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STUDIES ON THE NON-LINEAR OSMOTIC PRESSURE-VOLUME RELATIONSHIP IN MITOCHONDRIA AND ENTRY OF SUCROSE INTO THE MATRIX SPACE DURING CENTRIFUGATION

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The two-compartment sucrose-space hypothesis was refuted recently (Sitaramam, V. and Sarma, M.K.J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3441–3445), using the novel technique of enzyme osmometry of matrix enzymes based on the following premise: a shift in the discontinuity (break-point) of the activity profile of an occluded enzyme as a function of external osmolarity (i.e., osmotic profiles) would imply a shift in the internal solute content of the same compartment as that of the enzyme. A systematic re-evaluation of the osmotic profiles of mitochondrial enzyme systems has revealed that the activities of several matrix and inner membrane enzyme systems exhibited break-points larger than those of osmolysis (i.e., actual release of marker enzymes into the medium) of mitochondria. The experimental findings were consistent with (i) entry of sucrose across the inner membrane consequent to enhanced permeability effects by gravitational field, and (ii) dependence of the kinetic constant of several membrane-bound enzymes/carrier proteins, respiratory coupling and ADP/O ratio on the volume of mitochondria (i.e., osmotic stretch of the inner membrane).

Introduction

The sucrose-space hypothesis proposes that two anatomically distinct subspaces are present in subcellular organelles, one permeable to sucrose and the other, impermeable. This model was recently refuted by a novel observation that dramatic changes in permeability of biological membranes occur during centrifugation [1]. This study applied the physical theory of osmosis to the first-order kinetics of occluded-enzyme activities to evaluate the internal solute content of organelles [1,2]. In our studies the osmotic dependence of several enzyme activities was determined. Generally, the activity of occluded enzymes increased with decreasing osmotic pressure. At critcal external osmotic pressure (defined in the text as the break-point), changes in activity with external osmotic pressure occur sharply.

A shift in the break-points of the activity of the matrix enzymes, fumarase (L-malate hydro-lyase, EC 4.2.1.2) and malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37), corresponding to the sucrose concentration in the isolation medium, was considered adequate to prove that sucrose in the medium quantitatively equilibrates with the matrix compartment, for the following reasons: (i) a shift in the break-point (vide infra) would indicate a corresponding magnitude of shift in the solute content in the same compartment as that of the occluded enzyme, (ii) the validity of enzyme osmometry was demonstrated using single membrane structures, syn-

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aptosomes, which have occluded lactate dehydrogenase, (iii) unequivocal evidence was obtained in synaptosomes that sucrose equilibrates with the interior exclusively during centrifugation.

Examination of the literature shows that the sucrose-space estimates based on sucrose content are unduly large and varied, and that mitochondrial sucrose varied in relation to sucrose in the isolation medium [1–4]. The sucrose-space hypothesis, as originally proposed by Werkheiser and Bartley [5] and extended by De Duve [6], essentially holds that the presence of sucrose in subcellular organelles (which are isolated in sucrose media by differential centrifugation) argues for the presence of an anatomical subspace, of which only the limiting membrane is permeable to sucrose. The paradoxical behaviour of mitochondria, which behave as true osmometers in the presence of external sucrose and yet exhibit rapid entry kinetics, could only be resolved by the postulation of the sucrose-space hypothesis. However, this reasoning has been questioned previously by others (cf. Refs. 3 and 4). It should be emphasized that the sucrose-space hypothesis was postulated not for mere kinetic convenience, but indeed as a physical reality of two distinct compartments with an equilibration time of less than 2 min! Therefore, the problem of the sucrose-space hypothesis relates to the localization of sucrose in specific compartments and not to a measurement of rates of permeabilities by uptake studies.

However, the technique of enzyme osmometry is essentially indirect and is valid only if all its predictions hold true and any singularities are crictically accounted for. For instance, we have verified that a homology exists between osmolysis (i.e., release of enzymes, due to physical disruption) and enzyme osmometry (vide infra), since the two approached identical break-points in synaptosomes [1], but not for mitochondrial enzyme systems. In the present study, we have undertaken a systematic re-evaluation of this approach in mitochondria and we have found that the two techniques are not in agreement quantitatively. Despite this discrepancy, the earlier refutation of the sucrose-space hypothesis remains valid, both on theoretical and experimental grounds. Further, a systematic comparison of the osmometric profiles of several mitochondrial enzyme systems and

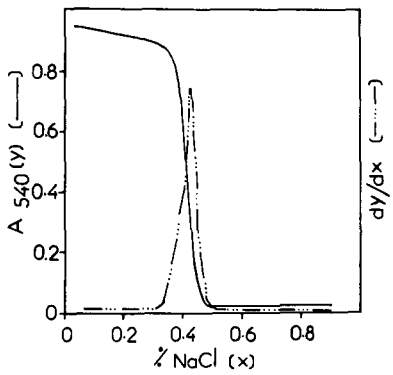

Fig. 1. Osmolysis of rat erythrocytes: Rat erythrocytes were obtained from heparinized blood and were washed twice in 0.9% NaCl. Erythrocytes equivalent to 20 μl of whole blood per assay were suspended in various concentrations of NaCl, and 3 mM sodium phosphate buffer, pH 7.4, for 15 min at room temperature, centrifuged at $1500 \times g$ for 10 min and the absorbance at 540 nm was measured in the supernatants. Absorbance (y) was plotted as a function of NaCl concentration (x) (with 19 data points; omitted in the figure for clarity). The derivative (dy/dx) was calculated and plotted as a function of NaCl concentration.

indices of oxidative phosphorylation has revealed the dependence of several of these activities and indices on the osmotic stretch of the inner membrane.

In the ensuing discussion, we introduce the following terminology: (1) Osmolysis refers to experiments wherein particles are exposed to different concentrations of external solute, and enzyme activities/protein in the particulate-free supernatants are measured. (2) Enzyme osmometry pertains to direct measurements of activities of enzymes in particles suspended in media of varied external osmolarity. (3) Osmotic profiles refer to plots of activities (of both osmolysis and osmometric experiments) as a function of external osmolarity. (4) Break-point * is the concentration of external solute at which a sharp discontinuity exists in the osmotic profiles (vide infra). (5) High-amplitude swelling refers to metabolically dependent high-amplitude swelling (originally referred to as

* Break-point is defined in the mathematical context and should not necessarily be equated with external osmolarity at which the particle disrupts (breaks).

'spontaneous swelling' [7]). (6) Osmometry-linked turbidimetry refers to experiments wherein maximal rates of change of turbidity are obtained as a function of external osmolarity. (7) Gravity-mediated entry of sucrose implies a primary interaction between the gravitational field (i.e., centrifugal field) and the biological membranes, causal to the enhanced permeability of such membranes to external solutes.

