OSMOTIC BEHAVIOR AND PERMEABILITY OF OSMOTICALLY LYSED MITOCHONDRIA

CHARLES L. BOWMAN, HENRY TEDESCHI, BETH J. DiDOMENICO and FRED D. TUNG

From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222. Beth DiDomenico's present address is the Whitman Laboratories, University of Chicago, Chicago, Illinois 60637. Dr. Tung's present address is the Division of Biologic Products, Bureau of Laboratories, Department of Public Health, Lansing, Michigan 48914.

ABSTRACT

Experiments were carried out with water-treated isolated rat liver mitochondria (mitochondrial ghosts) previously studied by Caplan and Greenawalt (Caplan, A. I., and J. W. Greenawalt. 1966. *J. Cell Biol.* **31**:455-472) and Vasington and Greenawalt (Vasington, F., and J. Greenawalt. 1968. *J. Cell Biol.* **39**:661-675). The ghosts have permeability properties and osmotic behavior comparable to those of isolated mitochondria. Although they have lost most of their internal contents, they must have resealed. Four properties were found which have not been previously described in systems derived from biological membranes: (a) an osmotic behavior in the virtual absence of internal components. (b) a self-arranging property in the formation of invaginations corresponding in morphology to the cristae. The results suggest that the assembly of the molecular components of the inner membrane is sufficient to specify the morphology. Hence the surface area to volume ratio of the vesicles may specify the presence or absence of cristae-like folds. (c) an increase in the permeability of the membranes to sucrose in the presence of iso-osmotic concentrations of sucrose. (d) an independence of the light transmitted by suspensions of the vesicles from the refractive index of the external medium. This observation runs counter to the general previous experience with either mitochondria or liposomes.

Water-treated mitochondria (mitochondrial ghosts) have been found to be useful experimental subjects (3, 20). The preparations may be of further use since they are capable of phosphorylation and Ca^{2+} transport but are significantly different from intact, isolated mitochondria at least in ionic content and metabolically induced responses to fluorescent probes.[1] The preparations are composed of inner mitochondrial membrane vesicles (about 1.5 μm in diameter) and a population of smaller vesicles (about 0.2-0.4 μm) formed from the outer mitochondrial membranes (3). The preparatory procedures result in the loss of approximately 50% of the total mitochondrial proteins (3). The results suggest that most of the membrane proteins are retained whereas as much as two-thirds of the matrix proteins are lost (20).

Despite these losses, the respiratory rate of ghosts per milligram protein is 1.5-2 times that of

[1] Kinnally, K. Walsh, and H. Tedeschi. Unpublished observations.

mitochondria, with a reduction of the phosphorylation to about one-half that of isolated mitochondria on a milligram protein basis (indicating an actual reduction to one-quarter) (3). In addition, the ghosts are capable of showing an ATP-induced condensation (3) and, on a milligram protein basis, an undiminished ability to accumulate divalent cations energized either by oxidation or by ATP-hydrolysis (20). Accordingly, the membranes of the ghost vesicles have been considered to have an orientation identical to that of the membranes of intact mitochondria and to be capable of energization for either phosphorylation or active transport. We have verified the phosphorylative ability which we found to be 56 ± 27 nmol $\sim$P min^{-1} mg^{-1} protein (mean of 15 independent experiments $\pm$ SD) for ghosts and 104 ± 34 nmol $\sim$P min^{-1} mg^{-1} protein for mitochondria for succinate oxidation in approximate agreement with the values reported by Caplan and Greenawalt (3).

The present study indicates that the ghosts are osmotically responsive in sucrose although the osmotic behavior is complicated by a number of factors. Since the ghosts are virtually devoid of internal solutes, the mechanism of this osmotic behavior is not immediately apparent. The osmotic behavior is indicated either by photometric measurements or by direct weighing of pellets at various concentrations of sucrose. The evidence suggests that shrinkage also induces a configurational change corresponding to invaginations of the inner membrane resembling cristae. Accordingly, the pellet space penetrated by [¹⁴C]carboxydextran or [¹⁴C]carboxyinulin generally, but not always, increases twofold (with no significant change in one experiment). Electron microscope observation also shows the formation of crista-like invaginations.

In their permeability to sucrose the ghosts exhibit anomalous behavior. When the ghosts are in distilled water, the sucrose-penetrated space is most frequently almost identical to the [¹⁴C]carboxydextran space. However, they are essentially completely penetrated by [¹⁴C]sucrose when they are suspended in 0.3 osmolal sucrose in 10 mM Tris buffer. In contrast, the [¹⁴C]glycerol space shows complete penetration even in distilled water. The relatively high permeability to glycerol was confirmed using photometric determinations. In addition, the ghosts appear to be highly permeable to propylene glycol. The permeability of the ghosts to malonamide was found to be of the same order of magnitude as that of mitochondria.

