure of hydrophobic acyl chains to the aqueous domain [7–9]. The problem of energy-mediated structural perturbations in biological membranes is more general than oxidative phosphorylation per se [9,10]. Oxidative phosphorylation, however, has specific advantages in the study of the role of membrane perturbations in energy-transduction mechanisms, with particular reference to the chemiosmotic hypothesis of Mitchell [11]. The major virtue of the chemiosmotic hypothesis lies in that it permits critical experiments to evaluate its central premise that the physical integrity of the membrane is an obligatory requirement for oxidative phosphorylation. Further, the thermodynamic basis of this transmembranous mechanism is understood adequately to delineate the empirical observations that it can or cannot handle (cf. Refs. 12–14). For instance, the chemiosmotic hypothesis cannot explain the variable porosity to polyols induced by respiration and ATP hydrolysis [3,15]. Protonmotive force would be inconsistent with a membrane whose permeability to sucrose or mannitol is considerable. Existence of $\Delta\tilde{\mu}_{H^+}$ would be inconceivable in a membrane whose porosity would actually increase with rate of respiration such that the highest rate of ATP synthesis and the largest porosity would actually coincide (i.e., at the height of state 3 respiration!). The chemiosmotic hypothesis cannot explain the ubiquitous phenomenon of a direct regulation of the activity of hydrophobic proteins by osmotic stretch/compression [2–4,9,10,15]. Besides these considerations, equilibration of sucrose with the matrix space invalidated, both during centrifugal phase separations and during respiration

Abbreviations: RCR, respiratory control ratio; Hepes, 4-(2-hydroxyethyl)-1-piperazineeethanesulfonic acid; P_i, inorganic phosphate; CTAB, cetyltrimethylammonium bromide; DNP, 2,4-dinitrophenol; PP_i, inorganic pyrophosphate.

Correspondence: V. Sitaramam, Department of Zoology (Biotechnology), University of Poona, Pune-411 007, India.

and ATP hydrolysis, the methodological basis of $\Delta\psi$ and ΔpH measurements in respiring mitochondria [3,15,16].

A significant number of these exceptional observations from our laboratory were based on the novel technique of osmometry (cf. Refs. 17 and 18), i.e., a systematic evaluation of the non-linear osmotic responses of cells and organelles to external osmolality. We undertook a systematic survey of the influence of osmotic pressure on various aspects of mitochondrial function vis-à-vis its physical integrity. The results show that osmotic pressure is a major determinant that influences various activities associated with oxidative phosphorylation, with the important exception of cytochrome oxidase activity. Unlike the preceding segments of respiratory chain which are sensitive to variations in osmotic pressure, cytochrome oxidase activity appears to be primarily modulated by ionic strength in intact mitochondria as well as in reconstituted cytochrome oxidase vesicles. The importance of these novel physical observations emerges from the fact that any mechanism of oxidative phosphorylation requires to be deeply embedded in physical theory. Therefore, a systematic evaluation of the influence of physical parameters would be critical to delineate the boundaries of any model that attempts to account for energy transduction.

Materials and Methods

Materials

Sucrose, ADP, ATP, Tris, various inhibitors, ionophores, detergents and related biochemicals were all obtained from Sigma. Sodium dodecylsulfate (SDS) was from Bio-Rad, cetyltrimethylammonium bromide (CTAB) from BDH, U.K., and Ammonyx LO from Onyx Chemical Co., USA. All reagents were of analytical grade. [^{32}P]orthophosphate (carrier-free) was obtained from Bhabha Atomic Research Centre, Bombay, India.

Methods

Rat liver mitochondria were isolated from Wistar male albino rats, as described previously, in 0.25 M sucrose media prepared from freshly boiled and cooled water [1–3,15]. These mitochondria were immediately tested for their functional integrity by assaying for ADP/O ratio and respiratory control ratio (RCR) polarographically. The oxygen electrode was calibrated by standard methods [19,20] at the required temperatures as well as at various osmolyte concentrations to ensure the calculations of specific activity of respiration in various preparations. Briefly, the preparations compare well with our previously published values as well as those reported from most other laboratories [2,3,15,20, 21].

Purification of cytochrome oxidase and reconstitution of cytochrome oxidase vesicles [22,23]

Cytochrome oxidase was purified from pig kidney and bovine heart as described earlier and was reconstituted into asolectin liposomes as described by Casey et al. [23]. The vesicles, which constituted of 70% orientation outside out, consisted of defined internal milieu, as specified in the legends. The activity of cytochrome oxidase was determined polarographically at 25 °C as specified in the legends.

Osmometric measurements of various activities were as described earlier [2,3,15,18]. Briefly, the technique of osmometry implies that various activities, including physical integrity and volume of a population of particles, vary as a function of external osmotic pressure linearly only within critical boundaries (limits). These limits, represented as intersections of independent regression lines (i.e., break-points), represent the true signatures of osmotic behaviour of particles and not the slope of activity (volume) vs. $1/\Pi$ plots. Such critical osmotic pressures include material constants such as the onset and completion of lysis, onset of activation of membrane-bound enzyme/transporter activities etc. The methodological, kinetic and statistical considerations have been described in detail in earlier publications [1–4,10,15–18]. In general, each osmometric profile is representative of at least two independent experiments and break-points are generally compared with controls carried out with the same preparation on the same day. Additional details were specified in the legends to individual figures.

Results

Osmometric analysis of indices of oxidative phosphorylation

Fig. 1 shows the summary of the osmometric behaviour of mitochondria with regard to site I, II and III substrates. The polarographic assays of oxidative phosphorylation do not permit an evaluation of ADP/O ratio for all the three sites. Therefore, the osmometric behaviour of respiration was compared with that of inorganic [^{32}P]phosphate esterification (ATP synthesis). Clearly, State 3 respiration for glutamate + malate and succinate were comparable in that osmotic compression of the bilayer led to progressive inhibition of respiration in an osmolality range significantly above that for the onset of lysis (not less than 0.1 M sucrose). Respiratory control ratio, phosphorylation and ADP/O ratio also exhibited an osmotic inhibition at higher osmolalities. The osmometric profiles of respiration and other indices were evaluated as a function of the osmolality of the total medium rather than the concentration of the external sucrose alone. This, in turn, revealed that the upper break-point for the maximal respiration with glutamate + malate as well as succinate-dependent respiration was

