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MINI-REVIEW

Determination of the Orientation of Membrane Vesicles Derived from Mitochondria

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Abstract

Membrane vesicles of physiological as well as inverted orientation can be isolated from mitochrondria. The presence of these vesicles in a membrane can be determined and quantitated by determining the differences between the two vesicle types in terms of rates of NADH oxidation, rates of oxidation of tricarboxylate cycle intermediates, rates of ATP hydrolysis and sensitivity to inhibitors, stimulation of respiration by exogenous cytochrome *c*, inhibition of respiration by polycationic proteins, and visualization of the ATPase by electron microscopy. Procedures to isolate the two membrane types and characteristics of homogeneously oriented preparations are described. Differences in data obtained with homogeneous vesicle preparations and with vesicles of mixed orientation are illustrated. Nonhomogeneously oriented preparations can be enriched in the desired vesicular type by the use of immunoprecipitation, affinity chromatography, and differential centrifugation. The concept of a hybrid vesicle containing oppositely oriented regions is not supported by experimental data.

Key Words: Mitochondria; submitochondrial particles; cytochromes; proton-motive force; membranes; orientation.

Introduction

Intact mitochondria from numerous sources are used to determine aspects of respiration, oxidative phosphorylation, membrane structure, mechanism of action of particular enzymes and enzyme systems, and the structure/organization of multi-subunit protein assemblies such as cytochrome oxidase or the bc_1 complex. Biological membranes possess two important characteristics, their impermeability to large or charged molecules and their structural

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and functional asymmetry, that are among the basic tenets of the chemiosmotic theory [proton impermeability and vectorial (asymmetric) transport]; these characteristics are utilized extensively in the determination of membrane/protein structure (topography) and function.

The mitochrondrial membrane has two planar surfaces, one facing the cytoplasm and one facing the matrix. The topography of the membrane has been discussed earlier (Harmon *et al.*, 1974; DePierre and Ernster, 1977) and while related to membrane orientation, will not be duplicated here. The determination of membrane topography relies heavily on the inability of inhibitors, substrates, and probes to cross the membrane. A permeant molecule is capable of reacting with both planar surfaces while an impermeant molecule will react only with those sites exposed to it on a single planar surface. As a result, the investigator is required to present one surface and then the other to the molecule by using oriented membrane vesicles.

In this work, the orientation of membrane vesicles and techniques to determine the orientation of vesicles will be discussed. While other membrane systems will not be mentioned, the concepts presented are applicable to other systems where inversion of the membrane is possible and desirable.

Possible Orientations of Membranes

As a vesicular structure, the membrane presents only two surfaces; in the mitochondrial system there are the cytoplasmic (C-side) and the matrix (M-side) faces. The orientation of the faces in vesicular or nonvesicular membranes allows six theoretical classifications of membranous populations or preparations (cf. Fig. 1).

Mitochondrially oriented membranes are isolated mitochondria of predominantly ($>95\%$) physiological orientation that can be isolated from heart, brain, liver, kidney, or other tissue from ox, rat, chicken, pigeon, pig, turkey, sheep, or other animals, and even from tissue culture cells. These vesicles have their cytoplasmic face containing cytochrome *c* exposed to the medium and the ATPase-containing matrix face sequestered in the vesicle interior. The reader is referred to the extensive compilation by Nedergaard and Cannon (1979) and the numerous procedures in *Methods in Enzymology*, Volume 55.

Inverted vesicles with orientation opposite to that of mitochondria can be isolated from mitochondria; they are commonly referred to as ETP (electron transport or transfer particles) or SMP (submitochondrial particles) and usually, but not always (Albracht and Heidrich, 1975), lack the matrix proteins and enzymes and the outer membranes. Numerous procedures outline their isolation (Crane *et al.*, 1956; Hansen and Smith, 1964; Lee, 1979; Harmon, 1982).