Theoretical Considerations

Erythrocyte osmolysis offers a simple model to explain much of the osmotic behaviour of subcellular organelles. Fig. 1 shows a typical osmolysis curve of rat erythrocytes. The curve is typified by two discontinuities: the lower reflecting beginning of lysis and the upper reflecting its completion. The slope represents a cumulative integral of the frequency distribution of the particles, as a function of osmotic pressure difference across the membrane. Thus, the derivative of the osmolysis curve reflects the heterogeneity of the particles with regard to their osmotic succeptibility. For a single erythrocyte, the osmolytic curve would necessarily be a step function, i.e., the derivative has an infinite slope. The lower break-point corresponds to maximal volume expansion prior to bursting and is alone relevant for the ensuing discussion. At a specified internal solute content of a given organelle, the lower break-point represents a material constant related to the elastic limits of the membrane. Since isotonicity represents osmotic equivalence across the membrane, the ideal osmotic pressure-volume relationship is:

$$\frac{\Pi_1}{\Pi_2} = \frac{V_2}{V_1} \tag{1}$$

where Π_1 corresponds to the osmotic pressure of the solutes at isotonic concentration, Π_2, osmotic pressure corresponding to maximal volume expansion (lower break-point), V_1 and V_2 the corresponding volumes of erythrocytes. Thus, the break-point is a good index for gross changes in internal solute content of organelles. *

Release of an occluded enzyme from a membranous particle can be monitored without phase separation by centrifugation (unlike in the case of osmolysis-type experiments), provided the substrate has low permeability and initial velocity conditions are adhered to. The rate of reaction of an occluded enzyme under initial velocity conditions, is,

$$Jr = KA_o/(1 + K/P) \tag{2}$$

where K is the kinetic constant of the enzyme and P the permeability of the membrane to the external substrate, A_o. Thus, osmotic titration of occluded-enzyme activities (enzyme osmometry) should yield activity profiles identical to osmolysis curves with reference to the break-points. We have shown that such indeed is the case with lactate dehydrogenase activity in synaptosomes [1]. Obviously, the break-point of osmometry cannot be less than that of a corresponding lysis experiment. However, it can be larger only if either the kinetic constant of the enzyme, or the permeability to the substrate (or both), for whatever reason, depends on the volume of the particle, i.e., osmotic stretch of the membrane *.

The interaction energy between lipids has a minimum at a certain head-group area, a_o, about which the energy varies parabolically, i.e., elastically [10]. The elastic compressibility modulus, K, is of the order of 10^2 dyne/cm for cell membranes [11] and is given by

$$\text{elastic energy} = 1/2\, K \frac{(a - a_o)^2}{a} \tag{3}$$

where a is the interfacial area.

The lateral pressure (external and internal) of the bilayer can be varied by osmotic imbalance

* While human erythrocytes expand 1.7-fold prior to bursting [8], break-point analysis based on the above Boyle-Van't Hoff relationship yields a value of 1.63. This 4.0% deviation of the osmotic pressure-volume relationship from ideal in erythrocytes is known to be due to osmotic dead space, elasticity of the membrane, etc. [9].

* Obviously, if the internal solutes leak out from the organelle rapidly during the assay itself, the requirement for external osmolarity for osmotic protection would also diminish as a function of time. This consideration becomes critical in assessing the sucrose content of the intermembranous space of mitochondria due to the leakiness of the outer membrane.

and the electrostatic repulsion of the head groups also contributes to the compressibility of the lipid moieties. If a_o corresponds to the interfacial area of the head groups at isotonicity (i.e., in the absence of a chemical potential difference for water across the membrane), the interfacial area, a, varies corresponding to the osmotic pressure difference. If a exceeds a_o, such that the elastic energy exceeds the intermolecular adhesive forces, the membranous structure bursts. Similarly, if a becomes smaller than a_o, the compressibility of the system should become non-linear below a critical value of a and the membrane may exhibit structural modifications beyond certain limits of compressibility.

Obviously, such considerations do not exclude non-spherical bilayers or bilayers with a significant protein component. * Thus, the volume curve of an organelle may be expected to exhibit two break-points – one corresponding to a critical osmotic pressure at which lysis begins (Π_L) and another corresponding to critical osmotic pressure beyond which the particle markedly deviates from the ideal osmotic relationship (Π_C) (Fig. 2). Thus, enzyme osmometry curves with break-points larger than the corresponding break-points of lysis curves would be compatible with the dependence of their activity on the volume of the particle and would constitute a special case of enzyme osmometry.

Since the internal osmotic pressure (which solely depends on osmotically active solutes within) is the decisive factor in determining the magnitude of the break-points of volume curves (in terms of external osmotic pressure), it should not matter whether or not osmolysis is homologous to osmometry. However, since enzyme osmometry is an indirect technique in the evaluation of solute content in specific compartments within the organelle, parallel evidence on the osmotic equivalence of compartmentalized enzymes is required. That is to say, the matrix enzymes and those facing the matrix side of the inner membrane should exhibit comparable osmometric curves. On the other hand, outer membrane enzymes may be distinctly different.

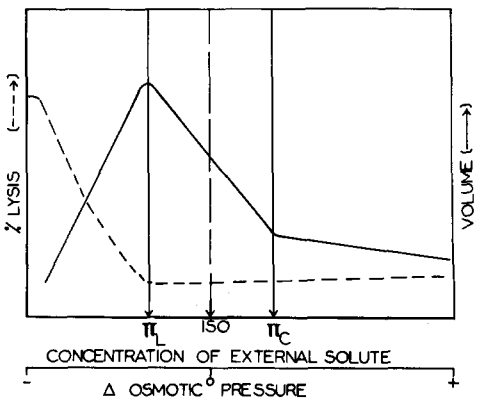

Fig. 2. Osmotic pressure-volume relationships in membranous particles: A schematic diagram. ISO, isotonic concentration of external solute. Volume change (———) and lysis (- - - - -) of a membranous particle are plotted vs. external solute concentrations (i.e., change in osmotic pressure across the membrane). By definition, the change in osmotic pressure across the membrane at isotonicity is zero. The volume curve of an organelle shows two break-points: Π_L is the critical osmotic pressure at which lysis of the particle begins and Π_C is the critical osmotic pressure beyond which the particle markedly deviates from the ideal osmotic relationship. Note: Π_L corresponds to the maximal volume of the particle as well as to the beginning of lysis.

Materials and Methods

Materials. Sucrose, Tris, NADH, oxaloacetic acid, cytochrome *c*, rotenone, L-glutamic acid, L-malic acid, ATP, ADP and Triton X-100 were obtained from Sigma. All other reagents were of analytical grade.

In all experiments, male albino rats from a locally inbred stock of Wistar/NIN strain (200–250 g) were used.

Preparation of mitochondria. Twice-washed mitochondria were prepared from livers of rats fasted overnight by conventional procedures [12]. The isolation medium was 0.25 M sucrose pre-

* The shape of the organelle or the convolutedness of its membranes need not be considered here, since neither the physical theory of osmosis (which is restricted primarily to the relative flow of solutes and solvent across the interface) nor the derived theory of enzyme osmometry (which is restricted to an interpretation of the break-points obtained) requires such geometric considerations. On the contrary, such considerations become crucial if one attempts to account for the entire osmometric curve quantitatively, or if quantitative comparisons are to be made between different organelles. Enzyme osmometry per se would be inadequate for such studies.

pared in freshly boiled and cooled water. All experiments were routinely completed within 5–6 h after the isolation of mitochondria.