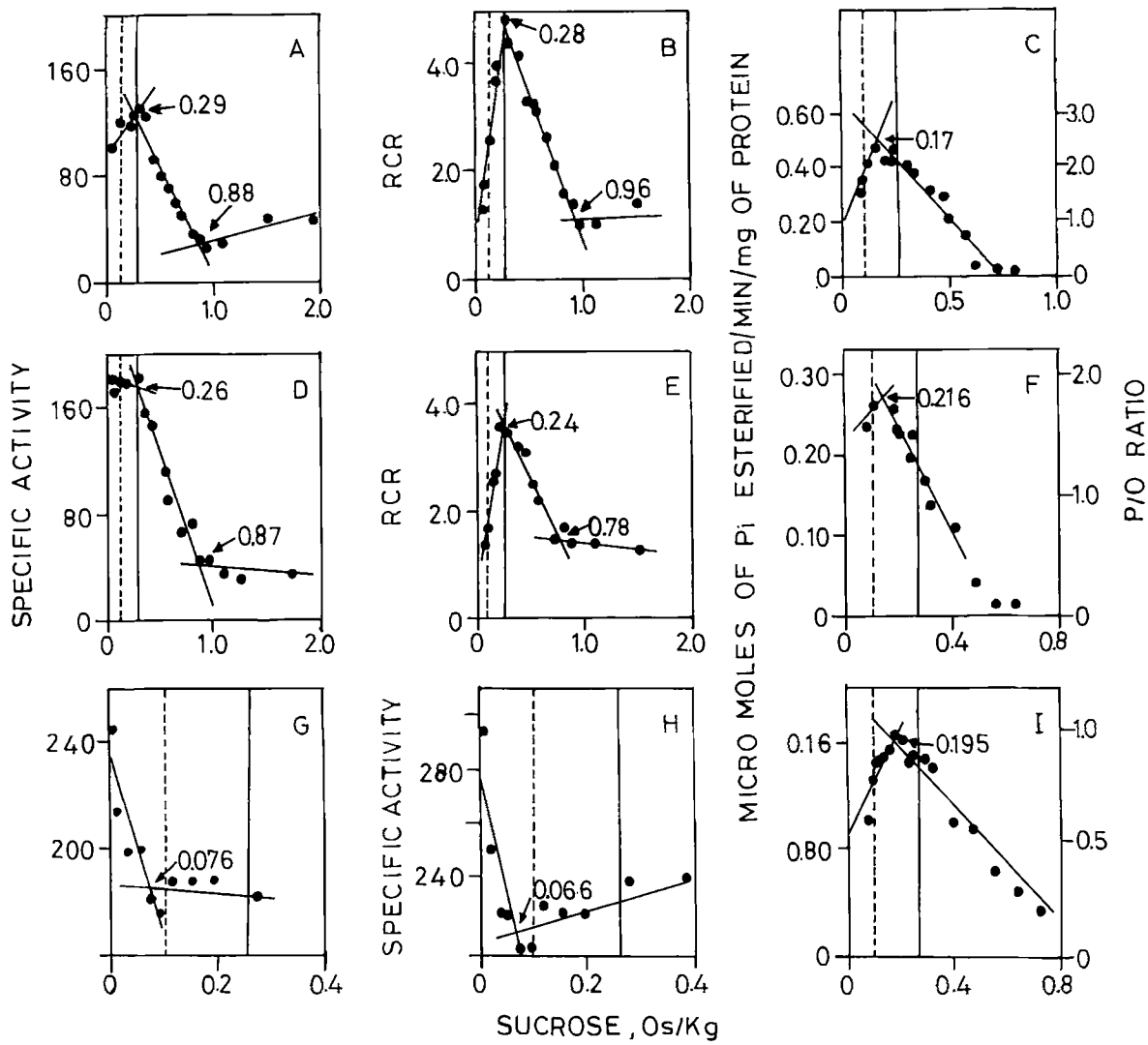

Fig. 1. Osmometric studies on oxidative phosphorylation in rat liver mitochondria for various substrates. The assay media consisted of 10 mM potassium phosphate buffer (pH 7.4)/3.3 mM $MgCl_2$/0.2 mM EDTA (pH 7.4)/0.15 mM ADP/10 mM Tris-HCl buffer (pH 7.4). Glutamate and malate as substrates for respiration (A–C) were 10 mM each as also succinate (D–F). For site III respiration (G–I), ascorbate (30 mM) with 1.0 mM TMPD was used along with 50 μM cytochrome *c* in rotenone-pretreated mitochondria (as in the case of succinate respiration). Respiration was monitored polarographically (specific activity, natoms/min per mg protein) as a function of external osmolality varied by the addition of sucrose. Osmolality of the assay media was monitored by Wescor vapour pressure osmometer. Phosphorylation was measured as esterification of [^{32}P]orthophosphate in the presence of 1.0 mg hexokinase and 1.5 μC_i of carrier-free [^{32}P]orthophosphate as described earlier [3]. A,D,G: State 3 respiration for different substrates. B,E: respiratory control ratio; C,F,I: phosphorylation (P/O ratios). H: ascorbate respiration in mitochondria pre-treated with 0.2 mM 2,4-dinitrophenol (DNP). Vertical dashed line at approx. 0.1 Os/kg corresponds to the onset of hypotonic lysis (cf. Ref. 3). Continuous vertical line represents the isotonic osmolality of 0.25 M sucrose (0.27 Os/kg), the medium of isolation. The data points in each osmotic domain were fitted independent regression lines as described in Materials and Methods. Break-points, the points of intersection of these regression lines were specified in each instance.

actually closer to isotonicity rather than to the onset of lysis. Thus the osmometric influence may arise from a more general case of the parabolic influence of osmotic pressure/volume on the elastic energy of the membrane (cf. Ref. 15). Inhibition of activity due to hypertonicity per se would be a rather restricted definition. These quantitative descriptions are not mutually exclusive, but depend on the domain of external osmolality being emphasized. It was clear that the notion of isotonicity in a membrane with variable induced porosity should be handled with extreme caution.

The osmometric behaviour of cytochrome oxidase was altogether different: (i) in an osmotic range comparable to site I and II substrates, though the rate of respiration was very high, osmotic inhibition of site III respiration was conspicuously absent; (ii) the onset of lysis actually stimulated site III respiration, unlike in the case of site I and II substrates; (iii) this stimulation had little to do with uncoupling of mitochondria incidental to lysis, since the stimulation was also seen in mitochondria uncoupled with DNP. The data clearly showed that state 3 respiration with site III substrates

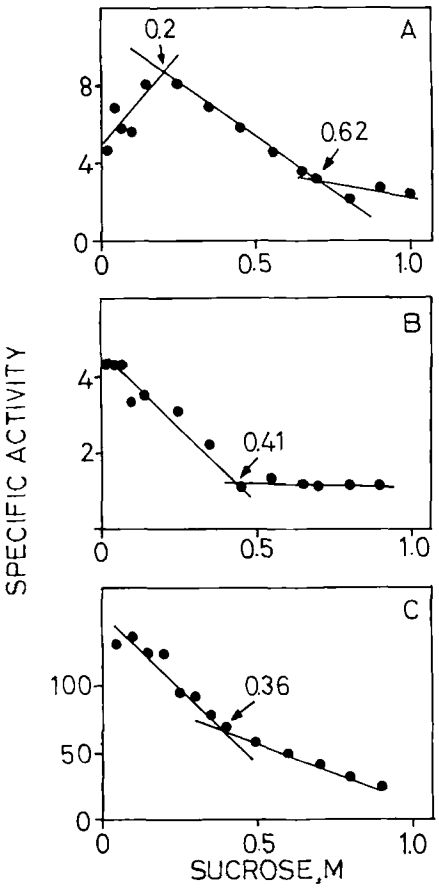

Fig. 2. Osmometry of respiration in rat liver mitochondria: endogenous respiration (state I) (A). Endogenous respiration in DNP-treated mitochondria (B). Respiration in the presence of glutamate + malate in DNP-treated mitochondria (C). Osmometric analysis of data by fitting to individual regression lines as in Fig. 1.

was different from that with other substrates. Even more striking was the possibility that the activity of cytochrome oxidase was not under the exclusive control of coupling, since lysis promoted the activity independent of the state of coupling. Phosphorylation was uniformly inhibited at all sites.

Osmotic inhibition of endogenous respiration

A simple explanation for the unique osmometric behaviour of site III respiration could lie in the fact that the substrate need not cross the inner membrane whereas the substrates for sites I and II require transport and some of these transporters were shown to be osmotically modulated (e.g., dicarboxylate transporter [2,3], the ADP/ATP carrier [15], etc.). State I respiration in mitochondria pertains to respiration exclusively due to endogenous substrates, which permits an evaluation of respiration uninfluenced by the transport of the substrates. Therefore, the osmometric profiles were determined in the presence of DNP as well as in the presence of glutamate + malate and DNP to exclude the role of coupled respiration directly or indirectly (Fig. 2): (i) osmotic compression of mitochondria led to inhibition of state I respiration; (ii) this inhibition was also present in uncoupled mitochondria; (iii) osmotic compression also led to inhibition of uncoupled respiration in the presence of glutamate + malate. Respiration in state 1 was primarily due to site I substrates, since it was abolished up to 95% on treatment with rotenone (1 μg per mg of protein). Therefore, neither the transport of substrates nor the state of coupling accounted for the observed osmotic modulation of respiration with site I and site II substrates. It was interesting to note that DNP actually inhibited state I respiration both in the osmotically sensitive and insensitive domains and also led to a significant inhibition of osmotic modulation of state I respiration as manifest by a shift in the breakpoint to left (confirmed in three independent experiments).

These results indicated that the observed inhibition of respiration by an increase in external osmotic pressure is a primary attribute of the respiratory chain itself and is not secondary to either the coincident transport or coupled transduction processes. Similarly, the lack of inhibition of cytochrome oxidase activity by osmotic pressure and activation on lysis indicated the influence of lysis on cytochrome oxidase activity by a mechanism distinct from either coupling and related processes or the osmotic integrity. Inhibition of osmotic modulation of site I respiration appeared to be another attribute of the action of the uncouplers, such as DNP. Inhibition of state 1 respiration by DNP remained enigmatic, since the inhibition would not be consistent with either the uncoupler action or the state of coupling of mitochondria that would account for state I respiration.

Osmotic inhibition of segmental electron transport

Since exogenous ferricyanide can be reduced in the presence of glutamate + malate (rotenone and antimycin-sensitive) and succinate (only antimycin-sensitive) [24], osmotic sensitivity of segmental electron transport could be evaluated in the presence (equivalent to state 3) and absence (equivalent to state 2) of exogenous ADP (Fig. 3). Interestingly, the segmental electron transport was osmotically inhibited both in the presence and absence of exogenous ADP. Phosphorylation also exhibited osmotic inhibition under these conditions (data not given). However, a respiratory control ratio which was significantly greater than 1.0 could be demonstrated only for site I, but not for site II, substrates.