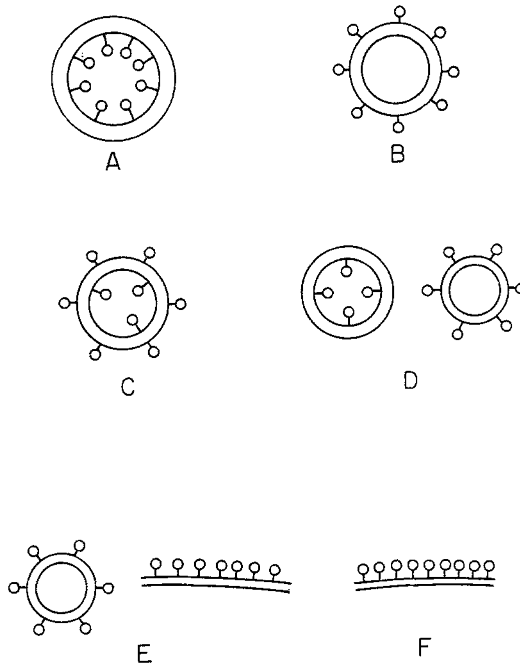

Fig. 1. Six general types of membrane preparations derived from mitochondria. (A) Totally intact mitochondria; (B) totally inverted submitochondrial particles or electron transport particles with orientation opposite to that of A; (C) "scrambled" or hybrid vesicles that display both the matrix and cytoplasmic face in the same vesicle; (D) membrane preparation composed of a mixture of intact mitochondria and inverted vesicles in varying proportions; (E) membrane preparation composed of a mixture of nonvesicular membrane "sheets" and either mitochondria or inverted vesicles; (F) membrane preparation composed solely of nonvesicular membranes.

Scrambled vesicles are vesicular membranes in which the external surface presents both the C- and M-sides. It is a mosaic composed of regions of the inner membrane that have been flipped 180° in the plane of the membrane. Numerous reports suggest their existence (Chance *et al.*, 1970; Huang *et al.*, 1973; Rosier *et al.*, 1980), but we shall provide evidence in this work that their existence is highly unlikely.

Mixtures of vesicles of opposite orientations are possible and probable. Evidence for mixtures will be presented; each vesicle is of mitochrondrial or inverted orientation while the overall preparation contains both in varying proportions (Malviya *et al.*, 1968; Harmon *et al.*, 1974; Astle and Cooper, 1974; Smith *et al.*, 1980).

Mixtures of vesicles and nonvesicular membranes or "sheets" are possible. The "open" vesicular sheets have both surfaces exposed. Vesicles of normal or inverted orientation may be present in varying proportions. Evidence for

mixtures has been presented by Scholte *et al.* (1973) and suggested by the data of Huang and Lee (1975).

Nonvesicular membranes or membrane fragments can be isolated that fully present both surfaces to the suspension medium. The existence of "open" membranes of this sort has been proposed (Harmon *et al.*, 1974; Storey *et al.*, 1980).

Techniques Used to Determine Orientation

The determination of the composition of a population and the evaluation of its utility in certain studies is achieved via the following assays that measure the impermeability of the membrane to the substrate/reactant used and that determine the asymmetric location of the enzymatic reactive site on only one membrane surface. It will become apparent that no single procedure will give definitive or unambiguous indication of the orientation and relative exposure of each membrane surface, but that multiple assays or reactions involving both sides of the membrane are needed for definitive characterization.

As shown by Lehninger (1955) and substantiated by others since (Crane *et al.*, 1956; Harmon and Crane, 1974; Rasmussen, 1969), intact mammalian mitochondria cannot reduce exogenous NAD^+ or oxidize exogenous NADH via an oxidative phosphorylation-linked pathway because of mitochondrial impermeability to these charged molecules. In cases where exogenous NADH oxidation is observed (Rasmussen, 1969), ADP is not phosphorylated and respiratory carriers may not be totally reduced; the noncoupled NADH oxidation could be due to somewhat permeable membranes or broken mitochondria that are uncoupled. Plant mitochondria have NADH dehydrogenases on both sides of the membrane (Moore and Bonner, 1981) precluding the use of this assay in determining orientation in this or similar systems.

As shown in Tables I and II, mitochondria exhibit slow respiration with NADH as substrate since the NADH-reactive site is on the matrix face. Intact mitochondria isolated by the procedure of Harmon and Crane (1974) can be "opened" by brief sonication after which a 15-fold increase in NADH oxidase activity is observed (868 ng-atoms O/min/mg vs. 58 ng-atoms O/min/mg). This "opening" phenomenon illustrated in Fig. 2 can be used as a good thumbnail indication of the orientation of mitochondria: the greater the stimulation by sonication, the greater the mitochondrial orientation of the preparation.

