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# Gravitational field enhances permeability of biological membranes to sucrose: An experimental refutation of sucrose-space hypothesis

(synaptosomes/mitochondria/peroxisomes/enzyme osmometry/rippled membrane)

V. SITARAMAM AND M. K. JANARDANA SARMA

National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania P. O., Hyderabad, A. P., India PIN-500 007

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**ABSTRACT** Isotonic conditions for the integrity of subcellular organelles are shown to be remarkably influenced by the concentration of sucrose present during their isolation by centrifugation. Using the technique of enzyme osmometry, we show that the content of sucrose in synaptosomes reflects nearly total equilibration across the membrane during centrifugation, due to altered permeability of membranes. Presence of sucrose in the matrix space of mitochondria, as demonstrated by enzyme osmometry of matrix enzymes, indicates that the sucrose-space hypothesis is invalid.

Synaptosomes and mitochondria are known to behave as osmometers due to the relatively impermeable nature of their limiting membranes to a variety of ions and nonelectrolytes (1–4). Entry of sucrose from the medium into subcellular organelles isolated by centrifugal methods remains an unsolved riddle in biochemical methodology (5, 6). The two-compartment or the sucrose-space hypothesis holds that the presence of sucrose in an organelle indicates the existence of a subspace, whose limiting membrane alone is permeable to sucrose (5, 7). It is difficult to visualize such a subspace in organelles with a single limiting membrane, such as synaptosomes, lysosomes, and peroxisomes. Even in mitochondria, the evidence is far from conclusive that sucrose is limited to the intermembranous space alone, which is generally believed. Verification of the sucrose-space hypothesis requires both the demonstration of sucrose in the matrix space of mitochondria and an explanation for the altered permeability of membranes during centrifugation, the latter being a necessary condition.

Membranous structures swell on exposure to hypotonic media and release their contents on bursting (e.g., erythrocyte osmolysis). Release of latent marker enzymes into the medium can be directly monitored by measuring their activity, provided the substrate is relatively impermeable to the membrane (1, 8). The resulting osmotic curves of the activity of latent enzymes are strikingly discontinuous (as opposed to those of soluble, free enzymes) and yield information, under appropriate conditions, regarding the total internal solute content in the same compartment (Fig. 1). We refer to this approach as "enzyme osmometry." The theoretical formalism of enzyme osmometry in relation to sucrose-space hypothesis will be published elsewhere.

We report here the validity of the technique of enzyme osmometry with lactate dehydrogenase (LDHase; L-lactate: $NAD^+$ oxidoreductase, EC 1.1.1.27) as a marker enzyme in synaptosomes, and we establish that breakpoints in the enzyme activity profiles reflect the internal sucrose content. By an extension of enzyme osmometry to mitochondrial matrix enzymes, sucrose is shown to enter the matrix space during centrifugation.

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## MATERIALS AND METHODS

**Materials.** Sucrose, sodium pyruvate, NADH, Tris, L-malic acid, oxalacetic acid, and Triton X-100 were obtained from Sigma, hydrogen peroxide from Merck, [$U$-$^{14}C$]sucrose from Bhabha Atomic Research Centre, Bombay, India, and Millipore filters (0.45-$\mu$m pore size) from Millipore. All other reagents were of analytical grade. In all experiments, the locally inbred stock of Wistar/NIN strain of male albino rats (150–250 g) were used.

**Isolation of Synaptosomes.** Synaptosomes from rat neocortex were isolated by the method of de Robertis *et al.* (9). The purpose of these studies being the correlation of sucrose content in subcellular organelles with that of the isolation medium, the synaptosomal fractions C and D were subjected to a centrifugation/lysis/centrifugation sequence in varying concentrations of ambient sucrose as follows. Aliquots of fractions C (isolated at 1.0–1.2 M sucrose interface) and D (at 1.2–1.4 M sucrose interface) were diluted slowly with cold glass-distilled water to 0.32 M sucrose with continuous mixing; these were then washed twice in 0.32 M sucrose by centrifugation, as in the case of the crude mitochondrial fraction, $P_2$; aliquots of the resulting fractions $C_L$ and $D_L$ were again centrifuged on seperate discontinuous sucrose density gradients to obtain fractions CII and DII, corresponding to the original C and D fractions, respectively.