Table I summarizes the osmotic results obtained in this and previous published papers from our laboratory [2,3,15]. The major results were: (i) state 2 and 4 respiration were osmotically insensitive; (ii) similar was the case with respiration with site III substrates, ascorbate and TMPD; (iii) electron transport in the absence of involvement of site III was uniformly inhibited by osmotic compression. Fig. 4 depicts the osmotically sensitive and insensitive patterns of responses of respi-

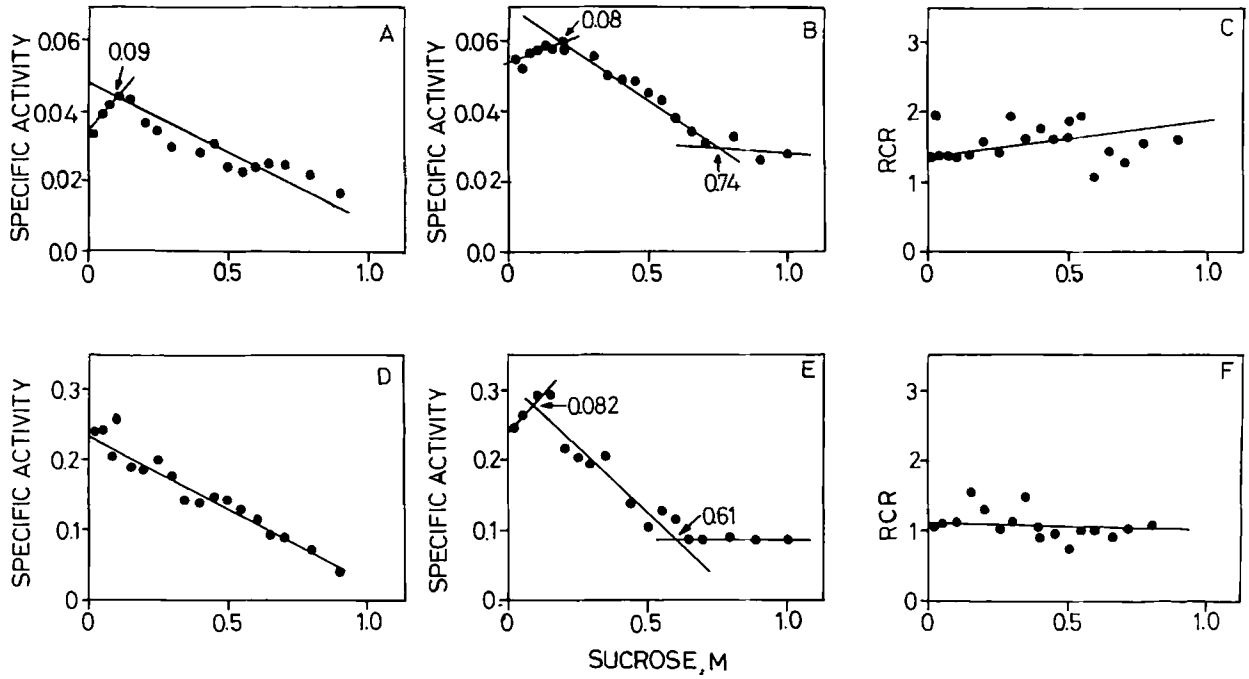

Fig. 3. Osmometry of segmental electron transport in rat liver mitochondria. Glutamate/malate: ferricyanide oxidoreductase activity (A–C) and succinate: ferricyanide oxidoreductase activity (D–F, in rotenone (1.0 μg/mg of protein)-treated mitochondria) equivalent of the absence (A,D) and presence (B,E) of ADP. Respiratory control ratio was computed similar to respiratory rate in the presence and absence of ADP as in Fig. 1 (C,F). The assay system comprised of 10 mM of glutamate and malate each or 10 mM succinate, 10 mM phosphate, 20 mM Tris-HCl, 1.5 mM $MgCl_2$, 1.0 mM EDTA and 3 mM KCN with specified amounts of sucrose. Instead of ADP as in polarographic assays, the spectrophotometric assays for the reduction of ferricyanide required the addition of 2 mM ATP and hexokinase, 1.5 mg, for an assay with 1 mg of mitochondrial protein in 1.5 ml assay volume. All reagents were adjusted to a pH of 7.4. Osmometric analysis of the data points by regression analysis and evaluation of break-points, cf. Fig. 1. Specific activity, μmoles of ferricyanide reduced/min per mg of mitochondrial protein.

ration to interpret the osmotic responses summarized in Table I. Respiratory states 2 and 4 would be associated with the inhibited respiration in the absence of exogenous ADP and the internal ATP would be high under these conditions; in states 1 and 3, the matrix ATP levels would be low. Two intriguing questions arise. (i) Is the osmotically insensitive cytochrome oxidase the rate-controlling step in state 2 respiration which also

TABLE I

Summary of osmotic responses of mitochondrial states of respiration

+, Indicates inhibition with hyperosmolality; −, indicates nonresponsivity to external osmotic pressure; n.r. Indicates not relevant, for reasons discussed in the text. Refer to Fig. 4.

Substrate	Osmotic sensitivity					
	respiratory states				RCR	phosphorylation
	1	2	3	4		
Glutamate + malate respiration	+	−	+	−	+	+
Succinate respiration	n.r.	−	+	−	n.r.	+
Ascorbate/TMPD respiration	n.r.	−	−	−	n.r.	+
Glutamate/malate: ferricyanide oxidoreductase	n.r.	+	+	+	−	+
Succinate: ferricyanide oxidoreductase	n.r.	+	+	+	n.r.	+

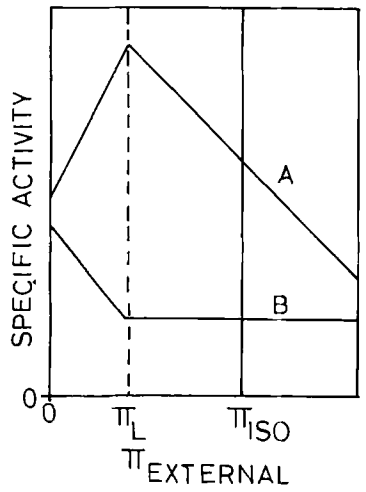

Fig. 4. A summary of osmometric profiles of respiration in mitochondria. Profile A represents inhibition of respiration at the onset of lysis as well as on increase in external osmolality. Profile B represents an activity essentially independent of osmolality in intact mitochondria and stimulation of respiration, if any, on the onset of lysis. Broken vertical line represents the onset of lysis and the vertical continuous line represents the tonicity of the isolation medium. The upper break-point for profile A could vary from Π_L to Π_{iso} as discussed in the text.

TABLE II

Summary of mitochondrial activities in 0.25 M sucrose media: a comparison of absolute rates.

Figures in parentheses represent P/O ratio measured by esterification of orthophosphate.

	Substrate used		
	glutamate + malate	succinate	ascorbate + TMPD
Rate of respiration [a] (−ADP)	31	29	270
Rate of respiration [a] (+ADP)	92	121	270
Rate of phosphorylation [a]	254	217	270
RCR [b] (range)	4–6	3–4	1
Rate of ferricyanide reduction [a]	48	204	–
RCR [c]	1.4	1.0	–
P/O ratio (theoretical)	3	2	1
ADP/O ratio (range)	2.2–2.8	1.5–1.8	(0.8–0.95)

[a] Specific activity, natoms (or equivalents)/min per mg of protein.
[b] State 3/state 4 for respiration.
[c] Rate of ferricyanide reduction in the presence/absence of ADP.

exhibits the lack of osmotic response? (ii) Can cytochrome oxidase be inhibited by endogenous ATP to account for this rate-limiting behaviour, since site III respiration would be otherwise the highest rate of respiration?

Both these questions run counter to current thinking. Firstly, the idea of a rate-limiting step in a complex enzyme system may appear to be at variance with the idea of the highly complex and interactive coupled processes. Secondly, inhibition of respiration by ATP would mean end product inhibition as an alternative to respiratory control which is traditionally visualized as an exhaustion of an intermediate or build-up of passive force [25]. In the absence of clear reasoning (as opposed to convention, however current) that forbids such thinking, we explored the consequences of these questions.

* Some degree of caution is necessary in making this assumption. For instance, ADP-mediated stimulation of respiration can be observed only if more than one site is involved, either in case of respiration or in case of segmental electron transport. Addition of ADP essentially alters, among others, the kinetic constant associated with the substrates for respiration. If the number of sites involved in electron transport matters for the phenomenon of respiratory control (i.e., the perturbation of the kinetic constants associated with substrates for respiration depends on the length of the respiratory chain mobilized for catalysis by free-energy generators), one cannot exclude further interactions among the complexes. In principle, if the kinetics of inhibition of cytochrome oxidase by ATP differ when electron transport occurs through other sites as well (as with NADH-dependent or succinate respiration), the site of inhibition of respiration by endogenous ATP could still be cytochrome oxidase. Under these conditions, site III respiration per se would still be unaffected by the addition of ADP.In order to exclude this, it would be critical to understand the allotopic regulation of cytochrome oxidase.

Nature of the putative rate-limiting step

The nature of the putative rate-limiting step could be examined by a critical comparison of the rates of respiration in various states and substrates at a single osmolality of 0.25 M sucrose, as in Table II. It was clear that the cytochrome oxidase activity represented the highest rate and was totally independent of the presence of exogenous ADP. Further, regardless of the rate of respiration, rate of phosphorylation was nearly the same with substrates for all the three sites. If we assume that the tandem process of electron transport occurs across respiratory complexes which are otherwise non-interactive *, we would need to identify a site which is at once osmotically sensitive but inhibited by endogenous ATP to account for respiratory control. This permits only one possibility, i.e., the site of inhibition could be prior to cytochrome oxidase, e.g., complex III (ubiquinone : cytochrome *c* oxidoreductase) activity. This would mean that the osmotic inhibition would reside in the respiratory segments prior to complex III, whereas complex III per se would be osmotically insensitive and ATP sensitive.