In contrast, electron transport particles (ETP) or submitochondrial particles (SMP) exhibit manyfold higher NADH oxidase activities than are observed with mitochondria in the absence of sonication or membrane disruption (cf. Table II). We have observed 33-fold greater rates in ETP than

Table I. Results of Techniques Used to Determine Orientation of Membranes of Various Preparation Types[a]

Technique/assay	Side being tested[b]	Preaparation type						
		Mitochondria	ETP/SMP	Scrambled	Vesicle mixture	Vesicle/sheet mixture: Mitochondria	Vesicle/sheet mixture: Inverted	Sheet
NADH → O_2 activity	M	Low to Zero	+	+	+	+	+	+
Malate + glutamate oxidation	C	+	0	+ → 0	+	+	0	0
Stimulation of respiration by cyt c_a	C	+	→0	+	+	+	+	+
Inhibition of respiration by protamine	C	+	→0	+	+	+	+	+
Ascorbate-driven oxidase activity	C	+	→0	+	+	+	+	+
ATPase activity/ATPase inhibition	M	→0	+	+	+	+	+	+
90 Å spheres visible by EM	M	→0	+	+	+	+	+	+
ATPase inhibition by atractyloside	C	+	0	+	+	+	0	0
β-OH-Butyrate oxidation	M	0 → +	+	+	+	+	+	+
Glycerophosphate oxidation	C	+	0	+	+	+	+	+
Proton translocation		Out +	In +	?	?	Out + → 0	In + → 0	0

[a] Not observed unless membranes are washed with high ionic strength medium.
[b] Significant effect indicates this side is exposed.

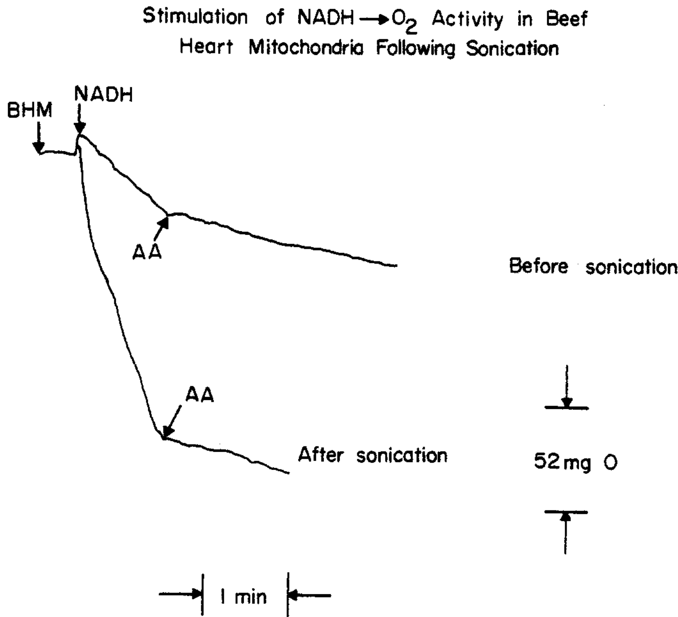

Fig. 2. Effect of sonication on antimycin A-sensitive (AA) NADH oxidase activity in beef heart mitochondria (BHM).

Table II. NADH Oxidase and Gluatmate + Malate Oxidation Rates in Oriented Vesicles (μg-atoms O/mg/min)[a]

Assay	Mitochondria	ETP/SMP	References/comments
NADH–O_2	0.79	3.74	BMH + 200 μg cyt *c*; Harmon and Basile, 1982
	0.43	8.36	BHM + cyt *c*; Harmon and Crane, 1974
	0.3–0.7	7.2	BMH + cyt *c*; Harmon and Crane 1976
	0.05	–	Pigeon heart; Rasmussen, 1969
	0.72	5.10	Beef, alkaline ETP; Crane *et al.*, 1956
Mal + Glu	0.2–0.3	–	Beef heart; Smith *et al.*, 1980
	0.059	–	BHM + ADP; Harmon, unpublished
	0.073	–	BHM + FCCP; Harmon, unpublished

[a]BHM = beef heart mitochondria; ADP = adenosine diphosphate; FCCP = carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; ETP = electron transport particle.

in intact mitochondria (Harmon and Crane, 1976). Unfortunately, any membrane preparation with substantial exposure of the matrix face, as would occur in "scrambled" vesicles or sheets, will exhibit high NADH oxidase activities due to exposure of the NADH dehydrogenase. As a result, comparison of NADH oxidase activities alone cannot be used as an indicator of orientation.