Preparation of mitoplasts. Mitoplasts were prepared by digitonin solubilization of the outer membrane [13] of mitochondria suspended in 0.25 M sucrose. The specific activity of rotenone-insensitive NADH : cytochrome *c* oxidoreductase was less than 15% of that of intact mitochondria in these preparations.

Isolation of mitochondria in different concentrations of external sucrose. Twice-washed mitochondria, isolated in 0.25 M sucrose, were layered on 30 ml of specified concentrations of sucrose and were centrifuged at 25 000 × *g* for 30 min. The pellets were resuspended in the same concentrations of sucrose as those of the isolation media and are referred to accordingly (e.g., 0.25 M mitochondria, 1.0 M mitochondria, etc.). All centrifugation steps were carried out at a sample temperature of 2–4°C, unless specified otherwise.

Osmolysis of mitochondria. 1-ml aliquots of mitochondrial suspension (15 mg protein) were individually pelleted at 25 000 × *g* for 10 min and the supernatants were drained off. Each pellet was resuspended in 5 ml of 10 mM Tris-HCl, pH 7.4, containing varying concentrations of sucrose, in individual centrifuge tubes by gentle hand homogenization and incubated for 15 min at 0°C. The suspensions were recentrifuged at 25 000 × *g* for 30 min at 4°C. The supernatants were assayed for protein and various enzymes.

Enzyme assays. Table I lists the reaction conditions used for various enzyme assays in the study. Briefly, the buffer and substrate concentrations were reduced so as to minimize their influence on the osmolarity of the external medium, such that it could be selectively modified by incorporation of sucrose at various concentrations. In osmolysis experiments, the relevant assays were carried out without additional sucrose in the assay medium. For enzyme osmometry (the term being restricted to assays of particulate enzymes as a funtion of osmolarity of the medium), aliquots of mitochondria were intially preincubated in the cuvette at various concentrations of sucrose. Assays were then started by direct addition of the substrate to the cuvette after changes in turbidity were stabilized. Initial velocities were determined for specific activity calculations; reaction velocities were first order with regard to mitochondrial protein. Mitochondria were solubilized in 0.1% (v/v) Triton X-100 (final assay concentration) to determine the effect of sucrose on solubilized enzymes wherever relevant.

TABLE I

ASSAY CONDITIONS EMPLOYED FOR ENZYME OSMOMETRY

(a) Assays using Gilford spectrophotometer with automatic cuvette positioner and recorder or Beckman DB spectrophotometer. (b) Specific activity expressed as μmol cytochrome *c* reduced/min per mg protein. (c) Specific activity expressed as μmol NADH oxidized/min mg protein. (d) Specific activity expressed as μmol fumarate formed/min mg protein. (e) Specific activity expressed as μmol P_i released/h per mg protein. (f) Gilson oxygraph with Clark-type oxygen electrode. Specific activity expressed as ngatoms oxygen consumed/min per mg protein. Glutamate and malate neutralized directly with Tris base. (g) Activity expressed as $(dA/dt)_{max}$ (min^{-1}).

Enzyme system	Conditions
Rotenone-insensitive NADH : cytochrome *c* oxidoreductase EC 1.6.99.3) [a,b]	20 mM PO_4^{3-} (K^+), pH 7.4; 0.14 mM KCN; 42 μM NADH; 0.037 mM cytochrome $c_{(ox)}$; A_{550}; 37°C [14]
Sulfite : cytochrome *c* oxidoreductase (EC 1.8.3.1) [a,b]	15 mM Tris-HCl, pH 8.5; 0.3 mM Na_2SO_3; 0.4 mM KCN; 0.07 mM cytochrome $c_{(ox)}$; A_{550}; 37°C [15]
Malate dehydrogenase (L-malate : NAD^+ oxidoreductase EC 1.1.1.37) [a,c]	10 mM Tris-HCl, pH 7.4; 0.09 mM NADH; 0.25 mM Tris-oxaloacetate; A_{340}; 37°C [16]
Fumarase (L-malate hydro-lase, EC 4.2.1.2) [a,d]	33 mM Tris-malate, pH 7.4; A_{250}; 37°C [17]
ATPase (EC 3.6.1.3) [e]	50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 75 mM KCl; 5 mM ATP; ±0.2 mM 2,4-dinitrophenol; 30°C [18]
Polarigraphic measurement of oxygen comsumption [f]	10 mM PO_4^{3-} (Na^+); 10 mM Tris-HCl, pH 7.4; 10 mM glutamate; 10 mM malate; 0.2 mM EDTA; 270 nmol ADP, 3.3 mM $MgCl_2$; 30°C [19].
High-amplitude swelling [a,g]	10 mM Tris-HCl, pH 7.4; mitochondrial protein 0.2 – 0.5 mg/ml; A_{520}; 37°C [20]

Mitochondrial protein was assayed by the biuret method [21], in the presence of 0.33% (w/v) sodium deoxycholate. Protein released into supernatants in osmolysis experiments was assayed by the method of Lowry et al. [22]. Break-point analysis was carried out as discussed in Appendix A.

Results and Discussion

The experiments reported here are of the following categories. First, the break-point for lysis was established in mitochondria; second, the nonlinear osmotic pressure-volume relationship was explored; third, the break-points of various selectively compartmentalized mitochondrial marker enzymes were critically compared with the above; and fourth, the indices of oxidative phosphorylation were evaluated osmometrically in the light of the above experiments.

Osmolytic behaviour of mitochondria isolated in 0.25 M sucrose

Release of protein and specific marker enzymes, i.e., sulfite : cytochrome *c* oxidoreductase (intermembranous space marker [15]), malate dehydrogenase and fumarase (markers for matrix space [16,17]) from mitochondria suspended in bufferd sucrose solutions of varying tonicity, was measured to determine the external osmotic pressures at which the mitochondrial membranes burst. The presence of two membranes with variable elasticity and/or variable reflection coefficients for the same external solute could, in principle, result in differential osmotic requirements for bursting. The data in Fig. 3 show that the release of these markers begins between 0.091 and 0.126 M external sucrose. The technique was not sensitive to differentiate between the differential osmotic requirements for bursting of inner and outer membranes, if any. However, it is clear that approx. 0.1 M external sucrose contributes to an osmotic pressure difference at which maximal expansion of mitochondria occurs prior to bursting (Π_L, the critical osmotic pressure of approx. 0.1 M external sucrose corresponding to the breakpoint of lysis). At hypertonic concentrations of sucrose larger than 0.4 M, release of these marker enzymes and protein was uniformly seen.