METHODS

Isolation of Ghosts

The ghosts were isolated basically by the method outlined by Caplan and Greenawalt (3), although some of the details differ. Mitochondria were isolated from livers of male rats of the Holtzman strain typically weighing from 200 to 350 g. The rats were not fed for 12–16 h before the isolation and were killed by cervical fracture. The livers were homogenized in 0.25 M sucrose (pH adjusted between 6.5 and 7.5 with NaOH) with a Teflon and glass, motor-driven Potter-Elvehjem homogenizer. Throughout all isolation procedures the preparations were maintained below 5°C.

The mitochondria were isolated by centrifuging with a Sorvall RC2-B refrigerated centrifuge (SS-34 or HB-4 rotors, Dupont Instruments, Sorvall Operations, Newton, Conn.) at 8,200 g for 10 min after a centrifugation at 600 g for 15 min to remove larger particles. They were then washed twice. The mitochondrial pellet was treated with water by mixing first with a Teflon-coated stirring bar and then homogenizing in a Teflon and glass homogenizer of the Potter-Elvehjem type. The mitochondria from each rat were suspended thoroughly in 15 ml of chilled water and centrifuged at 105,000 g for 30 min, usually, in an A-321 rotor of a model B-60 International ultracentrifuge (International Scientific Instruments Inc., Mountain View, Calif.). The procedure was repeated once more with 10 ml of water (wash II ghosts) and a third time to produce the final preparation (wash III ghosts). Unless otherwise specified, the results refer to wash III ghosts.

Where necessary, the ghosts were sedimented by centrifugation either at 105,000 g for 30 min (rotor SB 283 for model B-60 International ultracentrifuge) or, at 60 min at 49,500 g (rotor SM-24, Sorvall refrigerated centrifuge).

Use of Radioactive Compounds

The radioactive compounds used were as follows: [¹⁴C]carboxydextran, mol wt 15,000–17,000 (5 μCi/mg); [¹⁴C]carboxyinulin, mol wt 5,000–5,500 (2.6 μCi/mg); [1,3-¹⁴C]glycerol (135 μCi/mg); and uniformly labeled [¹⁴C]sucrose (12 μCi/mg) all from New England Nuclear (Boston, Mass.). Except for glycerol, the radioactive compounds were dissolved in glass-distilled water. The radioactive labels were added in solution to the ice-cold vesicle suspensions. 50-μl portions (0.5 μCi) were added to the samples. After centrifugal sedimentation of the ghosts, 50-μl aliquots of the supernates were counted directly. The pellets were extracted in 10 ml of water overnight with shaking, and 0.5-ml aliquots were counted after centrifugal removal of the debris. The [¹⁴C]glycerol space was estimated in the presence of 10 mM unlabeled glycerol. The sucrose concentration, in the absence of added unlabeled sucrose, was approximately 70 μM. The radiojctivity was estimated with a Unilux I model no. 6850 scintillation counter from Nu-

clear Chicago (Des Plaines, Ill.) using Liquiflor (New England Nuclear; final concentration of 4 g PPO/liter (2,5 diphenyloxazole) 0.050 g/liter *p*-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) in a mixture of 50% toluene and 50% Methyl Cellosolve. The counts recorded were corrected by either the ratios method (2) or the internal standard method. When compared, the two methods were found to be in good agreement.

Calculations of Spaces

The penetrated space was calculated from the radioactivity of the pellet, P, and the concentration of counts per milliliter of the supernate (S); $P/(S)$ = penetrated space. The equivalent calculation was carried out for the K^+ space using the atomic absorption estimates of the K^+. The water space was determined by drying the samples for 48 h at 50-60°C in a drying oven.

Photometric Measurements

Photometric measurements were carried out at a wavelength of 520 nm with a Coleman Junior II spectrophotometer model 6/20 (Coleman Instruments Div., Perkin Elmer Corp., Oak Brook, Ill.) with 3 ml of suspension during the measurements. The suspensions were maintained at 25°C. Matched round glass cuvettes with an optical path of 15 mm were used.

Atomic Absorption

Analyses of the ionic content of the ghosts or the appropriate sogutions were carried out with an atomic absorption spectrophotometer model AA 120 of Varian Techtron (Varion Associates, Palo Alto. Calif.). The ghost or mitochondrial pellets were extracted overnight in 10% perchloric acid with shaking. After removing the precipitate by centrifugation, the resulting extract was analyzed by atomic absorption.