**Isolation of Mitochondria.** Twice-washed mitochondria, isolated from rat liver in 0.25 M sucrose by the usual procedure (10), were layered on 30 ml each of 0.25 M, 1.0 M, and 1.3 M sucrose solutions and sedimented at 22,000 × $g$ for 30 min. The pellets were resuspended in corresponding sucrose solutions.

All centrifugation steps were carried out at 4–6°C (sample temperature) unless specified otherwise and were used within 3 hr of preparation.

**Enzyme Assays.** Assays generally were carried out at 37°C in a 3-ml vol, were started by the addition of the substrate, and utilized a Gilford-250 spectrophotometer with automatic cuvette positioner and recorder. Specific activities were expressed as $\mu$mol of substrate consumed per min per mg of protein. Total activity was determined in the presence of 1% Triton X-100. Synaptosomal protein was assayed by the method of Lowry (11), and mitochondrial protein was determined by the biuret method (12) in the presence of 0.33% sodium deoxycholate, with bovine serum albumin as a standard.

LDHase was assayed by the method of Whittaker and Barker (13) in 50 mM Tris·HCl, pH 7.4/0.36 mM sodium pyruvate/0.1 mM NADH. Catalase (hydrogen-peroxidide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) was assayed in the presence of 20 mM Tris·HCl, pH 7.0/12 mM hydrogen peroxide (14). Because of large turbidity changes under varied osmotic con-

Abbreviations: LDHase, lactate dehydrogenase; MDHase, malate dehydrogenase.

ditions, which could not readily be minimized, fumarase (L-malate hydro-lyase, EC 4.2.1.2) was assayed in a 1.5-ml vol by a modification of the method of Racker (15) in 33 mM malate (pH 7.4) neutralized directly with Tris base. Mitochondria were incubated with substrate for 60 min, the reaction was terminated with 4 M NaOH, and the absorbance was measured at 250 nm after dilution to 0.25 M NaOH with distilled water. Malate dehydrogenase (MDHase; L-malate:$NAD^+$ oxidoreductase, EC 1.1.1.37) was assayed in 90 $\mu$M NADH/250 $\mu$M oxalacetate/10 mM Tris·HCl, pH 7.4 (16).

**Estimation of Sucrose in Synaptosomes.** Sucrose was estimated by the method of Kulka (17). Aliquots of each synaptosomal fraction ($\approx$150 $\mu$g of protein) were suspended for 2 min in 3.0 ml of ice-cold solutions of NaCl. The salt concentrations used were experimentally determined to be nonlysing (i.e., 0.4 M for $P_2$, $C_L$, and $D_L$; 1.0 M for C and CII; and 1.4 M for D and DII). Identical aliquots of each of the fractions were also suspended in ice-cold 10 mM NaCl, which caused lysis in all of the fractions. The fractions were rapidly washed five times under vacuum on Millipore filters with 10 ml of corresponding NaCl solutions. Efficacy of washes in the removal of external sucrose was determined with [U-$^{14}$C]sucrose. The radioactive sucrose that nonspecifically adsorbed on the filters was negligible, being less than 2% of the lowest intraparticulate sucrose value measured spectrophotometrically. Each filter was dried, extracted quantitatively with distilled water for 10 min at 60°C, and deproteinized by the addition of equal volumes of 0.15 M $Ba(OH)_2$ and 5% (wt/vol) $ZnSO_4$; the supernatant was evaporated and assayed for sucrose recovery along with external standards and internal standards. Sucrose internal to the synaptosomes was defined as the resorcinol-positive material retained on the Millipore filters under nonlysing conditions minus that retained in the presence of 10 mM NaCl.

Breakpoint analysis was carried out on all osmotic curves by obtaining individual slopes by regression analysis as in Fig. 1. The slopes were significant in all cases ($P < 0.02$).