The first step was to study the regulation of site III respiration (cytochrome oxidase activity) vis-à-vis its mutual influence on the mitochondrial inner membrane. However, cytochrome oxidase activity is known to be highly modulated, exhibiting some of the most complex kinetics known [22,26], which requires further examination (vide infra). In the case of respiration, the present data begin to distinguish between different types of osmotic susceptibility. Similarly, we should also perhaps begin to distinguish between various modes of respiratory control. In its elemental sense, respiratory control represents stimulation of respiratory activity on addition of ADP or uncouplers. Stimulation of activity of soluble enzymes could be due to the presence of activators of the removal of inhibitors. In the case of membrane-bound enzymes, a variety of additional causes could lead to stimulation of activity, e.g., detergents, ionic strength, physical state of the membrane including osmotic stretch, length of acyl chains, etc. [27]. The experimental results obtained thus far focus on the need to study these distinctions systematically without being influenced by any particular hypothesis. The cytochrome oxidase activity was investigated in greater depth as a model system for an osmotically insensitive activity and as a potential locus for ATP regulation in vitro.

Osmometry of cytochrome oxidase in mannitol media

The break-point for the onset of lysis was the same in sucrose and mannitol media in the absence of respiration (cf. Ref. 3). Fig. 5 shows the osmometric behaviour of site III respiration as well as phosphorylation as a function of external mannitol concentration. The break-point of respiration in mannitol media was nearly

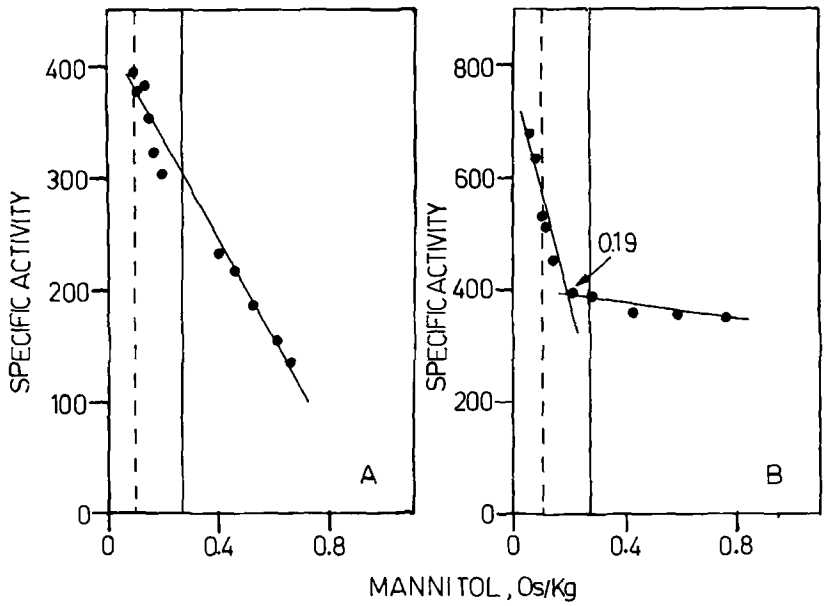

Fig. 5. Phosphorylation (A) (nmoles of P_i esterified/min per mg protein) and respiration (B) (natoms of O/min per mg protein) for ascorbate + TMPD respiration in mannitol media. The assay conditions and osmometric analyses were identical to those in Fig. 1 (G and I) except that mannitol was substituted for sucrose. Osmolality of assay media was monitored by a vapour pressure osmometer.

twice that in sucrose media, (cf. Fig. 1, G–I) in three independent experiments, confirming that the mitochondrial membrane was more permeable to mannitol in the presence of respiration at site III. Further, it would be correct to interpret the break-point as that of lysis even in mannitol media for site III respiration (cf. Fig. 4). It was shown earlier that even sucrose becomes permeable at the height of state III respiration for glutamate + malate, whereas ATP hydrolysis induced enhanced permeability to mannitol only [15].

Osmometry of phosphorylation exhibited osmotic inhibition. Since mannitol has limited solubility, it was not possible to test for the osmotic inhibition of phosphorylation by mannitol to obtain the precise break-point concentration. However, extrapolation of the slope of osmotic inhibition (which is equivalent to breakpoint concentration, since a more permeable solute would tend to cause less inhibition) even in sucrose media exhibited inhibition at approx. 0.9 M, whereas a similar inhibition was observed at 0.75 M for glutamate + malate or succinate respirations (Fig. 1). The inhibitory concentration in mannitol media (by extrapolation) was approx. 0.96 M, which was marginally larger than that for sucrose. Thus, while osmometry of respiration clearly indicated enhanced permeability to mannitol, the osmometry of phosphorylation was less definitive, due to relatively less susceptibility of site III phosphorylation to osmotic inhibition. Thus, permeability to mannitol itself being barely evident, enhanced permeability to larger polyols such as sucrose would be most unlikely due to site III respiration.

Osmometry of cytochrome oxidase activity in reconstituted vesicles

A major test for the validity of the technique of osmometry lies in whether the observations on mitochondrial activities can be reproduced using purified mitochondrial proteins of defined activity reconstituted into liposomes. Earlier studies showed that the osmotic inhibition of ATPase activity was due to the

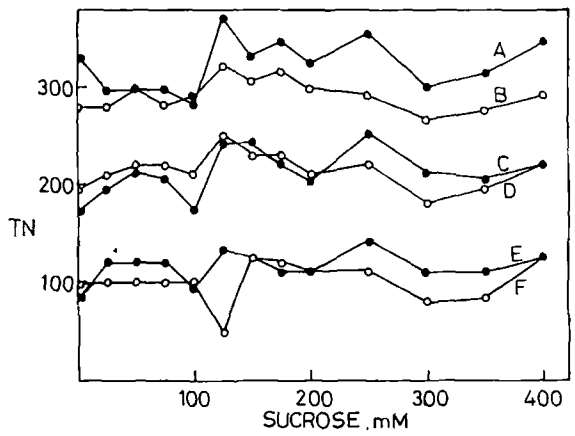

Fig. 6. Osmometric analysis of cytochrome oxidase activity in asolectin vesicles reconstituted with purified pig kidney cytochrome oxidase. Open circles, internal sucrose, 100 mM. Closed circles, internal sucrose, 200 mM. Activity represented as molecular turnover (TN = μmol e^- per nmol cytochrome aa_3 per s at 25 °C). Oxygen uptake was measured polarographically in the presence of 10 mM KCl, 10 mM potassium Hepes (pH 7.4), 7 mM Tris-ascorbate, 0.7 mM TMPD. Profiles a and b were in the presence of 9 μM FCCP, and 0.8 μM valinomycin and 40 μM cytochrome *C*; c and d with cytochrome *c* only; e and f without cytochrome *c*.

ATPase activity as well as the ADP/ATP carrier activity [2,3]. Osmometry in reconstituted vesicles showed that ADP/ATP carrier activity was indeed modulated by osmotic pressure [15]. The effect of osmotic pressure on cytochrome oxidase vesicles was studied using the pig kidney and bovine heart enzymes. Cytochrome oxidase vesicles exhibit 'respiratory control' in that the activity could be stimulated by detergents such as lauryl maltoside, uncouplers, and agents such as valinomycin + potassium, all of which should result in uncoupling. Fig. 6 shows the pig kidney cytochrome oxidase data, indicating the osmotic insensitivity of the enzyme in a population of vesicles which responded well to an uncoupler. Regardless of the source of the enzyme, cytochrome oxidase activity in reconstituted vesicles was insensitive to osmotic pressure, similar to the rat liver mitochondrial enzyme in situ. It should be particularly noted that this conclusion was found to be valid regardless of the method of assay (spectrophotometric or polarographic) or the substrate, i.e., cytochrome *c* and/or ascorbate + TMPD (data not given).

The results with reconstituted cytochrome oxidase vesicles brought to attention the need to re-evaluate the mechanism of stimulation of cytochrome oxidase activity, even on lysis, by uncouplers (Fig. 1G and H). Besides being protonophores, uncouplers could have a variety of effects including detergency, which have not been systematically evaluated thus far. As a prelude to these studies, ionic and non-ionic detergents were used to disrupt the mitochondrial membranes in a controlled manner using a variety of measurements that reflect the changes in the integrity of mitochondrial membranes (vide Appendix A). While the stimulatory and inhibitory effects of ionic and nonionic detergents were previously known (cf. Refs. 26 and 28), none of these studies attempted to interpret the effect of these agents on oxidase activity vis-à-vis the status of the membrane per se. Otherwise, one would be hard put to understand how the fallacy of equating the uncoupler stimulation of cytochrome oxidase activity in reconstituted vesicles with respiratory control persisted unexamined thus far!

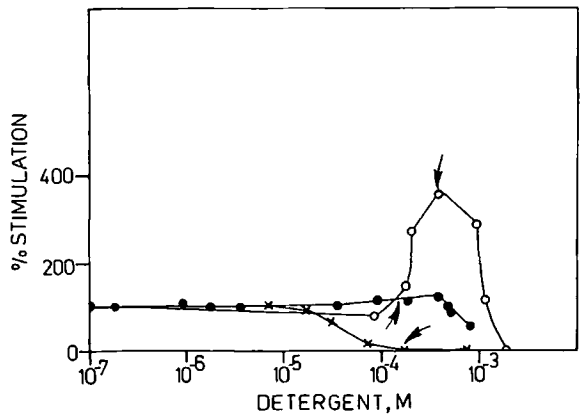

Fig. 7. Effect of Triton X-100 (●), SDS (○) and CTAB (×) on cytochrome oxidase activity. Assays were as in Fig. 1G, with comparable specific activities, represented as % of control activity in the absence of any detergent. The arrows represent the detergent concentrations for the onset of lysis as depicted from the experiments in Appendix A.