Phosphorylative Ability of Ghost Preparations

The ghost preparations were monitored continuously for phosphorylative ability in an automated system using a Technicon Autoanalyzer (Technicon Instrument Corp., Plainfield, N. J.). The process invogved stopping the reactions in 1.5 M H_2SO_4 and the assay of inorganic phosphate by the method of Hurst (5). The assay mixture contained 10 mM succinate, 1 mM glucose, 0.33 mg/ml hexokinase (Sigma Chemical Co., St. Louis, Mo., type VI), 1.7 mM sodium ADP (Sigma, grade 1), 2 mM $MgCl_2$ and 0.5 mM inorganic phosphate, 10 mM Tris (hydroxymethyl) aminomethane, pH 7.4.

Protein Concentrations of the Suspensions

The protein content was estimated by means of the Biuret reaction (4).

Electron Microscopy

The electron microscopy was done following the methods used by Caplan and Greenawalt (3) with a few alterations (see below). Well suspended ghosts were placed in equal volumes of distilled water or equal vol of 0.6 osmolal sucrose in 10 mM Tris, pH 7.4, and were mixed with equal vol of 12.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The two samples were centrifugednin a Beckman Microfuge (model 152, Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) in the cold at 8,800 g for 4 min. The pellets were removed, placed in 12.5% glutaraldehyde for 90 min, rinsed three times with 0.1 phosphate buffer, and postfixed overncght in 2% OsO_4 in 0.1 M phosphate buffer, pH 7.4. In addition, after centrifugation, some of the pellets were randomized by mixing in 1% agar and allowed to solidify (7) before further treatment. The results were approximately the same regardless of whether the agar technique was used. Dehydration was accomplished at room temperature at 15-min intervals through a routine alcohol series followed by embedding in resin developed by Spurr (16). The embedded pellets were then sectioned on an LKB Ultratome III microtome with a diamond knife and collected on uncoated grids. Double staining with uranyl acetate and lead citrate (21) preceded examination of the sections with an AEI Model 6B electron microscope.

RESULTS

The ability of ghosts to accumulate Ca^{2+} (20) suggests the resealing of the mitochondrial semipermeable membrane during the preparation of the ghosts. Two experimental approaches are possible to examine the question: *(a)* a study of the osmotic behavior of the ghosts and *(b)* a study of the permeability of the ghosts.

The osmotic behavior of the vesicles can be studied directly by examining in some manner the changes in internal water. A more indirect method, which has proven useful for both mitochondria (18, 19) and liposomes (12, 1), consists in measuring the light either transmitted or scattered by suspensions of the vesicles. Fig. 1 A depicts the dependence of the optical density of the suspensions on the osmotic pressure of the medium. Curve 1 represents the dependence of mitochondrial suspensions, curve 2 that of ghosts. The corresponding reciprocal plots are shown in Fig. 1 B (1/OD is directly proportional to volume in mitochondria and liposomes (18, 19, 12, 1)). The experiment shown in Fig. 1 was carried out with wash II ghosts (see Materials and Methods). However, the results are the same when the ghosts have been washed for a third time (e.g. in the experiments of Table II).
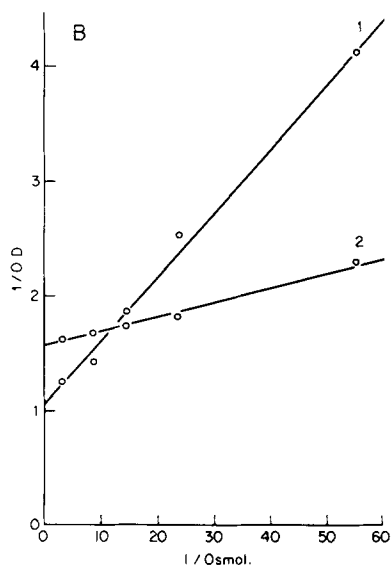
the preparations suggests that, at least under the conditions used, the vesicles are very permeable to sucrose. The apparent osmotic behavior could be explained alternatively by assuming that the vesicles are disrupted by the treatment in sucrose solutions. This explanation, however, is unlikely. The optical density of the suspensions increases with increasing sucrose concentration. In addition, the suspensions centrifuged in 0.3 osmolal sucrose have approximately the same protein content as those centrifuged in water. The ratio of protein content of the pellets of aliquots suspended in sucrose divided by the protein content of pellets of aliquots suspended in water was found to be 0.94, 1.10, and 0.88 ± 0.08 (n = 4) in three independent experiments.