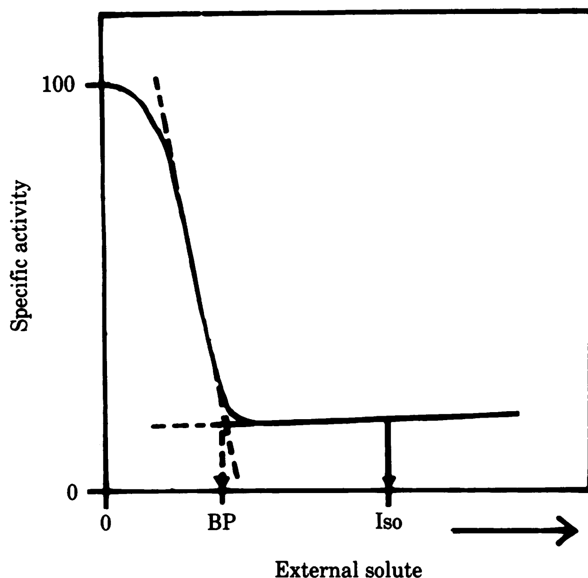


FIG. 1. Enzyme osmometry of an occluded (latent) enzyme. Activity of an occluded enzyme within a subcellular organelle, whose membrane is impermeable to the substrate, is determined at various concentrations of an osmotically active solute in the assay medium. The activity profile yields a breakpoint (BP) expressed as external solute concentration, which reflects the maximum expansion of the volume of the particle prior to bursting. Given the relationship of the slopes, $y = m_1x + c_1$ and $y = m_2x + c_2$, the breakpoint is obtained by computing $(c_2 - c_1)/(m_1 - m_2)$ from the individual slopes obtained by least squares regression analysis. Iso, Isotonic concentration of the external solute.

## RESULTS

Enzyme osmometry, an indicator of the internal solute content and, therefore, of the entry of sucrose during centrifugation, was initially tested in synaptosomes because these represent organelles with a single limiting membrane (18). Different fractions of synaptosomes, C and D, were held to represent different populations (9) and could be usefully compared. LDHase was chosen for synaptosomal studies because occluded LDHase activity is routinely used to determine the integrity of synaptosomes (1, 13, 18). For enzyme osmometry, soluble enzymes with impermeable substrates were preferred to membrane-bound enzymes, as the latter may respond directly to osmotic changes across the membrane without any disruption of the membranes—e.g., NAD(P)H oxidase of plasma membrane (19) and ATPase of mitochondria (20).

External solute concentration at breakpoint reflects the osmotic pressure difference across the membrane at which maximal expansion of the organelle occurs to the point of disruption, if the substrate remains impermeable. Therefore, it should depend on (*i*) the internal solute content, (*ii*) elasticity of the membrane, and (*iii*) permeability to the external solute.

**Osmotic Behavior of Occluded LDHase Activity in Synaptosomal Fractions.** The solubilized enzyme exhibited a monotonic profile of inhibition with increasing concentrations of sucrose in the assay medium. The activities in particulate fractions were clearly discontinuous as predicted (Fig. 2). Because LDHase is specific to the cytosolic compartment, the breakpoint of $P_2$ largely reflects that of its synaptosomal component. Density gradient centrifugation led to an increase in the breakpoints of fractions C and D, which was reversed on exposure to 0.32 M sucrose and reisolation by centrifugation. Thus, the observed changes in breakpoints reflect alterations in internal solute content as a consequence of centrifugation and not the variable elasticity of membranes of different fractions. Fraction $P_2$, when directly suspended in ice-cold 0.8 M and 1.2 M sucrose solutions and kept at 1 × *g* for the duration of centrifugation, did not show a change in breakpoints, indicating the absolute requirement for gravitational field for a change in breakpoint.

As synaptosomes have different permeabilities to various solutes, breakpoint analysis was carried out with different solutes with known permeabilities (sucrose < NaCl < KCl) (1). The breakpoints shifted correspondingly, showing the osmotic nature of the LDHase activity profiles (Table 1).

**Osmolytic Basis of LDHase Activity Profiles.** In principle, either the hypotonic lysis of synaptosomes or a primary osmotic sensitivity of substrate permeability could lead to the observed osmotic curves of LDHase activity. When washed on Millipore filters with media of various sucrose concentrations, LDHase was lost from synaptosomal fraction D; the breakpoint thus obtained was nearly identical to that obtained by LDHase osmometry (Fig. 3). Similar data were obtained for other fractions as well by using external sucrose, NaCl, and KCl (data not given). These experiments clearly indicate that the isotonic conditions for synaptosomal integrity vary directly with the sucrose concentration present during centrifugal isolation.