Based on elastic energy considerations (cf. Eqn. 3), structural disorganization of a lipid bilayer beyond critical limits of compressibility could be suggested as a possible mechanism for hypertonic disruption of mitochondria. The phenomenon of hypertonic disruption appears to be present in myelin particles isolated from primate cortical white matter [23] but was not evident in other organelles such as synaptosomes and lysosomes (Sitaramam, V., unpublished observations). Further, the phenomenon of hypertonic disruption does not occur in isopycnic density gradient centrifugation in sucrose, as its occurrence would result in bi- or multiphasic sedimentation profiles of various marker enzymes and protein [3]. Since the break-points of all the enzyme osmometric curves in 0.25 M mitochondria are less than 0.4 M (vide infra), the phenomenon of hypertonic disruption does not vitiate the experimental logic in the current studies. However, the underlying mechanism remains to be identified unequivocally.

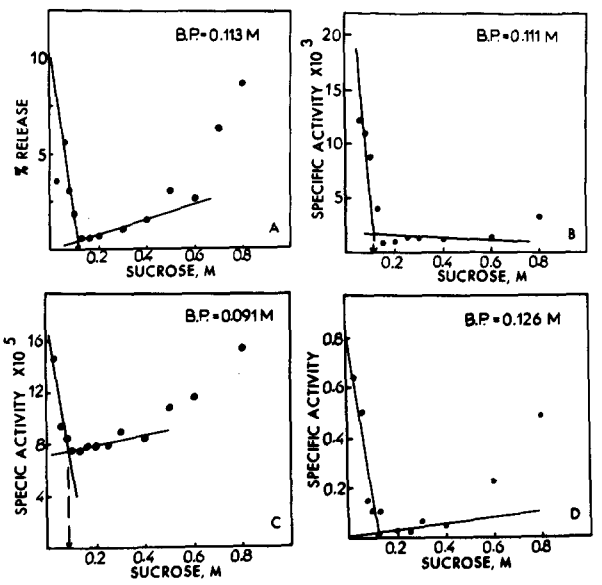

Fig. 3. Osmolytic behaviour of mitochondria isolated in 0.25 M sucrose. Release of protein and marker enzymes into the medium is plotted against ambient sucrose concentration in the medium. (A) Protein, (B) sulfite : cytochrome *c* oxidoreductase, (C) fumarase and (D) malate dehydrogenase. Specific activities were expressed per mg protein in the incubation medium. Break-point (B.P.) analysis was carried out as discussed in Appendix A.

Osmometric behaviour of high-amplitude swelling

Since enzyme osmometry is an indirect technique in the evaluation of osmotic pressure-volume relationships of organelles, it is necessary to monitor volume changes as a function of external osmotic pressure. Volume evaluation by light microscopy is highly unsatisfactory considering the size and shape of rat liver mitochondria (1–2 μm in diameter and oval in shape) and electron microscopy would involve fixation artefacts. Mitocrit determinations involve centrifugation and therefore would directly vitiate the current experimental logic. On the other hand, changes in light scattering have been successfully employed to evaluate volume changes [24]. It can be shown that the rate of change of absorbance should correlate with volume (vide, Appendix B)

Mitochondria exhibit the phenomenon of high-amplitude swelling as defined above (see Introduction) with the following features [7]:

(i) A 2–3-fold increase in volume occurs due to spontaneous swelling in buffered isotonic solutions of mitochondria (less than 1.0 mg protein/ml).

(ii) The phenomenon requires oxidation of endogenous substrates, it being inhibited by rotenone.

(iii) The time course of swelling is sigmoidal: the initial time lag (which possibly reflects depletion of ATP within mitochondria) varies directly with protein concentration, external osmolarity and a variety of agents including electrolytes (cf. Ref. 20).

(iv) The phenomenon represents irreversible damage to mitochondria which become uncoupled and ATP cannot contract such mitochondria.

(v) However, these organelles are osmotically intact in that, fumarase osmometry exhibits no significant shift in break-point (vide infra).

Irrespective of the mechanism of high-amplitude swelling, since it is swelling, it could be modified by external osmolarity. Thus, the break-point analysis of maximal rate of change of absorbance under these conditions should show: (i) pronounced osmotic behaviour, (ii) a break-point larger than that of lysis, and (iii) a shift in the break-point of a magnitude corresponding to that of sucrose in the isolation medium (owing to gravity-mediated entry of sucrose into mitochondria). The results in Fig. 4 confirm these predictions completely. In 0.25 M mitochondria (washed three

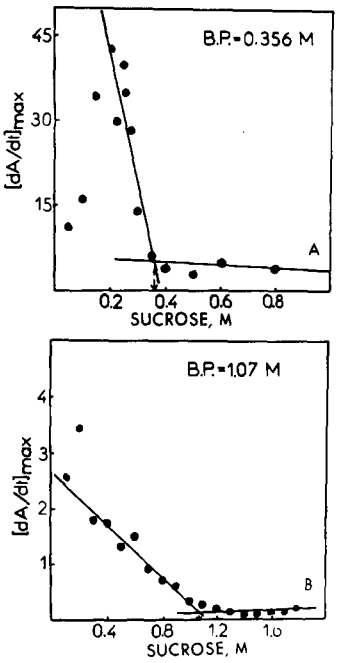

Fig. 4. Osmometric analysis of high-amplitude swelling in mitochondria. (A) 0.25 M mitochondria, (B) 1.3 M mitochondria. Maximal rate of change in absorbance due to spontaneous swelling of mitochondria at 520 nm ($(dA/dt)_{max}$) is plotted as a function of ambient sucrose concentration in the assay medium. Break-point (B.P.) analysis was carried out as in the case of enzyme osmometry. The break-points were essentially similar at 280 and 340 nm as well and were independent of protein concentrations (data not shown).

times in 0.25 M sucrose) two break-points could be discerned, one around 0.1 M sucrose and another around 0.36 M sucrose (Fig. 4A). The latter break-point shifted to 1.07 M in 1.3 M mitochondria (Fig. 4B). Thus, these data taken together with those on osmolysis (Fig. 3) show that ideal osmotic behaviour occurs between 0.1 M sucrose (Π_L, critical external osmotic pressure at which lysis begins) and 0.36 M external sucrose (Π_C, critical external osmotic pressure at which the osmotic pressure-volume relationship deviates acutely from ideal). It should be cautioned, however, that the coefficient relating volume changes with rate of change of absorbance would vary monotonically with the external solute concentration and is not an invariant. Thus, only the general shape of the volume curve and the

break-points would be the same as those of the turbidimetric profiles. The turbidimetric profiles do not indicate swelling of mitochondria corresponding to the osmotic range of hypertonic disruption of 0.25 M mitochondria, suggesting that structural disorganization at the limits of compressibility is not accompanied by swelling.

The experiments thus far have defined the boundary conditions of external osmotic pressure, Π_L and Π_C, within which mitochondria behave as ideal osmometers. Various mitochondrial enzyme systems and indices of oxidative phosphorylation were evaluated systematically in relation to these boundary conditions. For instance, one can predict that the latent ATPase activity in mitochondria, State 3 respiration, respiratory control ratio and ADP/O ratio should exhibit marked break-points corresponding to Π_L, as osmotic disruption of mitochondria would lead to loss of coupling.