The osmotic behavior of the ghosts is, to some extent, surprising since their lofs of internal protein suggests a leakage of smaller molecular weight solutes as well. The cationic composition of the ghosts was examined in the hope of throwing some light on this question. The results in Table I
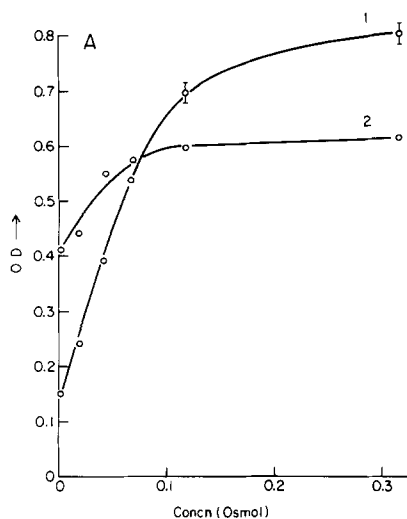

FIGURE 1 (A) Dependence of the optical density of mitochondrial (curve 1) or ghost (curve 2) suspensions on the sucrose concentration (abscissa). All sucrose solutions contained 10 mM Tris, pH 7.4. The protein contents of the ghosts and mitochondria were 0.635 mg/ml and 0.463 mg/ml, respectively. Where SD's are not given, the standard deviations were smaller than the diameter of the circles. (B) The reciprocals of the optical densities of Fig. 1 A plotted as a function of the reciprocals of the sucrose concentrations.

A direct evaluation of the ghost internal water (corrected for [^{14}C]carboxydextran space) is shown in Fig. 2. The dependence as a function of the reciprocal of the sucrose concentration is slight but significant. The limited osmotic behavior of
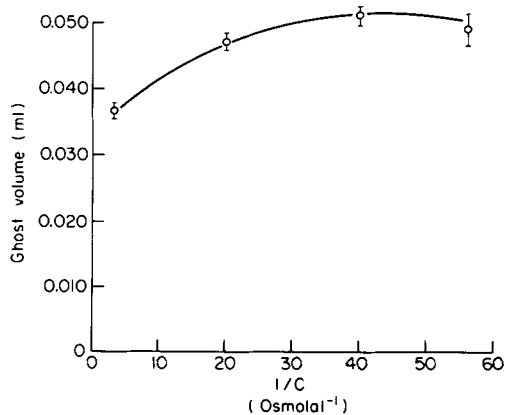
FIGURE 2 The volume of ghost pellets corrected for external space (assumed to equal the dextran space) as a function of the reciprocal of the sucrose concentrations. All sucrose solutions contained 10 mM Tris, pH 7.4. The protein content of the ghosts in each pellet was 8.9 mg.

TABLE I

Cationic Content of Ghosts ($nmol\ mg\ protein^{-1}$)

	K$^+$	Na$^+$	Mg^{2+}	Ca^{2+}
Exp 1	0.46 ± 0.07	0.34 ± 0.24	11.6 ± 0.3	2.6 ± 0.2
	(67 ± 10)	(50 ± 35)	(1700 ± 40)	(380 ± 30)
Exp 2	0.42 ± 0.02	1.35 ± 0.54	19.0 ± 0.1	6.0 ± 1
	(68 ± 3)	(220 ± 90)	(3100 ± 20)	(980 ± 160)

The values in parentheses represent the concentration (μM). The volumes were not corrected for external space.

show that the cationic content is much reduced. In fact, an estimate of the concentration of the ions in the ghosts (the values in parentheses) suggests that the total of molality of all the cations is less than 4 mM. The actual concentration is likely to be much lower since the ions may be present in bound form (e.g., see reference 6, 11, 13, 14). Intact mitochondria have a much greater concentration of cations: (in our hands, nmol/mg protein: K^+, 180 ± 40; Na^+, 11 ± 3; Ca^{2+}, 18 ± 6; Mg^{2+}, 40 ± 9 mean ± SD, six independent experiments).

The permeability to sucrose or other solutes can be tested directly by using the labeled compounds. The pellet water external to the vesicles can be estimated by using either [14C]carboxydextran or [14C]carboxyinulin. Table II, part B, shows that the space occupied by these probe molecules is 11-26% of the pellet when the ghosts are in water (column 2). However, it is significantly higher in the presence of sucrose. Although the external space of the ghosts in sucrose measured with the macromolecules may be as low as 15% (exp 10), more generally it is two to three times the carboxydextran space of the ghosts suspended in water (e.g., see exp 6). The results suggest that either (a) the ghosts become permeable to the macromolecules in high concentration of sucrose or (b) they have a configurational change which increases the amount of external fluid trapped in the pellet. A configurational rearrangement could take the form of crista-like invagination.