**Acquisition of Sucrose During Density Gradient Centrifugation.** As the breakpoint would be less than the isotonic concentration, the observed large changes in the breakpoints could be only due to variations in internal sucrose acquired during centrifugation. Indeed, the internal sucrose content varied with the concentration of sucrose in the isolation media in each of

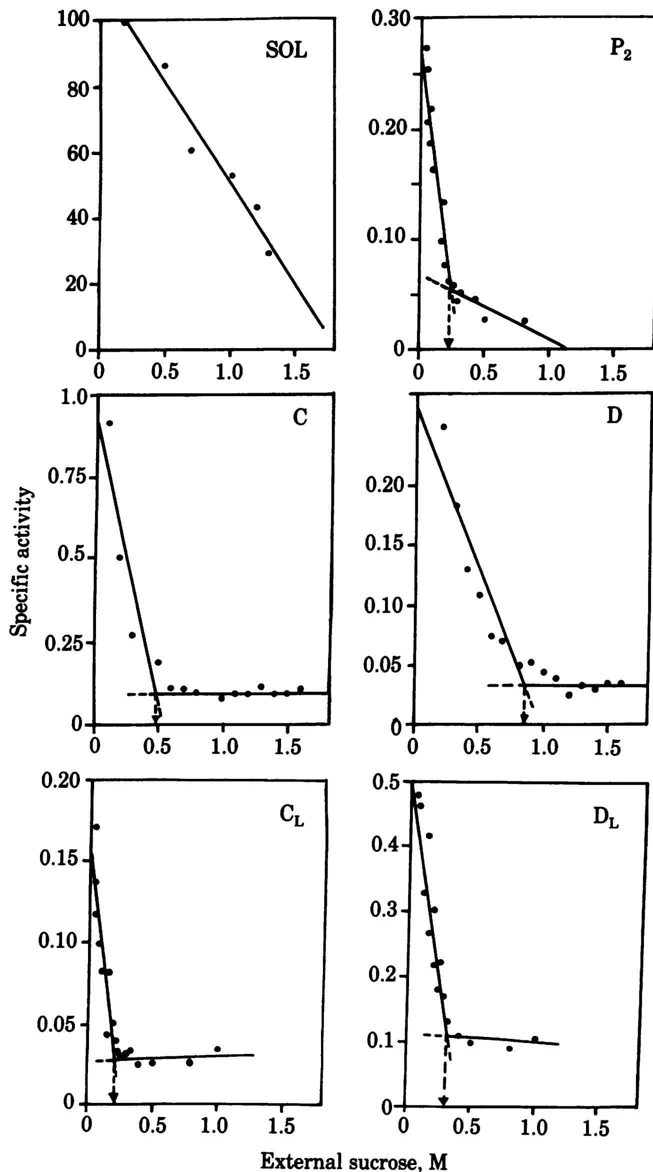


FIG. 2. Enzyme osmometry of LDHase in synaptosomal fractions. LDHase was assayed in the presence of various concentrations of sucrose in synaptosomal fractions $P_2$, C, D, $C_L$, and $D_L$ and in Triton-solubilized enzyme (SOL) obtained from fraction C. The specific activity of the soluble enzyme was 0.82 unit. Breakpoints, determined as in Fig. 1, were: $P_2$, 0.22 M sucrose; C, 0.49 M sucrose; D, 0.86 M sucrose; $C_L$, 0.2 M sucrose; and $D_L$, 0.31 M sucrose.

the fractions (Table 2). Hypotonic rupture of synaptosomes led to a loss of both protein and LDHase to different extents, as judged by variations in LDHase specific activity in various fractions (Table 2). The observed changes in internal sucrose content were much larger than the variations in LDHase specific activity.