Enzyme osmometry of marker enzymes of mitochondria

The critical evidence for the entry of sucrose into the matrix compartment during centrifugation was based on shifts in the break-points of fumarase and malate dehydrogenase osmometric curves (Fig. 5A–D) because: (i) these soluble marker enzymes are osmotically equivalent to lactate dehydrogenase in synaptosomes [5]; (ii) these enzymes, on solubilization of mitochondria with Triton X-100, showed no discontinuity, but only a monotonic profile of inhibition by sucrose (Fig. 5E, similar data for malate dehydrogenase not shown); (iii) the presence of outer membrane does not vitiate the experimental logic, since mitoplasts exhibited comparable break-points (Fig. 5F); and (iv) the presence of phosphate in the assay medium is known to enhance permeability to dicarboxylates [25] and therefore no osmometric behaviour was observable in the presence of phosphate buffer due to a poor signal-to-noise ratio (represented by m_1/m_2, the individual slopes of the regression lines) (data not shown).

Break-points of these enzymes were closer to Π_C and were twice as large as Π_L. Since these are soluble enzymes, the kinetic constans of the enzymes are not likely to be affected and therefore the data would argue for a direct influence of the

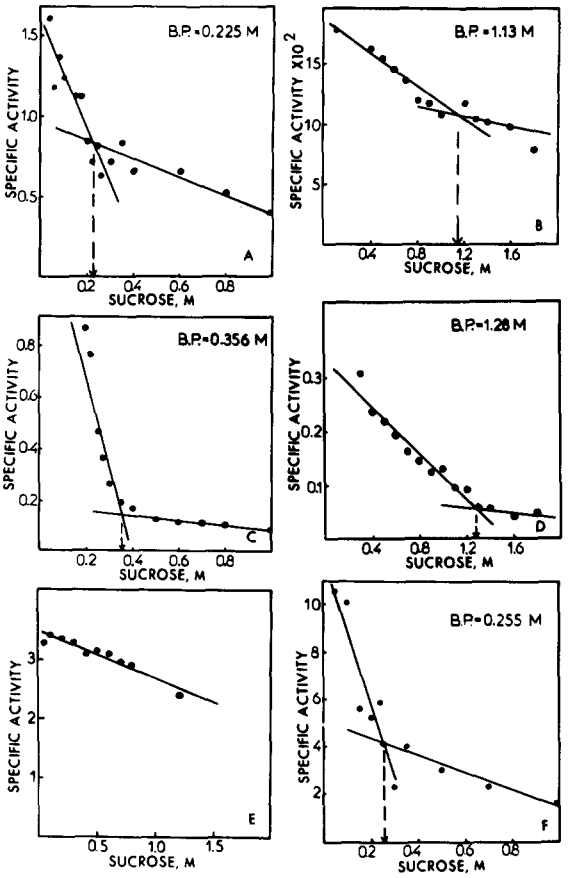

Fig. 5. Enzyme osmometry of the soluble matrix enzymes, fumarase and malate dehydrogenase. Enzyme osmometry of the matrix enzymes was carried out as described in Materials and Methods, in intact mitochondria isolated in 0.25 and 1.3 M sucrose, as well as in mitoplasts isolated in 0.25 M sucrose. Specific activities are plotted as a function of sucrose concentration in the assay medium. Break-point (B.P.) analysis was carried out as discussed in Appendix A. Malate dehydrogenase in 0.25 M mitochondria (A) and 1.3 M mitochondria (B); fumarase in 0.25 M mitochondria (C) and 1.3 M mitochondria (D); fumarase in Triton X-100 solubilized mitochondria (E) (comparable data for malate dehydrogenase not shown) and fumarase in 0.25 M mitoplasts (F).

osmotic stretch of the inner membrane on the activity of the dicarboxylate transporter. This would account for the lack of homology between the lysis curves and osmometry curves for these matrix enzymes, unlike lactate dehydrogenase in synaptosomes. The dependence of dicarboxylate-transporter activity on the osmotic stretch of the

membrane (since the latter would affect the lateral pressure within the membrane which in turn could effect the boundary lipid of the hydrophobic protein [26]) is not surprising, since the K^+-H^+ exchanger in mitochondria [27] and NAD(P)H oxidase of plasma membrane [28] are also known to be osmotically sensitive.

Osmometry of the intermembranous space marker, sulfite : cytochrome *c* oxidoreductase exhibited: (i) a break-point larger than Π_L in 0.25 M mitochondria, similar to that of matrix enzymes (Fig. 6A) and a comparatively smaller increase in the break-point in 1.3 M mitochondria (Fig. 6B); (ii) a monotonic profile of inhibition of the solubilized enzyme with sucrose (Fig. 6C). The absence of cyanide in the osmometric assays of sulfite : cytochrome *c* oxidoreductase did not affect the break-points (data not shown). Break-point analysis would underestimate the internal solute content of a compartment if the osmotically active internal solute were to leak out during the enzyme osmometric assays. The outer membrane is known to be leaky to small molecular weight substances [29], and therefore, it would be logical to expect the lower break-point obtained for sulfite : cytochrome *c* oxidoreductase in 1.3 M mitochondria.

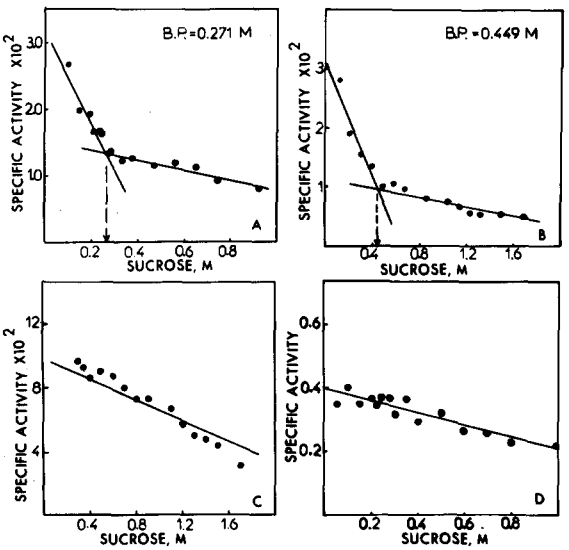

Fig. 6. Enzyme osmometry of sulfite : cytochrome *c* oxidoreductase, an intermembranous enzyme and rotenone-insensitive NADH : cytochrome *c* oxidoreductase, an outer membrane enzyme. Sulfite : cytochrome *c* oxidoreductase activity in 0.25 M mitochondria (A) and 1.3 M mitochondria (B). Effect of sucrose on sulfite : cytochrome *c* oxidoreductase activity in solubilized mitochondria (C); rotenone-insensitive NADH : cytochrome *c* oxidoreductase activity in 0.25 M mitochondria (D). Enzyme osmometry and break-point analysis were carried out as discussed in Appendix A. B.P., break-point

Since the break-point for osmometry in 0.25 M mitochondria is larger than that of lysis, it may be argued that the permeability to cytochrome *c* is also sensitive to osmotic stretch of the outer membrane, an argument comparable to that in relation to the dicarboxylate transporter and matrix enzymes. The break-point of osmolysis curves for matrix enzymes can only be smaller than or equal to those of intermembranous enzymes, since matrix enzymes cannot leak out without the disruption of the outer membrane. However, a leaky outer membrane that loses sucrose from the intermembranous space would result in a break-point lower than that expected in both osmometric and osmolytic experiments. Thus, the true break-point for lysis of outer membranes may be larger than the observed approx. 0.1 M external sucrose and therefore it would be hazardous to speculate upon the osmotic dependence of the permeability for cytochrome *c* across the outer membrane based on our data.