Electron microscopy confirms the view that exposure to sucrose at higher osmotic pressures leads to invaginations of the ghost membrane. Part A of Fig. 3 shows water-treated ghosts. The profiles are much more irregular than those reported by Caplan and Greenawalt (3). Although we have occasionally noted more spherical vesicles, the view shown in this figure is much more representative of our preparations. The same preparation is shown after sucrose treatment in part B of the figure. A number of invaginations resembling cristae can be observed (labeled C). These are much narrower than the finger-like projections previously reported for ATP-contracted preparations (3). In randomized sections, we have observed 202 crista-like invaginations among 160 water-treated ghost vesicles, and 991 crista-like invaginations among 186 sucrose-treated ghost vesicles (1.3 ± 0.9 and 5 ± 1, cristae per vesicle). These results are most easily interpreted by assuming that the shape of the ghosts is affected by osmotic shrinkage.

TABLE II

Pellet Water and Spaces of Mitochondrial Ghosts Penetrated by Labeled Probe Molecules

	1	2	3	4
		Solutions used to resuspend pellet		
Exp.	Probe	Water	Tris	Sucrose-Tris
Part A				
1	[14C]Glycerol	104 ± 3%	–	–
		0.127 ml	–	–
Part B				
2	[14C]Carboxyinulin	26%	–	–
		0.220 ml	–	–
3	[14C]Carboxyinulin	24%	–	–
		0.154 ml	–	–
4	[14C]Carboxyinulin	17%	–	–
		0.185 ml	–	–
5	[14C]Carboxydextran	24%	–	–
		0.232 ml	–	–
6	[14C]Carboxydextran	22 ± 1%	–	63 ± 2%
		0.164 ml	–	0.084 ml
7	[14C]Carboxydextran	–	57 ± 7%	63 ± 5%
		–	0.148 ml	0.0915 ml
8	[14C]Carboxydextran	14 ± 1%	–	29 ± 2%
		0.098 ml	–	0.082 ml
9	[14C]Carboxydextran	15 ± 1%	–	31 ± 2%
		0.099 ml	–	0.082 ml
10	[14C]Carboxydextran	11 ± 1%	–	15 ± 1%
		0.172 ml	–	0.143 ml
Part C				
11	[14C]Sucrose	17 ± 1%	–	93 ± 4%
		0.200 ml	–	0.098 ml
12	[14C]Sucrose	58 ± 1%	–	94 ± 7%
		0.108 ml	–	0.071 ml
13	[14C]Sucrose	78 ± 1%	–	109 ± 8%
		0.125 ml	–	0.128 ml
14	[14C]Sucrose	24 ± 4%	–	105 ± 8%
		0.104 ml	–	0.077 ml
15	[14C]Sucrose	43 ± 5%	39 ± 5%	–
		0.106 ml	0.120 ml	–

The results are listed as means of four determinations ± SD. Typically, 10-20 mg of protein per pellet was used. Pellet water is given in milliliters. Penetrated space is given as per cent of pellet water. "Sucrose-Tris" is 0.30 osmolal sucrose containing 10 mM Tris. "Tris" is 10 mM Tris. All solutions were at pH 7.4 (except when in water). Temperature was maintained below 5°C. Where no SD's are shown, the results were obtained from two-three determinations, which agreed within 10%.