The sucrose content of mitochondria has similarly been shown to vary with the concentration of sucrose in the isolation medium (4, 5). However, the shortest time required for the entry of sucrose was less than 2 min (5). It was possible to separate fractions C and D reasonably well by centrifugation for 0.5 hr in discontinuous sucrose density gradients. Data in Table 3 indicate that equilibration with external sucrose was complete by 0.5 hr. Further, no such equilibration appeared to occur with centrifugation at 27–29°C, as opposed to the usual centrifugation at 4–6°C. This suggested that the gravity-mediated entry

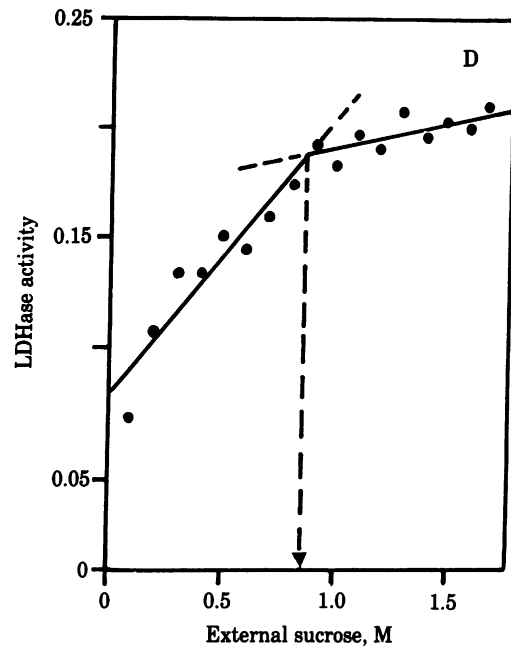


FIG. 3. Loss of LDHase from fraction D due to osmolysis. Aliquots of fraction D (200 μg of protein) were suspended in 3.0 ml of various concentrations of sucrose at 37°C for 15 min. Each aliquot was washed with 10 ml of corresponding sucrose solution on a Millipore filter presoaked in appropriate sucrose solutions containing 10 mg of bovine serum albumin per ml. LDHase was assayed in the extracts after each filter was extracted with 0.3 ml of ice-cold 10% (vol/vol) Triton X-100. LDHase activity was expressed per mg of protein loaded on the filters. The breakpoint, determined as in Fig. 1, was 0.87 M sucrose.

of sucrose has a negative temperature coefficient.

**Localization of Sucrose in the Matrix Space of Mitochondria.** Having established the close parallelism between breakpoints, sucrose concentration during isolation, and the internal sucrose content in synaptosomes, the technique of enzyme osmometry was extended to the mitochondrial matrix enzymes fumarase and MDHase (21) to find out whether sucrose enters the matrix space during centrifugation. Clearly, changes in the breakpoints for matrix enzymes should reflect changes in the solute content of the matrix space, whether the osmotic curves represent osmolysis or osmotic sensitivity of substrate permeability. The breakpoints increased with increasing concentrations of sucrose during isolation, showing that sucrose does enter the mitochondrial matrix space during centrifugation (Table 4). Mitochondrial fractions are usually contaminated with peroxisomes (22); catalase is considered to be exclusively localized in peroxisomes (23). Enzyme osmometry of catalase in mitochondrial fractions showed similar shifts in breakpoints.

We have obtained further experimental evidence that the gravity-induced entry of sucrose into matrix space also exhibits

Table 1. LDHase osmometry in synaptosomal fractions C and D with different external solutes

| External solute | Breakpoint, [external solute] Fraction C | Fraction D |
|---|---|---|
| Sucrose | 0.49 | 0.86 |
| NaCl | 0.46 | 0.59 |
| KCl | 0.51 | 0.80 |

Breakpoint analyses of fractions C and D were carried out by measuring LDHase activity in the presence of various concentrations (M) of sucrose, NaCl and KCl as in Fig. 2. The salt solutions exert nearly twice the osmotic pressure of sucrose because of ionization.