TABLE III

Effect of ionic detergents on the activity of cytochrome oxidase activity in the intact and Triton X-100 solubilized mitochondria

Triton X-100 was used at a concentration of $4.7 \cdot 10^{-4}$ M, corresponding to approx. 70% decrease in turbidity. n.d., not detected. Specific activities were comparable to those reported in other figures.

Detergent concentration	Cytochrome oxidase activity	
	− Triton	+ Triton
Nil	100	100
SDS ($2 \cdot 10^{-4}$ M)	300	144
CTAB ($1.6 \cdot 10^{-4}$ M)	n.d.	12.5

Cytochrome oxidase activity in detergent-treated mitochondria

The non-ionic detergent, Triton X-100 is a weak stimulator of cytochrome oxidase activity, which it inhibited at solubilizing concentrations (Fig. 7). The cationic detergent, cetyltrimethylammonium bromide fully inhibited the enzyme activity well before the solubilization. On the other hand, the anionic detergent, sodium dodecylsulfate was a potent stimulator of the activity at concentrations required for the onset of lysis. It should be noted that some detergent effects in mitochondria were clearly biphasic. Experimental results reported in Appendix A identify the critical concentrations required for each of these detergents for the onset of specific effects.

Table III shows direct experimental evidence that distinguishes the detergency from ionic effects. SDS and CTAB resulted in monotonic stimulation and inhibition of cytochrome oxidase activity, respectively, on prior solubilization of mitochondria by Triton X-100, showing that the ionic detergents exert specific and opposing effects on the cytochrome oxidase molecule depending on the net ionic charge these detergents possess, whereas non-ionic detergents only stimulated the activity.

Table IV shows the effect of three non-ionic detergents on ascorbate respiration at critical concentrations wherein turbidity changes (as a measure of lysis) and DNP stimulation of cytochrome oxidase activity were measured, in three independent experiments. The three non-ionic detergents stimulated the activity to a variable degree such that Lubrol > triton > ammonyx. Careful titration of changes in turbidity with occluded fumarase activity revealed that (vide Appendix A) a change in turbidity greater than 70% was consistent with lysis. Under these conditions, the data showed that lysis did not abolish stimulation by DNP. Further,

TABLE IV

Effect of non-ionic detergents and DNP on the cytochrome oxidase activity in mitochondria

The activity of ascorbate respiration was expressed as natoms O/min per mg protein.Protein per assay; I,0.65 mg/ml; II, 0.49 mg/ml; III, 0.66 mg/ml. These rates were obtained by careful deduction of non-enzymatic rates in the presence of detergents in every instance. The sequence of addition of either DNP (0.2 mM) or detergent had no influence on the final rates obtained. Triplicate measurements indicated as mean ± S.D.

	Control	Triton	Lubrol	Ammonyx LO
Expt. I				
(a) Concentration of the detergent (v/v)	nil	0.028	0.028	0.028
(b) % decrease in turbidity at 520 nm	nil	70	73	36
(c) Ascorbate respiration				
−DNP	270	405	533	281
+DNP	321	453	534	346
Expt. II				
(a) Concentration of the detergent (v/v)	nil	0.022	0.011	0.022
(b) % decrease in turbidity at 520 nm	nil	76	80	62
(c) Ascorbate respiration				
−DNP	264 ± 8.6	375	528	167
+DNP	321	440	528	361
Expt. III				
(a) Concentration of the detergent	nil	0.028	0.028	0.028
(b) % decrease in turbidity at 520 nm	nil	72	75	41
(c) Ascorbate respiration				
−DNP	146 ± 4.4	231 ± 7.3	279 ± 9.6	120 ± 4.4
+DNP	296 ± 11	280 ± 18	271 ± 13	183 ± 6.3

despite large daily variations in the detergent effects on activity vis-à-vis the turbidity changes, there appeared to be an inverse correlation between the stimulation by a nonionic detergent and stimulability by DNP. That is, an already maximally stimulated preparation was not quite suitable for the demonstration of stimulation by DNP.

The day-to-day variation was, however, problematic in observing the stimulation of site III respiration by DNP in mitochondria by detergents. The polarographic assays were sensitive enough to detect reliably changes of up to 5% of the activity. The effect of various concentrations of DNP was measured in mitochondria pre-treated with lytic concentrations of Triton X-100 (Fig. 8). The results clearly indicated that DNP stimulated, with a biphasic effect, the solubilized cytochrome oxidase activity. Since protonmotive force cannot exist in a solubilized system, the stimulatory effect of DNP must be entirely due to a direct interaction and not due to its uncoupling activity. What kind of the molecular interaction would be responsible for stimulation of site III respiration by DNP?

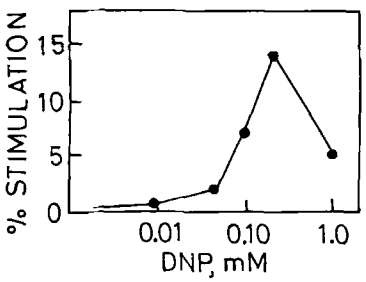

Fig. 8. Effect of DNP on solubilized mitochondria. The activity of ascorbate+TMPD respiration was determined as in Fig. 1G in mitochondria pretreated with Triton X-100 (0.47 mM, corresponding to 70% decrease in turbidity and nearly 100% abolition of the latency of occluded fumarase activity, vide Appendix), as a function of the concentration of 2,4-dinitrophenol. Data representative of two independent experiments.

Discussion

Modulation of cytochrome oxidase activity by ions

Detailed osmometric evaluation of mitochondrial respiration revealed that site III respiration was osmotically insensitive. The osmotic insensitivity of reconstituted cytochrome oxidase activity, demonstrated here for the first time, led to a number of new observations with regard to the nature of regulation of cytochrome oxidase activity in mitochondria: (i) uncoupler stimulation of oxidase activity was present regardless of whether the mitochondrial inner membrane was intact or lysed; (ii) site III respiration in intact mitochondria was partly activated by the lysis of the inner membrane such that lysis (and presumably release of endogenous substrates) resulted in uncoupler-independent stimulation of the activity; (iii) cytochrome oxidase activity was stimulated by detergency per se; however, (iv) the anionic and cationic detergents appear to interact with the respiratory complex differentially. Interaction of CTAB with the cytosolic aspect of the oxidase molecule was inhibitory to the enzyme, and similarly, interaction of SDS with the matrix aspect of the molecule was also inhibi-

tory and this inhibitory effect was distinct from the stimulatory effect of detergency per se.; and (v) the classical uncoupler, 2,4-DNP, also exhibited a biphasic effect on the oxidase activity. Interestingly, the stimulatory effect of DNP in solubilized mitochondria required that detergent per se should not have maximally stimulated the oxidase activity. Different detergents appeared to stimulate the oxidase activity differently though the degree of solubilization of mitochondria was comparable.

Taken together, these experiments, replete with a systematic documentation of the degree of disintegration of mitochondrial membranes (Appendix A), permit a simple topological model for the ionic modulation of cytochrome oxidase activity. The oxidase molecule exhibits charge anisotropy such that the cytosolic aspect has net negative charge and the matrix aspect has net positive charge. Clearly, expression of the fixed charges is one of the determinants of the activity of the enzyme, in order to account for the inhibition of the enzyme by the ionic detergents.

Two lines of evidence support this model: the nuclear coded subunits indeed exhibit charge anisotropy [29] though not the mitochondrial subunits (Kadenbach, B., personal communication), the former being essential for the activity in all species. Kadenbach and co-workers reported recently [30,31] using the solubilized, purified cytochrome oxidase from a variety of species, that oxidase activity was initially stimulated by anions and subsequently inhibited at higher concentrations. This biphasic effect of anions followed a precise rank order of the required external anionic concentrations (for both activation and inhibition) vis-à-vis their ionic charge, i.e., ATP < ADP < AMP < PP_i < P_i < Cl < Hepes. The cations were ineffective. Thus, these results supported the possibility that endogenous nucleotides (anions) could inhibit respiration by a switch in the matrix space from the less potent ADP to more potent ATP. The site for such a control could be the bc_1 complex, which is currently being investigated for comparable ionic interactions.