The space penetrated by [14C]sucrose in ghosts suspended in water varies (Table II). In some experiments, it approximates the space penetrated by the macromolecules (e.g., exp 11 and 14). In other experiments, the penetrated space is somewhat larger (exp 12, 13, 15), suggesting that the ghosts are leaky to sucrose. In contrast [14C]glycerol, which penetrates mitochondria rapidly, permeates the entire ghost space (Part A, column 2). However, the ghosts become completely or almost completely permeable to [14C]sucrose when suspended in 0.3 osmolal sucrose (column 4). The results show that the presence of sucrose has increased the permeability of the vesicles to sucrose.
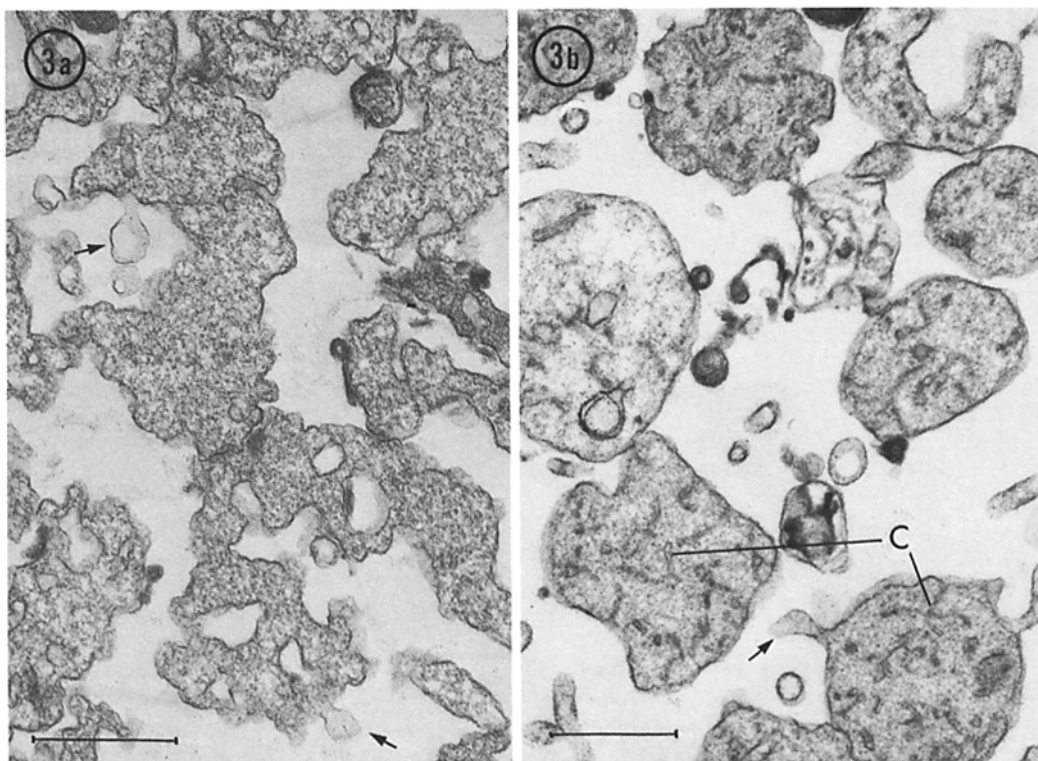
FIGURE 3 Osmotically lysed mitochondria (ghosts); fixed with glutaraldehyde and postfixed with OsO₄; doubled stained with uranyl acetate and lead citrate. (A) Ghosts in distilled water and then fixed. (B) Ghosts subjected to 0.30 osmolal sucrose (in 10 mM Tris, pH 7.4) and then fixed. Generally, the shape of the ghosts in water (A) is more irregular than that of those in sucrose (B). Crista-like invaginations (C) are readily apparent in the sucrose-treated ghosts and are virtually absent in ghosts resuspended in water. The arrows indicate blebs where the membrane appears more diffuse. The scale in the lower left hand corner of each micrograph indicates 1.0 μm. × 18,750.

With the exception of experiment 13 of Table II, the total pellet water of the ghosts suspended in sucrose is less than when they are suspended in water (columns 2 and 4 of exp 6–14). This result agrees in a qualitative way with the idea that the ghosts behave osmotically as shown in Fig. 2.

A similar study was carried out on the permeability to K⁺. The content of the ghost pellets was examined as a function of external concentration as shown in Fig. 4. The slope of the line × 100 corresponds to the percent of the space penetrated by K⁺. The results are summarized in Table III. The proportion of the space penetrated by K⁺ does not change with concentration (Fig. 4), and the results show that at least a large portion of the ghosts is essentially impermeable to K⁺.

The permeability of the ghosts can be studied further by the use of the photometric technique. Table IV, experiment 1 shows how the technique
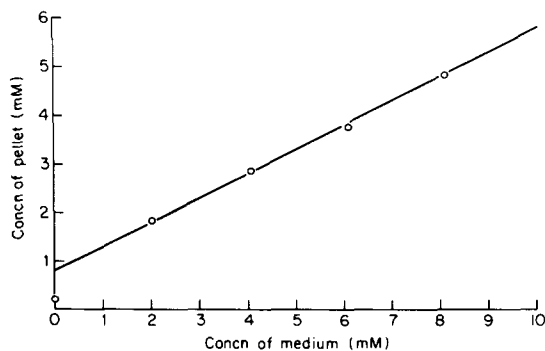

FIGURE 4 Concentration of K⁺ in the pellet (not corrected for external space) as a function of K⁺ in the supernate.

in its simplest form can be applied to mitochondria. The optical density of the mitochondria or the ghosts in buffer [10 mM Tris (hydroxymethyl)

TABLE III

The K⁺ Space of Ghosts Suspended in Various Concentrations of KCl (See Fig. 4) Ranging between 0 and 10 mM

Exp.	K⁺ Space	Protein	Pellet volume
	%	mg	
1	32	17.5	0.0874
2	33	24.4	0.0653
3	51	27.7	0.0998