Table 2. Internal sucrose content of synaptosomal fractions

| Synaptosomal fraction | External sucrose, M | Internal sucrose, nmol | | LDHase specific activity |
|---|---|---|---|---|
| | | Per unit of LDHase | Per mg of protein | |
| C | 1.1 | 193 ± 3.15 | 164 ± 2.7 | 0.843 |
| $C_L$ | 0.32 | 56 ± 2.3 | 48 ± 2.0 | 0.864 |
| CII | 1.1 | 133 ± 39.7 | 141 ± 42.1 | 1.060 |
| D | 1.3 | 234 ± 4.9 | 167 ± 3.5 | 0.714 |
| $D_L$ | 0.32 | 94 ± 10.9 | 51 ± 5.9 | 0.540 |
| DII | 1.3 | 164 ± 7.4 | 187 ± 8.4 | 1.140 |

Internal sucrose in various fractions was determined as described; data are means ± SD of three experiments. In both C and D series, fractions $C_L$ and $D_L$ and CII and DII differ from the original fractions and from each other significantly ($P < 0.01$). LDHase specific activity (μmol of NADH oxidized per min per mg of protein) was determined in Triton-solubilized fractions.

a negative temperature coefficient; radioactive sucrose trapped within $C_L$ and $D_L$ fractions readily equilibrates with the medium on centrifugation; and $K^+$ within synaptosomes also behaves in a similar fashion (data not given).

## DISCUSSION

The experimental evidence presented here indicates that (*i*) sucrose enters into subcellular organelles during centrifugation, (*ii*) the entry of sucrose appears to reflect nearly quantitative equilibration across biological membranes during centrifugation, (*iii*) the isotonic requirements for subcellular organelles change drastically depending on the external sucrose concentration during centrifugal isolation, and (*iv*) inattention to the altered isotonic requirements could lead to uncontrolled bursting and resealing of organelles. A variety of "beneficial" and "deleterious" effects of sucrose or centrifugation on biochemical parameters related to subcellular organelles reported in literature can be directly linked to this phenomenon (20, 24–27).

It appears to be proper to generalize the gravity-mediated entry of sucrose in subcellular organelles in view of the following observations: (*i*) the sucrose-space hypothesis has been extended to several subcellular organelles (7); (*ii*) besides synaptosomes, mitochondria, and peroxisomes, lysosomes also have been shown to exhibit similar shifts in osmotic curves of occluded enzyme activities dependent on sucrose concentrations during isolation (8, 28); and (*iii*) centrifugation was shown to cause loss of urea, glucose, and $Na^+$ from isolated hepatocytes (29). Therefore, it is necessary to establish that low molecular weight solutes do not equilibrate with the medium during centrifugation of organelles in all uptake studies. If membranes become permeable to sucrose, they cease to provide osmotic protection to the organelles during centrifugation. Consequently, there would be osmolysis of the organelles, unless other internal solutes were simultaneously lost. Concurrent increase in hydrostatic pressure (up to 300 atm under our centrifugal conditions; 1 atm = $1.013 \times 10^5$ Pa) may further help to stabilize the organelles.

A comprehensive analysis of sucrose-space hypothesis by Tedeschi (6) has led him to suggest that a single compartment model would account for all of the experimental data, given the solution for the paradoxical observations of rapid-entry kinetics (4) and simultaneous osmotic behavior of sucrose in mitochondria (3). Gravity-mediated changes in the permeability of biological membranes, as indicated by our data, resolve this paradox.

Four distinct, possible mechanisms may be considered to explain the entry of sucrose during centrifugation.

(*i*) A trivial explanation would be that these organelles break and reseal during centrifugation. This is unlikely as indicated by the accumulated experience of over two decades of centrifugation by several workers (6, 7).

(*ii*) Enhanced hydrostatic pressure was considered to cause damage to organelles during centrifugation, as indicated by high basal activity of occluded enzymes and by electron microscopy (26). Inattention to the altered isotonic requirements was largely responsible for this conclusion. Hydrostatic pressure *per se* without concomitant increase in gravitational field was shown to decrease permeability of liposomes to a variety of solutes (30).

(*iii*) Enhanced gravitational field could, in principle, raise the potential energy of sucrose molecules to overcome the activation energy barrier for crossing the phospholipid bilayer (31). Sucrose is inert and foreign to most organelles, with the exception of the intestinal brush border (32). Thus, the entry of sucrose needs to be explained in terms of its partition across the lipid membrane. The potential energy, $\phi$, of a solute of mass, $m$, in a centrifugal field is given by

$$\phi = \frac{m\,\omega^2\,r^2}{2}, \qquad [1]$$

in which $\omega$ is the angular velocity and $r$ is the radius. Even erythritol, which is more permeable than sucrose, requires an activation energy of ≈25 kcal/mol to cross the lipid domain (33).