The procedure of Crane *et al.* (1956) provides large quantities of mitochondrial protein that exhibit considerable NADH oxidase activity that is stimulated at least twofold by exogenous cytochrome *c* (Harmon *et al.*, 1974). This procedure, while providing excellent starting material for SMP, ETP, or protein isolation, does not yield homogeneous physiologically oriented mitochondria.

Mild protease treatment of tissue is used to isolate coupled intact mitochondria from the hearts of numerous animals including rats (Vercesi *et al.*, 1978), pigeons (Chance and Hagihara, 1963), and chickens (Harmon and Sanborn, 1982; see Nedergaard and Cannon, 1979; for a review on mitochondrial isolation from various tissues). While the protease treatment yields excellent mitochondria that exhibit high RCR (respiratory control ratio) values and P/O (ADP/O) ratios approaching 2 or 3 for succinate and malate plus glutamate (respectively) as substrates, the yield of mitochondria can be limited. Coupled beef heart mitochondria can be isolated by homogenization of the tissue without proteolysis by procedures similar to those of Smith (1967) or Hatefi and Lester (1958); these mitochondria exhibit very low NADH oxidation rates and have ADP/O ratios approaching 2 and 3 as in pigeon heart mitochondria. In coupled mitochondrial preparations, the ability to phosphorylate and exhibit respiratory control while exhibiting low NADH oxidase activity is taken as strong evidence that the membranes are physiologically oriented and apparently intact, since they can generate and sustain pH and potential gradients.

The lack of NADH oxidase activity alone is not substantial proof for the existence of a physiological membrane orientation. Intact vesicular mitochondria are capable of oxidizing exogenous malate, glutamate, or pyruvate to generate NADH intramitochondrially while ETP and sheets of membrane, following washing to remove the soluble matrix enzymes, are incapable of utilizing tricarboxylic acid (TCA) intermediates. A preparation can be claimed to be intact if malate plus glutamate (or other TCA substrates) oxidation is present but NADH oxidase activity is not. If both activities are present, scrambled vesicles (if they exist) or mixed populations are present; high NADH oxidase but low malate plus glutamate oxidation activity (due to loss of matrix contents during opening and inversion (Huang and Lee, 1975; Robinson and Srere, 1985) indicates either inverted vesicles and/or open sheets of membrane.

One can envision instances where mitochondrial membranes temporarily "open up" and lose their matrix contents; resealing of the mitochondria might then leave them unable to oxidize NADH as well as TCA intermediates. On the other hand, it is also highly likely that the mitochondria would reseal around the matrix proteins during vesicle closure. The entrapment of compounds from the medium is known (Harmon and Crane, 1976), and the "loading" of mitochondrial membrane vesicles with cytochrome *c* during sonication of

mitochondria has been demonstrated (Lenaz and MacLennan, 1966; Lee, 1971). These findings give credence to the possibility that TCA substrate-driven respiration would then occur within the resealed vesicles. If loss of matrix during resealing is suspected, the presence of mitochondria can be ascertained by measuring exogenous NADH oxidase following sonication or following solubilization of the membrane with detergents. An alternate assay to determine if mitochondria are present would be to measure cytochrome *c* stimulation of respiration or ascorbate-driven cytochrome *c* oxidase activity in those intact vesicles (to be treated below).

A last area of concern regarding the use of TCA intermediates such as malate or succinate involves the possible shortage of exchangeable metabolites within a very small mitochondrially oriented vesicle. The small internal volume would contain few metabolites; respiration would be limited by the transport rate which is in turn limited by the amount of exchangeable metabolite. Small vesicles the size of SMP, lacking 90 Å knobs, have been reported (Y-particles; Huang *et al.*, 1973). These vesicles lack ATPase activity and have cytochrome *c* reactivity, but retain malate dehydrogenase activity, NADH-cyt *c* reductase (95% rotenone-sensitive), and monoamine oxidase activity, suggesting that these are small mitochondrially oriented vesicles. These vesicles (Y-particles) did not give enhancement of anilinonaphthalene sulfonate fluorescence typical of inverted vesicles (Huang *et al.*, 1973; Huang and Lee, 1975). These Y-particles were shown to possess oligomycin-sensitive ATPase activity and 4.8-fold stimulation of NADH oxidase activity by exogenous cytochrome *c* (Huang *et al.*, 1973), indicating that these small vesicles of predominantly mitochondrial orientation can hydrolyze ATP with an internal ATPase and that their enzymatic activities are not limited by their internal volume.