The total vol were 10 or 13.5 ml, and each suspension contained 3 mM glycylglycine in addition to 3.3–5 μM antimycin A and 3.3–5 μM rotenone. The inhibitors were added to avoid a possible active transport of the cations. All suspensions were at pH 7.4. The pellet volumes are in milliliters. The K⁺ space is represented by the slope of the line in experiments similar to that of Fig. 4. It is expressed as percent of the total pellet volume.

aminomethane (taken arbitrarily as 1, in relative units)] can be compared to that in 0.30 osmolal of various solutes dissolved in the buffer. For mitochondria, the optical density in NaCl, mannitol, sucrose and sucrose-raffinose is much higher than that of the suspension in the tris buffer. The results agree with the interpretation that all of these solutes have not completely penetrated the vesicles during the period of incubation. For mitochondria, the small change in optical density from solute to solute can be quantitatively accounted for by the changes in refractive index of the medium (18, 19) (expressed in this Table as Δr, the difference in refractive index between the solution and water). In the same solutions, the ghosts also exhibit a higher optical density than when in the buffer. The lesser difference between the various solutes and the buffer are in agreement with the results shown in Fig. 1 A. Interestingly enough, there is little change in optical density with the refractive index of the medium (except for NaCl which may be partially penetrating). These results suggest that although the mitochondrial volume and the ghost volume are both inversely proportional to the optical density, the light-scattering phenomena have probably two entirely different underlying mechanisms. This independence from the refractive index of the medium is surprising since the optical density of the suspension of vesicles artificially formed from phospholipids (i.e. liposomes) do show a dependence (12) except perhaps when prepared in water (see Fig. 10 of reference 1).

The photometric results suggest that these same solutes do not completely penetrate the ghosts

either. In contrast, glycerol (Table IV, exp 2, 3) and propylene glycol (exp 2 and 4) act as penetrants for both mitochondria and ghosts since the optical density approximates that of the suspension in the buffer. The results suggest that propylene glycol penetrates faster than glycerol (the OD at 30 s is higher), in complete agreement with previous studies with mitochondria suspensions (17, 18).

The permeability can be more accurately estimated by evaluating the rate of swelling when the particles are suspended in a penetrant (17, 18). Although more precise permeability constants can be obtained by following the kinetics of the swelling (see e.g. reference 17), the time required to reach an arbitrary end point is useful in comparing two preparations. The time required to reach three-quarters of the optical density difference between the initial optical density of the suspension and the optical density of the buffer has been previously used as a convenient end point (see reference 18). The results of two independent experiments (six determinations per experiment) are displayed in Table V. The $t_{3/4}$ does not seem to differ significantly for the two cases. Since the difference in diameter between mitochondria and ghosts is not likely to be large (see reference 3), these results probably indicate that the isolated mitochondria and the ghosts have similar and perhaps identical permeability constants for malonamide.

DISCUSSION

The capacity of the ghosts to respond osmotically is not entirely surprising. Resealed red blood cell ghosts exhibit ideal osmotic behavior (8). However, the observation of a graded osmotic response is paradoxical on several accounts. First, the ghosts are almost entirely devoid of internal solutes (Table I). When exposed to a substantial osmotic pressure, the vesicles should collapse. Secondly, the ghosts appear to be leaky to sucrose at low osmotic pressures (Table II, part C, cogumn 2), and completely permeable to sucrose at high osmotic pressures (Table II, part C, column 4). Liposomes prepared in water (Fig. 10 of reference 1) also exhibit an osmotic behavior, suggesting that this observation may reflect a general phenomenon which is presently not understood within the usual framework. It is possible that the ghosts initially decrease drastically in volume, but then swell again as they become permeable to the external solute. The swelling would be faster the

TABLE IV

The Optical Density of Mitochondria and Ghost Suspensions as a Function of Solute and Solute Concentration