Table 3. Effects of temperature and duration of centrifugation on the breakpoints in synaptosomal fractions C and D by LDHase osmometry

| Exp. | Temperature, °C Initial | Temperature, °C Final | Duration of centrifugation, hr | Breakpoint, [external solute] C | D | $P_2$ |
|---|---|---|---|---|---|---|
| 1 | 4 | 5 | 2.0 | 0.49 | 0.86 | 0.22 |
| 2 | 4 | 6 | 0.5 | 0.55 | 0.88 | — |
| 3 | 27 | 29 | 0.5 | 0.19 | 0.20 | 0.217 |

Fraction $P_2$ was prepared in each experiment (Exp.) as described in text. Fractions C and D were isolated under conditions of centrifugation (sample temperature and duration of centrifugation) specified here. Breakpoint analysis was carried out by LDHase osmometry.

Table 4. Breakpoint analysis of mitochondrial matrix enzymes and catalase

| Concentration of sucrose in the isolation medium | Breakpoint, [sucrose] MDHase | Fumarase | Catalase |
|---|---|---|---|
| 0.25 M | 0.225 | 0.15 | 0.46 |
| 1.00 M | — | 0.75 | 0.82 |
| 1.30 M | 1.14 | 1.05 | — |

Activities of MDHase, fumarase, and catalase were determined in mitochondrial fractions that were isolated in different concentrations of external sucrose. Breakpoint analysis was carried out as with LDHase in Fig. 2. The inhibitory effect of sucrose on the enzymes was monotonic as with LDHase.

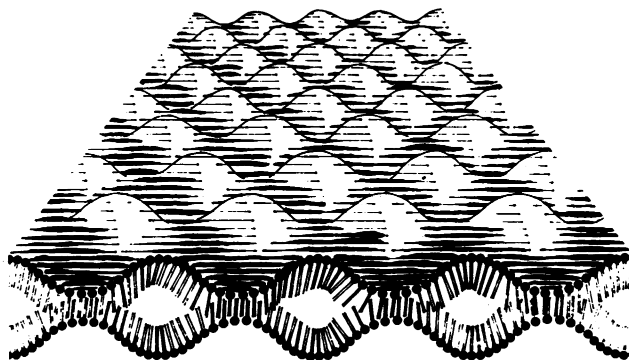

FIG. 4. A periodic model of "rippled membrane"—a consequence of being energized by an external field.

Assuming no change in entropy (i.e., $\Delta S = 0$), one can compute that an activation energy of, say, 100 kcal/mol would require the gravitational field to be $\approx 10^8 \times g$ or a rise in temperature of $\approx$300°C. The highest gravitational field used in our studies (namely, $50{,}000 \times g$) barely corresponds to 20 cal/mol!

(*iv*) The isoentropic assumption in the above calculations may not be justified, and it is possible that the order of the membrane could increase consequent to its being energized by the external field. The observed inhibitory effect of high temperature supports such an argument. Permeability, $P$, of a solute, $i$, across a membrane is determined by both interfacial and diffusional barriers to the solute so that

$$P_i = 2R + \int_0^{\delta} \frac{dx}{D_m(x)\, B(x)}, \quad [2]$$

in which $R$ is the interfacial barrier, $\delta$, the thickness of the membrane, $D_m(x)$, the diffusion coefficient, and $B(x)$, the partition coefficient at a distance, $x$, from the membrane surface (34). Clearly, the homogeneous assumption inherent to the Singer–Nicolson model (35) would not permit a ready explanation for sucrose entry in view of these barriers. The more recent models incorporating membrane heterogeneity (36, 37) could explain sucrose entry, provided one postulates increased heterogeneity on application of an external field. These models uniformly view the planar nature of the bilayer as an invariant property. Alternatively, one could visualize a "rippling" of the membrane layers on being energized, as shown in Fig. 4, with areas of lipid condensation and rarefaction. The rarefied trough regions, where the staggered head-groups of phospholipids result in the lowering of both the interfacial and diffusional barriers, can behave as pseudochannels for the entry of solutes. The gravity-mediated entry of sucrose, as demonstrated here, supports models incorporating heterogeneous lipid distribution in biological membranes.

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