In contrast, the outer membrane marker, rotenone-insensitive NADH : cytochrome *c* oxidoreductase, exhibited only a monotonic profile of inhibition in intact mitochondria (Fig. 6D). Monoamine oxidase using benzylamine as substrate [30] also showed only a monotonic profile of inhibition with sucrose (data not shown). These results are commensurate with the prediction that the osmometric behaviour varies with the compartmentalization of marker enzymes.

The observed lack of osmotic behaviour of the outer membrane marker, rotenone-insensitive NADH : cytochrome *c* oxidoreductase, suggests that (a) its kinetic constant does not vary significantly with the osmotic stretch of the outer membrane and (b) its active site faces the exterior, since cytochrome *c* has no apparent permeability barrier for this activity. The osmotic curves obtained with the intermembranous marker enzyme, which utilizes cytochrome *c* as an electron acceptor, indicate the role of the outer membrane as a relative permeability barrier to cytochrome *c* (cf. Ref. 31).

Since the invalidation of the sucrose-space hy-

pothesis would require the demonstration of osmotically active sucrose in the matrix compartment and, since an explicit homology between osmolysis and osmometry was lacking, the conformity of the predicted osmometric behaviour of mitochondrial marker enzymes of various compartments with these experimental data reaffirms our earlier conclusions. On the other hand, a rigorous comparison between osmolysis and osmometry of mitochondrial enzyme systems appears to offer a direct method to study the influence of osmotic stretch of the membranes on the activity of membrane-bound enzymes and carrier proteins.

Enzyme osmometry of indices of oxidative phosphorylation

The oligomycin-sensitive mitochondrial ATPase should be osmotically equivalent to matrix enzymes, in that the active site faces the matrix side of the inner membrane [32]. Osmometric analysis of ATPase in the presence and absence of the classical uncoupler, 2,4-dinitrophenol in 0.25 M (Fig. 7A and B) and 1.0 M mitochondria (Fig. 7D)

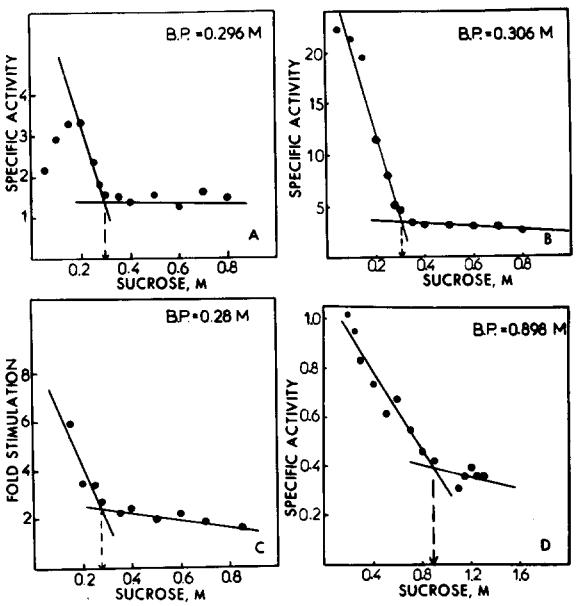

Fig. 7. Enzyme osmometry of mitochondrial ATPase: (A) Latent ATPase, (B) 2.4-dinitrophenol-stimulated ATPase and (C) -fold stimulation of ATPase activity by 0.2 mM 2,4-dinitrophenol in 0.25 M mitochondria. (D) 2.4-Dinitrophenol-stimulated activity in 1.0 M mitochondria. Enzyme osmometry and break-point (B.P.) analysis were carried out as discussed in Appendix A.

confirmed this prediction. The beak-points of latent and 2,4-dinitrophenol-stimulated ATPases were comparable and were larger than Π_L, indicating that the kinetic constant of ATPase per se or that of ATP/ADP translocase (or both) varies in response to the osmotic stretch of the inner membrane. However, unlike in the case of soluble matrix enzymes, it is not possible to distinguish between these alternatives without direct measurements of the nucleotide uptake. The osmometric profile of latent ATPase was comparable to that of high-amplitude swelling in that another break-point corresponding to Π_L was distinctly seen, owing (presumably) to the loss of Mg^{2+} due to lysis of mitochondria.

The osmotic behaviour of ATPase activity was confirmed by a shift in its break-point corresponding in magnitude to the sucrose in the isolation medium (Fig. 7B). It is particularly interesting to note that the extent of stimulation of ATPase activity by 2,4-dinitrophenol (which is considered to be a measure of coupling) also showed a distinct osmometric profile (Fig. 7C). The data would argue for the dependence of coupling directly on the osmotic stretch of the membrane. Therefore, the studies were extended to a systematic evaluation of the osmometric profiles of respiratory states described by Chance et al. [33].

Osmometric profiles of high-amplitude swelling (which occurs due to oxidation of endogenous substrates) are comparable to State 1 respiration [24]. The respiratory States 2 and 4 (i.e., in the absence of exogenous ADP and on depletion of exogenous ADP, respectively) showed break-points corresponding to the onset of lysis, Π_L, indicating that the permeability of exogenous substrates is the rate-limiting step (Fig. 8). The osmometric profile of State 3 respiration showed a consistently higher respiratory rate compared to either State 2 or 4 and the profile was similar to those of turbidimetry and latent ATPase activities, i.e., a break-point corresponding to Π_L was seen and yet another break-point near 0.35 M sucrose was consistently discernible in several experiments (data not shown). Polarigraphic measurements beyond 0.4 M external sucrose resulted in very noisy data and therefore further studies are now in progress using manometric methods.