Viscous regulation of oxidase activity and its implications

Stimulation of cytochrome oxidase activity on treatment with detergents indicated that, as common to several other membrane-bound proteins [27], the activity exists in an inhibited state in the presence of lipids. The experimental data clearly indicated that stimulation by DNP was not additive to that by detergents confirming that diverse agents modulate the activity from zero activity (e.g., SDS and CTAB) to maximal activity (e.g., laurylmaltoside). It may be, perhaps, instructive to seek an interpretation of this phenomenon from more fundamental considerations, e.g., Kramers' theory of chemical kinetics as applied to enzyme catalysis [32]. The relationship between K_{cat} and viscosity of the medium was derived to show that

$$K_{cat} = \left(\frac{A}{\eta_S^{\epsilon}} + A'\right) e^{-\Delta/kT} \quad (1)$$

where Δ is the activation energy, η_S is the viscosity, A is a function characterizing the potential energy profile, the exponent ($0 < \epsilon \leqslant 1$) relates to the attenuating influence of protein matrix on solvent viscosity, and A' is an empirical (viscosity-independent) parameter. It is intuitively obvious that viscous regulation should be maximally seen in hydrophobic proteins, as borne out by (i) stimulation by detergents (which perturb/remove the lipid domain) and (ii) osmotic inhibition of the activity (vide infra).

Yet another feature of the viscous regulation of oxidase was that the activity, in turn, affects the lipid bilayer so as to enhance its non-ohmic conductance as evidenced by enhanced permeation to mannitol. The importance of induced porosity of the inner mitochondrial membrane is two fold. Firstly, at steady state,

$$J_{ox} n = L \Delta P \quad (2)$$

where J_{ox} is the rate of respiration, n the stoichiometry (H^+/O ratio), L the leak conductance to protons and, ΔP, the protonmotive force. Enhanced leak conductance to large polyols such as mannitol, sucrose, etc., would constitute compelling evidence against the development any significant protonmotive force [3,15,33]. Interestingly, enhanced mannitol permeation was also reported from other laboratories (cf. Ref. 34), though its significance with regard to protonmotive force escaped their attention. Secondly, we have shown elsewhere that this enhanced polyol permeation requires dramatic lowering of interfacial and diffusional barriers of the lipid bilayer. This would be consistent with density fluctuations in the lipid bilayer such that the polyols permeate through the rarefied lipid domains [1–4]. The metabolically induced high-amplitude swelling of rat liver mitochondria was shown to be one of sequential disruption of mitochondrial membranes, as an extreme case of this variable porosity [3,33]. In any event, structural fluctuations in the membrane indicate corresponding variations in enthalpy and entropy [35] and the resulting lowering of entropy of the energized membranes (i.e., negentropy) would require considerable revision in the current models of transduction which uniformly assume invariant entropy of the system [36].

Consequences of density fluctuations in the energized membranes

It appears that, despite considerable efforts in measuring fluctuations incidental to enzyme catalysis, large changes in activity of globular proteins could not be

observed due to their relative incompressibility [37,38]. Membrane-bound enzymes appear to offer an ideal experimental system, hitherto unexploited, in order to probe the fluctuational basis of enzyme catalysis. Given the possibility of density fluctuations in the lipid bilayer, osmotic inhibition of the enzyme activities as well as coupled processes would be consistent with a statistical mechanical explanation. Compressibility of the bilayer relates to density fluctuations since

$$\frac{kT\beta_T}{V} = \frac{\overline{\rho - \bar{\rho}^2}}{(\bar{\rho})^2} \tag{3}$$

where k is the Boltzman constant, T the temperature, $\overline{(\rho - \bar{\rho})^2}/(\bar{\rho})^2$ the mean square relative deviation in density from mean density, V the volume, and β_T the isothermal compressibility, i.e.,

$$\beta_T = \frac{-1}{V}\left(\frac{\partial V}{\partial P}\right)_T \tag{4}$$

where P is the pressure [39]. Our results with polyol permeation as a marker for density fluctuations indicated that

$$\frac{\overline{(\rho - \bar{\rho})^2}}{(\bar{\rho})^2} = f(J_{ox}, J_{-ATP}, \ldots \text{etc.}) \tag{5}$$

Osmotic pre-compression of the bilayer could retard the activity of free-energy dissipators such as respiration (J_{ox}) and ATP hydrolysis (J_{-ATP}) as a consequence of increase in the mean density. Variable porosity across the bilayer induced by energization of the bilayer would indicate that the volume (V_M) and surface area (A_M) of the membrane also varies in the energized state, such that

$$V_M = V_L + V_D$$

and

$$A_L = A_L + A_D \tag{6}$$

where L pertains to the incompressible lipid domain and D, the compressible domain of pockets of free volume or lattice defects. We have shown elsewhere that variable porosity induced by gravitational fields exhibits a negative temperature coefficient for polyol permeation [1]. Recently, we also obtained evidence that ADP/O ratio was enhanced on lowering the temperature [36]. These observations suggest that as the lipid molecules tend to diffuse in the plane of bilayer, the probability of voids diminishes which in turn retards polyol permeation, i.e., an apparent negative temperature coefficient for the process. Similarly, the molecular dynamics associated with catalysis by enzymes/transporters could be dampened by the annulus lipid and even on energization, if the molecule partitions into the lipid-dense domains preferentially. Osmotic compression of the bilayer similarly could decrease the voids that permit molecular breathing contingent to catalysis thereby leading to inhibition of activity. Osmotic pressure, as a novel physical variable, introduces in the thermodynamic analysis of energy transduction phenomena an extensive (structural) property that relates to the amount of voids in the membrane phase, which in turn determines compressibility.

These arguments are consistent with the general observation that the activity of a variety of membrane-bound enzyme systems, transporters and channels was modulated by osmotic pressure such that the activity,

$$J = J_{max} - \tilde{K}\Pi \tag{7}$$

where $\tilde{K}$ is the newly described elastic/compressibility coefficient, J the coupling activity and Π the external osmotic pressure *. Thus, osmotic inhibition, respiratory control, detergent-mediated stimulation of activities of hydrophobic proteins could all focus on the lipid–protein interactions that underlie their viscous regulation. Concerted fluctuations of proteins and the lipid domain could mediate energy transduction and the phenomenon of respiratory control itself could arise from several reasons, individually or even all together, e.g., kinetic (end-product inhibition), viscous (non-dissipation of lipid 'pulses' in the annular lipid of the hydrophobic free energy generators, which inhibits K_{cat} for these enzymes by enhanced local viscosity) and thermodynamic (due to 'back pressure' of various kinds). The mechanism of action of uncouplers could be similarly manifold, e.g., ionic, detergent-like and as doping agents that dampen fluctuations. Ionic susceptibility of cytochrome oxidase as well as lack of osmotic 'compressibility' of its activity could be a direct consequence of its high charge anisotropy.

Osmotic pressure as a probe for the 'intermediate' in energy transduction

Osmotic susceptibility of the component reactions of energy transduction in mitochondria appears to offer a unique and novel approach to probe these complex reactions. Fig. 9 gives two plots, one relating P/O ratio with 1/RCR, the other relating the efficiency of oxidative phosphorylation for glutamate + malate and suc-

* Zimmermann [40] argued that osmotic modulation of the activities of enzymes would be forbidden on a priori thermodynamic grounds that chemical reactions would require very high pressures to be inhibited by compression. Volume work being negligible from an energetic point of view, it would be difficult to envisage conformational changes due to osmotic stretch/compression as the basis for the observed continuous modulation of these activities. A continuous tuning of microstates/protein dynamics by osmotic pressure would be more likely.

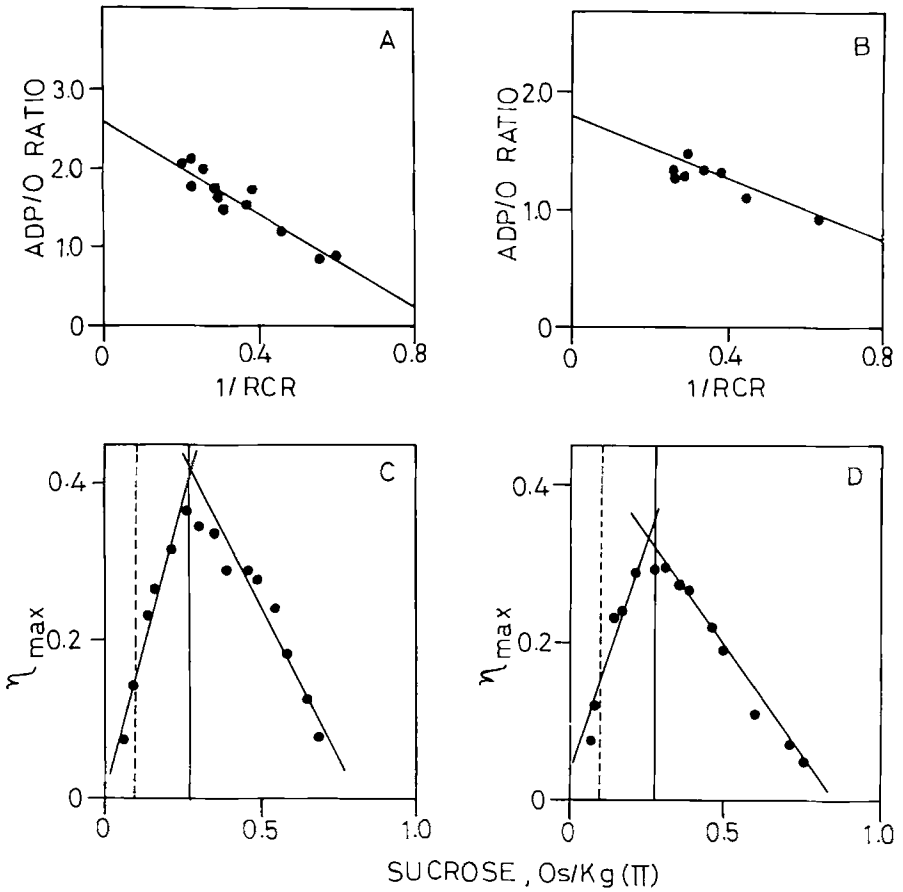

Fig. 9. A comparison of the effect of osmotic pressure on glutamate+malate and succinate-dependent respiration. Plots of ADP/O ratio vs. 1/RCR yield mechanistic stoichiometry (Z) of 2.7 for glutamate+malate (A) and 1.85 for succinate (B). η_{max} was computed from Eqn. 12 in the text and was plotted for glutamate+malate (C) and succinate (D) respiration. A comparable evaluation for site III respiration or segmental respiration was not possible since the RCR remained constant (cf. Fig. 1G and H and Fig. 3). Data points in A and B appear less in number due to multiple superimposition. Data were obtained from a single mitochondrial preparation subjected to a detailed osmometric evaluation ($n=15$); linear regression was by least squares method.