		Relative OD	
	$\Delta r \times 10^3$	Mitochondria	Ghosts
Exp. 1			
10 mM Tris	0.2	1.00 ± 0.03	1.00 ± 0.02
0.30 osmolal NaCl	1.8	3.36 ± 0.11	1.32 ± 0.03
0.30 osmolal mannitol	7.9	3.17 ± 0.06	1.44 ± 0.04
0.30 osmolal sucrose	14.2	2.95 ± 0.05	1.45 ± 0.03
0.20 osmolal raffinose	17.7	2.87 ± 0.03	1.46 ± 0.02
+ 0.10 osmolal sucrose			
Exp. 2			
10 mM Tris	—	—	1.00 ± 0.06
0.30 osmolal sucrose	—	—	1.56 ± 0.04
0.025 osmolal sucrose	—	—	1.25 ± 0.02
0.30 osmolal glycerol	—	—	1.21 ± 0.02
0.025 osmolal glycerol	—	—	0.985 ± 0.06
0.30 osmolal propylene glycol	—	—	0.985 ± 0.02
0.025 osmolal propylene glycol	—	—	0.977 ± 0.06
Exp. 3			
10 mM Tris	—	1.00 ± 0.03	1.00 ± 0.02
0.30 osmolal sucrose	—	2.70 ± 0.05	1.51 ± 0.04
0.025 osmolal sucrose	—	1.27 ± 0.07	1.12 ± 0.06
0.30 osmolal glycerol	—	1.27 ± 0.05	1.14 ± 0.02
0.025 osmolal glycerol	—	1.02 ± 0.04	1.04 ± 0.03
Exp. 4			
10 mM Tris	—	1.00 ± 0.09	1.00 ± 0.04
0.30 osmolal sucrose	—	3.00 ± 0.04	1.39 ± 0.03
0.025 osmolal sucrose	—	1.52 ± 0.05	1.26 ± 0.04
0.30 osmolal propylene glycol	—	1.06 ± 0.02	1.04 ± 0.02
0.025 osmolal propylene glycol	—	0.96 ± 0.03	1.04 ± 0.02

Results are means of four determinations ± SD. Δr corresponds to the refractive index of the solution minus that of water. All solutions contained 10 mM Tris at pH 7.4. Incubation was carried out for 30 seconds at 25°C. Mitochondria and ghost protein contents were: 1.42 and 1.96 (exp 1), 2.92 (ghosts, exp 2), 0.8 and 1.12 mg/ml (exp 4). Concentrations are expressed as osmolal, except for Tris buffer. The optical densities of mitochondria and ghosts suspended in 10 mM Tris buffer were: 0.290 ± 0.008 and 0.455 ± 0.009 (exp 1), 0.397 ± 0.025 (ghosts, exp 2), 0.301 ± 0.010 and 0.559 ± 0.009 (exp 3), 0.287 ± 0.026 and 0.356 ± 0.013 (exp 4). The relative OD column lists values and their standard deviation both resulting from a normalization of the optical density obtained in the solution to the optical density obtained using 10 mM Tris.

TABLE V

$t_{3/4}$ *for Malonimide Penetration (See Text)*

	Protein content		$t_{3/4}$	
Exp.	Mitochondria	Ghosts	Mitochondria	Ghosts
	mg/ml	*mg/ml*	*s*	*s*
1	1.4	1.4	117 ± 16	169 ± 7
2	1.7	1.8	102 ± 15	112 ± 11

greater the initial volume (e.g. see reference 17).

As already noted, the optical density of the ghost suspension is not affected significantly by changes in the refractive index of the medium. In contrast, most preparations of mitochondria (18, 10) and liposomes (12) generally show this effect. We believe, however, that liposomes prepared in water also do not show a dependence on the

refractive index of the medium (Fig. 10 of reference 1). This is surprising and cannot be easily explained from current theoretical analysis (9, 10).

The shrinkage induced by the sucrose solutions apparently produces invaginations of the surface membranes. This is shown by the increase in the proportion of the pellets occupied by [^{14}C]carboxydextran as well as by the electron microscopy. Fingerlike invaginations have been shown to occur when ghosts are shrunken in the presence of ATP (3). However, the ATP could well have had an effect independent of the volume change. The osmotic formation of crista-like invaginations suggests that it is solely the change in volume which is responsible for this effect. Hence, this observation suggests that the organization of the mitochondria themselves is responsible for the formation of the cristae and that no special information need be provided by the assembly process. In addition, the evaginations produced by the digitonin removal (15) of the external membrane must be the special consequence of the treatment and not the result of stripping the outer membrane.

As seen with the electron microscope, the size of the vesicles appears decreased by the sucrose treatment, in harmony with an osmotic behavior (although more work would be necessary to firmly establish this point since we do not know the shape of the ghosts). Many of the vesicles (Fig. 3 A and B) show blebbing (see arrows). We have made no attempts to study this phenomenon.

Our preparations show much more contrast than those of Caplan and Greenawalt (3). We ascribe this difference to some difference in electron microfcopy procedures. It is not the result of a less complete lysis. In our hands, the protein remaining in our ghosts is 40 ± 3% of the amount present in the mitochondria (five preparations), comparable to the 46 ± 2% reported previously (3). In addition, the optical densities of the ghost suspensions are much lower than those of the mitochondria from which they have been prepared even at equivalent protein concentrations, confirming that extensive loss of refractile material has taken place. For example, the protein concentration of the ghosts used in the experiment of Fig. 1 A is 1.4 times greater than that of the mitochondria. Yet the optical densities of the suspensions are lower.

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