The respiratory control and ADP/O ratios ex-

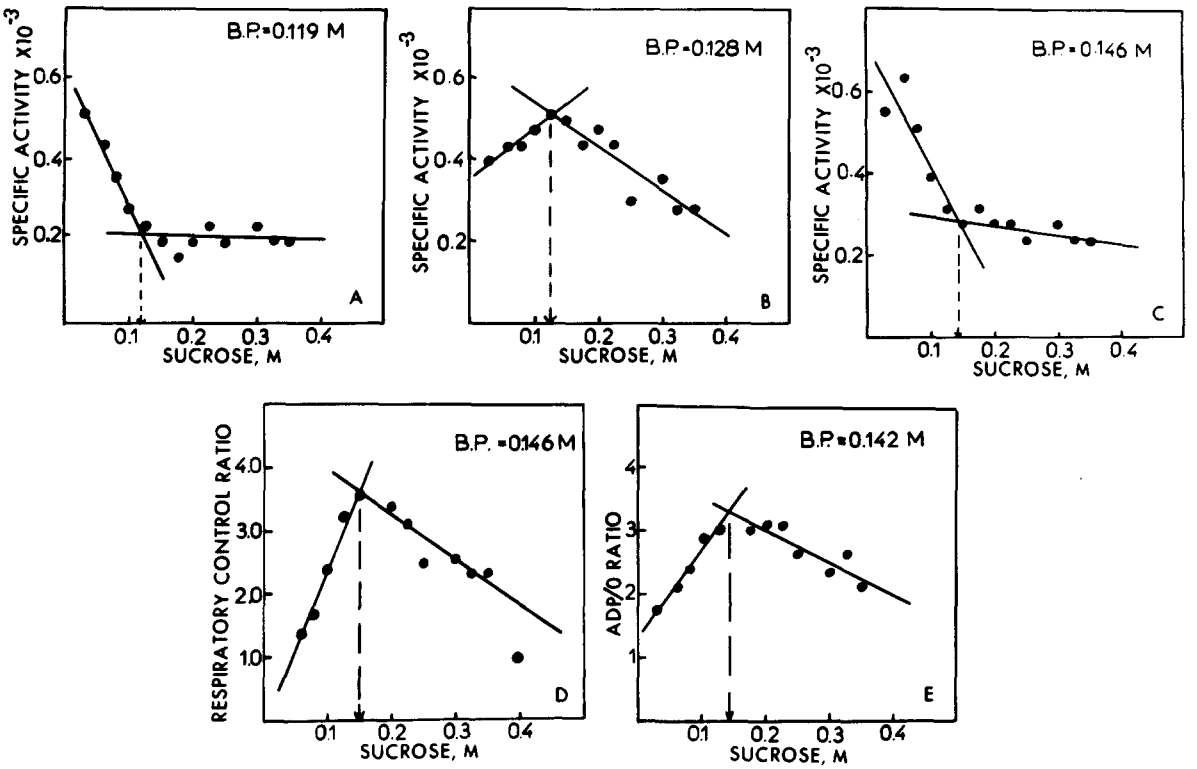

Fig. 8. Osmometric analysis of mitochondrial respiratory states: Rate of oxygen consumption/min per mg mitochondrial protein was determined polarigraphically in various respiratory states in 0.25 M mitochondria and plotted as a function of ambient sucrose in the assay medium. (A) State 2, (B) State 3, (C) State 4, (D) respiratory control ratio (State 3/State 4) and (E) ADP/O ratio (nmol ADP/ngatom oxygen consumed). Break-point (B.P.) analysis was carried out as discussed in Appendix A.

hibited osmometric profiles, as well as a definite negative slope in the Π_L–Π_C range. These findings were consistent with the data on osmometric profiles of the extent of stimulation of latent ATPase by 2,4-dinitriphenol. If the component enzyme systems exhibit a dependence on the osmotic stretch (i.e., lateral pressure within) of the inner membrane, it is to be expected that the derived indices of oxidative phosphorylation also depend on the physical state of the inner membrane. Thus, it is perhaps proper to consider coupling in mitochondria as a true variable, depending on the 'physical' state of the inner membrane.

Effect of temperature on gravity-mediated entry of sucrose in mitochondria

It was reported earlier from our laboratory that entry of sucrose into synaptosomes during centrifugation was inhibited at higher temperatures, suggesting a negative temperature coefficient [1]. In 1.3 M mitochondria isolated at 15 to 23°C, the break-points of fumarase and malate dehydrogenase, respectively, did not shift, compared to those isolated at 2–4°C (Fig. 9, cf. Fig. 5A–D). Thus, the gravity-mediated entry of sucrose in mitochondria is mechanistically similar to that in synaptosomes.

In conclusion, the relevance of our experimental observations to some of the existing controversies in studies related to subcellular organelles in general, and mitochondria in particular, may be highlighted.

(1) The phenomenon of gravity-mediated changes in the permeability characteristics of biological membranes renders quantitation of compartments using small molecular weight markers by centrifugal methods highly suspect. Specifically, [^{14}C]sucrose is currently recommended to

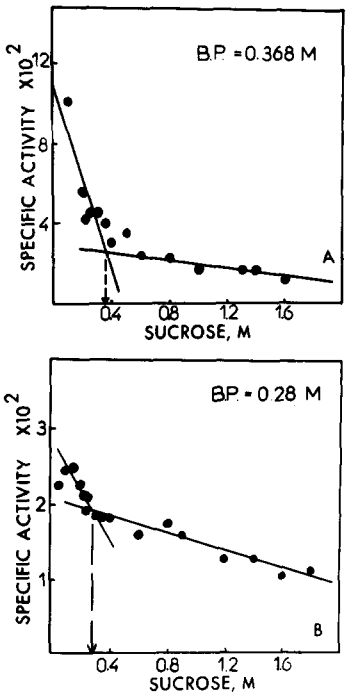

Fig. 9. Effect of temperature of centrifugation on the break-points of the matrix enzymes, fumarase and malate dehydrogenase. Mitochondria isolated in 0.25 M sucrose were layered on 30 ml of 1.3 M sucrose at specified temperatures and centrifuged at 25000 × *g* for 30 min. The pellets were resuspended in 1.3 M sucrose and enzyme osmometric studies were carried out as described in Materials and Methods. (A) Malate dehydrogenase activity in 1.3 M mitochondria isolated at 23°C; (B) fumarase activity in 1.3 M mitochondria isolated at 15°C. Break-points (B.P.) analysis was carried out as discussed in Appendix A.

subtract the intermembranous space from the total mitochondrial space in assessing the membrane potential changes consequent to oxidative phosphorylation [34,35]. Since sucrose equilibrates during centrifugation with the matrix space as well, estimates of the latter could be grossly in error. The use of centrifugal methods in uptake studies in general, irrespective of the organelle, should be viewed with caution.

(2) 'Polyol' inhibition of mitochondrial respiration was postulated earlier [7]. This phenomenon was observed with several sugars including sucrose at comparable concentrations and can now be accounted for in terms of a primary osmometric behaviour of mitochondrial enzyme systems.

(3) The classical theory of isopycnic density gradient centrifugation [3] needs to be modified as two of its basic assumptions, viz., subcellular organelles have an ideal osmotic pressure-volume relationship in the entire range of osmolarity of the continuous density gradients and the internal solute content is invariant under centrifugal conditions, are no longer tenable. While sucrose permeates inwards during centrifugation, other small molecular weight substances also could permeate outwards (cf. Refs. 6 and 36).

(4) The essential difference between our conclusions and those of Wattiaux-De Coninck et al. [37] is with regard to the relative influence of hydrostatic pressure and gravitational field on mitochondrial integrity. In the experiments reported in this paper, the maximal hydrostatic pressure encountered by the particles varied from 190 to 290 atm, depending on the rotor used (fixed-angle or swing-out).