cinate as substrates. For a two-way coupled process of respiration, the input (i), and phosphorylation (o), the output, forces (X) and fluxes (J),

$$J_i = L_{ii}X_i + L_{io}X_o$$

$$J_o = L_{oi}X_i + L_{oo}X_o \tag{8}$$

where L represents the phenomenological coefficients coupling the forces and fluxes [41]. The degree of coupling, q, would be,

$$q_{io} = \frac{L_{oi}}{\sqrt{L_{oo}L_{ii}}} \tag{9}$$

and the respiratory control ratio would be

$$1-q^2 = \frac{1}{RCR} \qquad 0<q<1 \tag{10}$$

such that when q tends to 1.0, P/O ratio tends to be the mechanistic stoichiometry, Z, such that

$$Z = \sqrt{L_{oo}/L_{ii}} \tag{11}$$

It can be clearly seen that as enhanced osmotic pressure led to decreased RCR, it also led to a corresponding decrease in the P/O ratio. The values for Z were well within the generally accepted limits for the two substrates. Similarly, η_{max} merely tracked RCR, since

$$\eta_{max} = q^2\left(1+\sqrt{1-q^2}\right)^2 \tag{12}$$

showing that the efficiency varied directly with the elastic energy (which varies parabolically with a deviation from isotonicity (cf. Ref. 15)).

Since RCR and P/O ratio were both inhibited by osmotic pressure (as expected of a tandem process), the target for inhibition would be the common coefficient that also imparts a change in the same direction to both these indices. This would be L_{ii} which represents the 'self coefficient' for respiration! Thus the thermodynamic considerations fully support the experimental observation that the respiratory chain per se is the primary site of action of osmotic pressure consistent with the inhibition of indices of oxidative phosphorylation.

The differential effects of osmotic pressure on oxidative phosphorylation also bring to fruition the long search for a clear demonstration of the non-equivalence of the sites in energy transduction. Osmotic pressure could be seen to interfere with the mechanism of energy transduction at the first two sites. Also, the maximal efficiency was seen to correspond to apparent isotonicity of 0.27 Os/kg in the case of site I and site II substrates. The data clearly showed that there exists an intimate relationship between the transduction mechanisms and the osmotic pressure of the external medium for the first two sites. On the other hand, site III respiration exhibited not only an insensitivity to variations in external osmotic pressure, but also exhibited no respiratory control. In fact, both site III respiration and succinate-ferricyanide oxidoreductase activity exhibited no respiratory control, though their osmotic susceptibility was markedly different. The site III respiration was indeed unique in several ways. Since respiratory control was absent (RCR = 1), plots as in Fig. 9 would not be possible, since a single value for RCR would be present rendering Z infinite! Similarly, maximal efficiency would not be calculable based on respiratory control for site III substrates. On the other hand, phosphorylation was shown to be fully sensitive to osmotic pressure, similar to that at other sites. Susceptibility of energy transduction to specific physico-chemical variables would be the first level evidence required in the identification of the nature of the intermediate. it was clear that this susceptibility varies with the site of energy transduction.

It is but a truism to state that the complexity of the mechanism of oxidative phosphorylation resides in the multiplicity of interactions between various components and phases. Thermodynamic approaches definitely aid in handling the complexity though the arguments would transcend detailed mechanisms. Thus, it is of critical importance to use probes that offer discrimination between alternative models. In our experience, a gentle, quantitative and defined physical probe, the osmotic pressure, appears to hold considerable promise to yield novel insights into the mechanism of oxidative phosphorylation.

Appendix A. Influence of detergents on the physical integrity of mitochondria: use of occluded fumarase activity as a probe

A definition of physical integrity, in a simplistic sense, merely requires to test whether the membrane is a barrier (or not) for the movement of molecules from within and without. A direct method to probe the integrity in the presence of detergents is to determine the behaviour of the activity of an occluded soluble enzyme such as fumarase. The initial velocity of activity is expressed in Eqn. A-1:

$$J_s = \frac{KS_0}{(1 + K/P)} \qquad \text{(A-1)}$$

where K is the rate constant of the activity and P is the rate constant of the permeability. An increase in the initial velocity at constant external substrate, S_0, represents either a change in K or in P. Addition of a detergent would remove the barrier and, therefore, P would increase, leading to stimulation of the occluded fumarase activity. This would be attendant with the release of enzyme/protein from within into membrane-free supernatants.

Solubilization of a membrane need not be the only mechanism by which the activity of an occluded enzyme could increase. Fig. A-1 shows the effect of Triton X-100 on: (a) the occluded activity; (b) release of fumarase and (c) release of protein into membrane-free supernatants, obtained by assaying for fumarase activity and protein as absorbance at 280 nm. A striking aspect of the data is that each of the parameters exhibited a different break-point for the onset, consistent with different levels of action of the detergent. Clearly, release of fumarase into supernatants would coincide with lysis of the particle, releasing its contents into the supernatants. Since a significant part of mitochondrial protein represents the membrane protein, the onset of release of protein at a higher detergent concentration would imply the onset of solubilization of membrane. Lysis would precede solubilization, since the action of detergent was shown to involve prelytic enhancement of permeability to external solutes (including substrates) such that lysis would be by colloidal swelling [4,42].

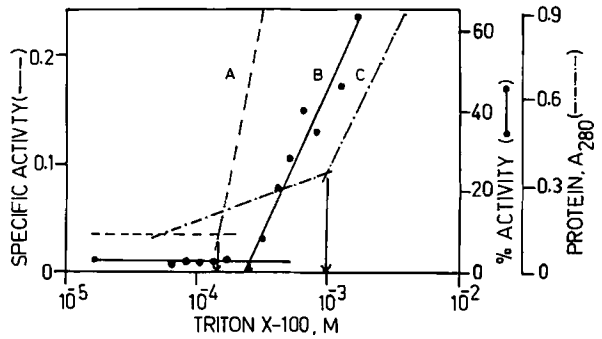

Fig. A-1. Effect of Triton X-100 on rat liver mitochondria. (A) occluded fumarase activity (measured directly in mitochondrial suspensions as A_{250} as fumarate formed in the presence of 33 mM Tris-malate, pH 7.4). (B) Release of fumarase (expressed as % of total mitochondrial fumarase) into membrane-free supernatants (obtained by prior centrifugation of the detergent-treated mitochondrial suspensions at 4°C and 25000 × g). (C) Release of protein into membrane-free supernatants (as in B) measured as A_{280}. Data points A and C omitted for clarity. The data were fitted to independent regression lines similar to osmometry, as in Fig. 1, to obtain the critical detergents concentrations for the onset of increase in each activity.

Detergents were shown to induce single channel conductance behaviour as well as porosity that can discriminate between different polyols [43,44]. This is, however, a non-specific enhancement of permeability, which is intimately related to lysis per se [42]. Besides solubilization and lysis preceded by non-specific enhancement of porosity of the bilayer, activation of the occluded enzyme at a distinct lower concentration for the onset of enhancement of activity would imply a third mode of enhancement of P (Eqn. A-1) viz, a detergent-mediated enhancement of the dicarboxylate transporter activity. This would be a necessary consequence of viscous regulation of the enzyme/transporter by the annulus lipid, shared by several membrane-bound enzymes, including cytochrome oxidase, which was also stimulated by Triton X-100 in the same concentration range (Figs. 7 and A-1). Preliminary results indicate that the occluded fumarase (dicarboxylate transporter) activity is isotonic media was inhibited by inhibiting mitochondrial respiration as also stimulation by detergents in this concentration range. Thus, the concentration-dependent action of detergents on mitochondrial inner membrane could also involve an initial activation of dicarboxylate transporter, besides lysis of membrane leading to the release of matrix proteins and finally, solubilization of the membrane itself.