The minimal hydrostatic pressure requirement for disruption of rat liver mitochondria was found to be approx. 900 atm [37]. Since equilibration of sucrose was demonstrated even at 8500 × *g* [5] (which barely corresponds to a hydrostatic pressure of 50 atm under these experimental conditions), it is unlikely that equilibration of sucrose could be effected by hydrostatic pressure-mediated phase transitions in membrane lipids. However, a critical reevaluation of the hydrostatic pressure hypothesis of Wattiaux-De Coninck et al. [37] would require a detailed analysis of sedimentation profiles of reference marker enzymes under varied conditions of temperature, hydrostatic pressure and gravitational fields and interpretation of the data in the light of the limitations of the classical theory of isopycnic density gradient centrifugation. These studies are now in progress.

Isotonic requirements of subcellular organelles cannot be assumed but need to be actively determined by the osmometric technique. Thus, optimal fixation conditions for electron microscopy of subcellular organelles need to be re-examined in the interpretation of the structural integrity of subcellular organelles.

(5) The parabolic relationship between elastic energy and the head-group area appears to be a particularly useful abstraction in understanding the osmotic phenomena. Osmotic stretch beyond

the limits of intermolecular adhesive forces of the membrane would result in lysis, a catastrophic phenomenon. Interestingly, an increase in elastic energy beyond a critical limit of compressibility could also be expected to lead to structural disorganization as evidence by hypertonic disruption of mitochondria. We have observed pronounced activation of 2',3'-cyclic nucleotide 3'-phosphohydrolase in myelosomes in the hypertonic range [23].

(6) The possible dependence of the kinetic constants of membrane-bound enzymes and carrier proteins on the osmotic stretch of the membranes offers, if one may speculate, a method to classify membrane proteins as those that respond to osmotic stretch and those that do not. Irrespective of the mechanism (e.g., exposure of buried active sites, allosteric transitions of membrane proteins consequent to changes in lateral pressure, altered lipid-protein interactions, etc.), such a phenomenon could imply an important role of mitochondrial volume and its regulation in relation to the stoicheiometry of ATP production.

(7) Finally, we have already emphasized [1] that the phenomenon of gravity-mediated entry of sucrose is not compatible with the homogeneous membrane assumption inherent to the membrane model of Singer and Nicolson [38] and could argue for heterogeneous membrane models [39,40]. Nor is gravitational field unique in rendering biological membranes permeable to sucrose. For instance, electrical fields were shown to lead to permeation of sucrose across macroliposomes, though not microliposomes, presumably owing to the differences in curvature [41]. We have thus far not succeeded in demonstrating entry of sucrose in microsomes and submitochondrial particles by the osmometric technique.

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Appendix A

Evaluation of break-points in osmometry curves:

The problem in break-point analysis is one of an objective assessment of individual slopes. Given the slopes ($Y_1 = m_1x + c_1$ and $Y_2 = m_2x + c_2$), the break-point may be estimated graphically or algebraically (break-point = $(c_2 - c_1)/(m_1 - m_2)$. The majority of the curves fall in the category of only two slopes. In such cases one can arrive at the break-point by two different methods.

(1) Start at either end of the osmometric profile and compute the coefficient of correlation of the least-squares linear-regression line by progressive incorporation of the data points. The value of the coefficient of correlation improves and begins to deteriorate across the break-point.

(2) Alternatively, the individual slopes may be visually determined and relevant data points may be fitted to obtain individual slopes.

In actual practice, the break-points obtained by visual fitting are indistinguishable from those obtained by iterative-regression fits. A number of precautions, however, were undertaken experimentally to ensure objectivity in the assessment of break-points, as indicated below.

(i) Each break-point was assessed in two to three independent experiments.

(ii) Break-points were essentially similar when plotted in log-log and semi-log graphs as well. The osmometric profiles were non-linear as no single polynominal (also that of a high order) would fit any or all the osmometric curves.

(iii) In all cases, a pilot experiment was conducted to determine the discontinuities and external solute concentrations were chosen to yield a significant number of data points for each slope.

(iv) Since the slope indicates the heterogeneity of the particles, repeated centrifugations in fixed concentrations of sucrose was seen to sharpen the discontinuity without a shift in the break-point.

(v) As a further precaution, most of the experiments were repeated using a more homogeneous fraction in terms of sedimentation of mitochondria. The mitochondrial fraction reisolated between 2000

and $4000 \times g$ yielded sharper discontinuities of the osmometric profiles than the routinely isolated mitochondria (at $8500 \times g$) though the break-points were comparable (data not shown).

(vi) All interpretations pertaining to break-point analysis were restricted to large changes in the break-point and further, only when a number of enzyme systems were compared to be mutually consistent with the predictions.

(vii) In all cases the individual slopes were significant ($P < 0.01$).

(viii) Since the relevant parameter on the ordinate is osmotic pressure, not concentration (which may not be related in a linear manner in the concentration ranges tested), break-point analysis was also carried out by plotting activities against freezing point depression of the external solute. The significance of individual slopes and the magnitude of shifts in break-points essentially remained the same.

Tedeschi [24], in his pioneering work on the osmotic behaviour of mitochondria, addressed himself primarily to a parallellism between osmotic pressure-volume relationships of mitochondria and the ideal Boyle-Van't Hoff relationship. He specifically cautioned that his methodology lacks precision in rigorous quantitative interpretation. The break-point analysis as outlined here offers a simple experimental strategy to evaluate boundary conditions, within which alone the nearly ideal osmotic pressure-volume relationship exists. In fact, an extension of the technique of enzyme osmometry to any other organelle should, we recommend, take all the experimental precautions indicated here to ensure an objective assessment.

Appendix B

A note on assessment of mitochondrial volume by turbidimetry

Light-scattering studies in rat liver mitochondria have shown the validity of the Jobst approximation [42]:

$$A = \frac{9}{2 \cdot 2.3} \cdot \left(\frac{dn/dc}{n_o} \right)^2 \cdot \frac{q^2 v}{r^2 \lambda'^2} \qquad \text{(A-1)}$$

where A is the absorbance, dn/dc the specific refractive index increment, n_o the index of refraction of medium, q the anhydrous mass of the materials, v the volume illuminated, r the radius of the spherical particle, and $\lambda' = \lambda/n_o$, i.e., the wavelength of light in the medium. In the turbidimetry of high-amplitude swelling, the maximal rate of decrease in absorbance was obtained from the time course of swelling at each sucrose concentration in the assay medium. While A varies with $V^{-2/3}$ (V, volume of the particle), in the time domain, ceteris paribus:

$$dA/dt = -2K/r^3 = -K'/V \qquad \text{(A-2)}$$

where K and K' represent appropriate constants related to the parameters listed above Eqn. A-1).

In the earlier studies on mitochondrial swelling, absorbance was related to volume and concentration of particles directly, though it was explicitly recognized that the variable refractive index of the medium would significantly affect the turbidimetric profiles [24]. The rate of change of turbidity, wherever measurable, is independent of these considerations and yields a sensitive measure of volume changes.

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