Fig. 2 shows a systematic comparison of three detergents and their concentration-dependent influence on three parameters, viz. turbidity, soluble fumarase and occluded fumarase activities. These effects were studied over a wide range of detergent concentrations viz. 0.1 μM–10 mM. Each detergent stimulated the activity of the occluded fumarase activity prior to its effect on turbidity, suggesting that the stimulation of dicarboxylate transporter is a common property of all detergents. This may be usefully contrasted with their effects on cytochrome oxidase (cf. main text). The inhibition of the occluded fumarase activity occurred in the same concentration range as that at which the soluble fumarase was inhibited. A comparison of Triton X-100 data from Figs. A-1 and A-2 showed that significant lysis coincided with a corresponding change in turbidity.

These results indicated that a simplistic argument that the action of detergents would be lysis/solubilization is not tenable, since the critical concentrations at which specific events occur differed. Activation of membrane-bound enzymes and transporters, lysis and solubilization would occur at different concentrations of the detergent. Great caution would be required to predict, except grossly, at which concentration a detergent acts from without or within the mitochond-

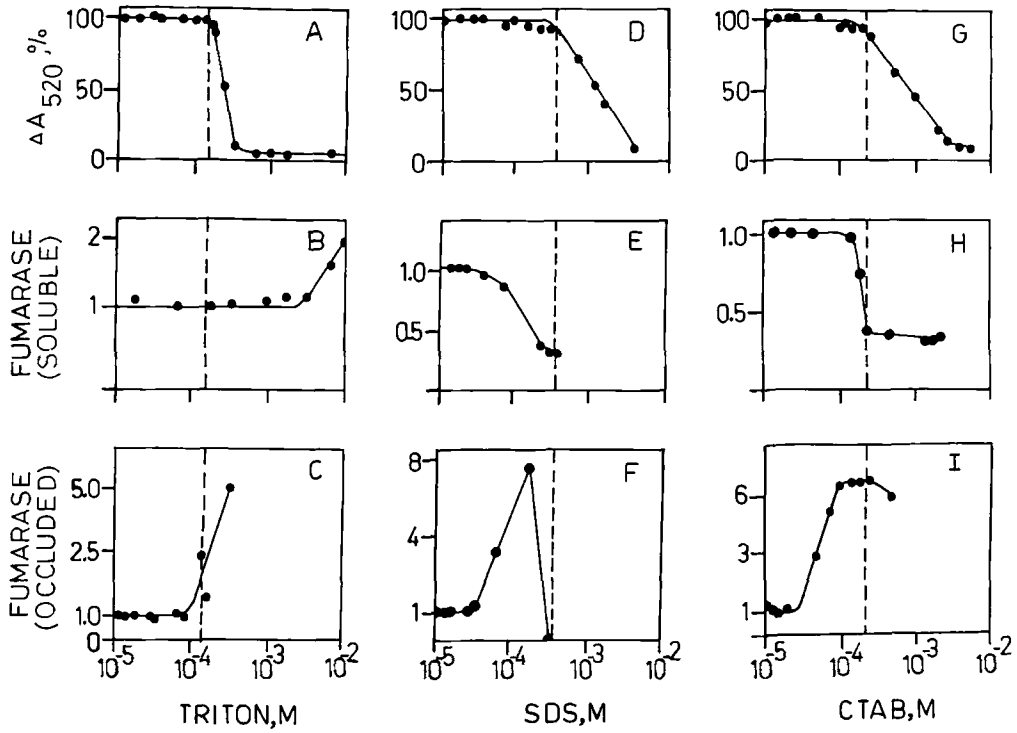

Fig. A-2. Titration of mitochondria with Triton X-100 (A–C), SDS (D–F) and CTAB (G–I). Vertical dashed line represent the onset of decrease in turbidity at 520 nm, monitored in various detergents (A, D and G) to facilitate comparison, taking the turbidity of the native preparation in 0.25 M sucrose media as 100%. The effect of detergents on fumarase activity per se in membrane-free, mitochondrial supernatants (obtained by an osmotic shock with ice-cold 10 mM Tris-HCl (pH 7.4), to obtain supernatants at 100000 × g) was determined in B, E and H (taking the activity of untreated supernatant as 1.0 unit). Effect of these detergents on occluded fumarase activity was determined at various detergent concentrations (C, F and I), expressed in the amount of stimulation over the activity of untreated mitochondria.

rion, due to large prelytic changes in permeability. The activity of occluded enzymes such as fumarase could be effectively deployed to demonstrate the concentration-dependent effects of detergents on membranes.

Acknowledgments

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References

1 Sitaramam, V. and Sarma, M.K.J. (1981) Proc. Natl. Acad. Sci. USA 78, 3441–3445.
2 Sambasivarao, D. and Sitaramam, V. (1983) Biochim. Biophys. Acta 722, 256–270.
3 Sambasivarao, D. and Sitaramam, V. (1985) Biochim. Biophys. Acta 806, 195–209.
4 Sambasivarao, D., Rao, N.M. and Sitaramam, V. (1986) Biochim. Biophys. Acta 857, 48–60.
5 Teissie, J. and Tsong, T.Y. (1979) Biochemistry 20, 1548–1554.
6 Haines, T.H. (1979) Biophys. J. 37, 147–148.
7 Hladkey, S.B. and Gruen, D.W.R. (1984) Biophys. J. 45, 645–646.
8 Miller, I.R. (1984) Biophys. J. 45, 643–644.
9 Sitaramam, V. (1987) in Interactions of Water in Ionic and Non-ionic Hydrates (Kleeberg, H., ed.), pp. 213–216, Springer-Verlag, Berlin.
10 Sitaramam, V. and Rao, N.M. (1986) Ind. J. Exp. Biol. 24, 615–623.
11 Mitchel, P. (1979) Eur. J.Biochem. 95, 1–20.
12 Nichols, D.G. (1982) Bioenergetics: An Introduction to Chemiosmotic Hypothesis, Academic Press, New York.
13 Ferguson, S.J. (1985) Biochim. Biophys. Acta 811, 47–95.
14 Slater, E.C., Berden, J.A. and Herweijer, M.A. (1985) Biochim. Biophys. Acta 811, 217–231.
15 Sambasivarao, D., Kramer, R., Rao, N.M. and Sitaramam, V. (1988) Biochim. Biophys. Acta 933, 200–211.
16 Sitaramam, V. and Sambasivarao, D. (1984) Trends Biochem. Sci. 9, 222–223.
17 Sitaramam, V. and Sarma, M.K.J. (1981) J. Theoret. Biol. 90, 317–336.
18 Sitaramam, V. and Sarma, M.K.J. (1987) J. Biosci. 11, 89–105.
19 Robinson, J. and Cooper, J.M. (1970) Anal. Biochem. 33, 390–399.
20 Beavis, A.D. and Lehninger, A.L. (1986) Eur. J. Biochem. 158, 315–322.
21 Beavis, A.D. and Lehninger, A.L. (1986) Eur. J. Biochem. 158, 307–314.
22 Buge, U. and Kadenbach, B. (1985) J. Bioenerg. Biomembr. 17, 375–384.
23 Casey, R.P., Chappel, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156.
24 Klingenberg, M. (1979) Methods Enzymol. 56, 229–241.
25 Lehninger, A.L. (1970) Biochemistry, Worth Publ. Inc., New York.
26 Kadenbach, B., Kuhn-Nentwig, L. and Buge, U. (1987) Curr. Top. Bioenerg. 15, 113–161.
27 Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209–237.
28 Melhorn, R.J. and Packer, L. (1976) Biochim. Biophys. Acta 423, 382–397.
29 Wikstrom, M., Saraste, M. and Pentilla, T. (1985) in The enzymes of Biological Membranes (Martonosi, A.N., ed.), Vol. 4, pp. 111–148, Plenum Press, New York.
30 Kadenbach, B. (1986) J. Bioenerg. Biomembr. 18, 39–54.
31 Reimann, A., Huther, F.-J., Berden, J.A. and Kadenbach, B. (1988) Biochem. J. 254, 723–730.
32 Gavish, B. and Werber, M.M. (1979) Biochemistry 18, 1269–1275.
33 Sambasivarao, D. and Sitaramam, V. (1986) EBEC Rep. 4, 334.
34 Whipps, D.E. and Halestrap, A.P. (1984) Biochem. J. 221, 147–152.
35 Sitaramam, V. (1988) J. Scient. Ind. Res. (India) 47, 395–403.
36 Sitaramam, V. (1988) EBEC Rep. 5, 114.
37 Somogyi, B., Welch, G.R. and Damjanovich, S. (1984) Biochim. Biophys. Acta 768, 81–112.
38 Gekko, K. and Hasegama, Y. (1986) Biochemistry 25, 6563–6571.
39 Hill, T.L. (1956) Statistical Mechanics, McGraw-Hill, New York.
40 Zimmermann, U. (1978) Annu. Rev. Plant Physiol. 29, 121–148.
41 Rottenberg, H. (1979) Biochim. Biophys. Acta 549, 225–253.
42 Seeman, P., Sauks, T., Argent, W. and Kwant, S.O. (1969) Biochim. Biophys. Acta 183, 476–489.
43 Schleiper, P. and De Robertis, E. (1977) Arch. Biochem. Biophys. 184, 204–208.
44 Rao, N.M. (1988) Ph.D. Thesis, Osmania University, Hyderabad, India.