An important aspect of this discussion is the inability of a single assay to definitely determine the orientation of a membrane preparation. We propose the use of multiple assays to characterize a preparation. An example is the case where low NADH oxidase activity is present which may be due to the presence of mitochondria or the presence of non-inner membrane vesicles. Disruption of the permeability barrier (a second type of assay) will show if mitochondria are present. This protocol has been used to localize dehydrogenases on the matrix face.

Another variety of substrate-dependent assays involves the activity of glycerol phosphate and β-OH-butyrate dehydrogenases. The membrane is impermeable to these substrates. Glycerol phosphate dehydrogenase is located on the C-side of the membrane and will display latent activity in ETP/SMP because of the impermeant nature of glycerol phosphate (Klingenberg and Buchholz, 1970; Donnellan *et al.*, 1970). β-OH-butyrate dehydrogenase displays latent activity in intact mitochondria and is located

on the M-side (McIntyre *et al.*, 1978). Thus, mitochondria will exhibit low β-OH-butyrate and high glycerol phosphate dehydrogenase activities in comparison to the activities in homogeneously inverted vesicles. Both enzymes would be active in sheets; intermediate activities will be observed with other membrane populations depending on the proportions of each membrane type in the preparation.

Cytochrome *c* (cyt *c*) is a basic impermeant extrinsic protein readily removed from the cytoplasmic surface by washing the membrane in 0.15 M KCl or equivalent ionic strength medium (Jacobs and Sanadi, 1960; Lee, 1971; Lenaz and MacLennan, 1966). Mitochondria isolated in low ionic strength medium will still contain most or all of their endogenous cyt *c* (Lee, 1971). If cyt *c* is partially removed from the membrane, respiration is apparently limited by the amount of cytochrome retained (Jacobs and Sanadi, 1960; Lenaz and MacLennan, 1966; Harmon, 1982). Succinate oxidase in intact cyt *c*-depleted mitochondria or open sheets should be markedly stimulated by addition of exogenous cyt *c*, while succinate oxidation in inverted vesicles should not be affected by exogenous cytochrome *c* (cf. Table III). Succinate is transported into the mitochondria via the dicarboxylate transporter to react with the dehydrogenase on the M-side.

Intact mitochondria that exhibit low NADH oxidation activities (cf. Tables I and II) also show marked stimulation of respiration by addition of exogenous cytochrome *c* with either NADH or succinate as substrates. "Y-particles" isolated following sonication of mitochondria (Huang *et al.*, 1973) exhibit NADH–cyt *c* reductase activity and a 4.8-fold stimulation of NADH oxidase activity following cyt *c* addition, indicating that these vesicles are predominantly oriented like mitochondria. Intact beef heart mitochondria with 20-fold stimulation by cyt *c* and low NADH oxidase activity (220 ng-atom O/min/mg protein) in the presence of cyt *c* have been reported (Harmon and Crane, 1976). Muscatello and Carafoli (1969) reported an 8-fold stimulation of respiration by cyt *c* in rat liver mitochondria. Conversely, inverted particles with 90 Å knobs exposed and high NADH oxidase activity are not stimulated by exogeneous cyt *c*. ETP isolated by alkaline homogenization (Crane *et al.*, 1956) can show 0–5% stimulation of respiration. SMP isolated by sonication of beef heart mitochondria (Harmon, 1982) exhibit less than 3% stimulation; "X-particles" isolated following sonication of beef heart mitochondria and density gradient centrifugation (Huang *et al.*, 1973; Huang and Lee, 1975) show less than 10% stimulation of NADH oxidase by exogenous cytochrome *c*. Thayer and Rubin (1979) isolated inverted vesicles from rat liver mitochondria that show less than 6% stimulation of NADH oxidase; Moury and Crane (1964) isolated an ETP from rat liver that is stimulated less than 10% by exogenous cyt *c* while D'Souza and Lindsay (1981) isolated SMP from rat liver mitochondria

Table III. Effect of Cytochrome c on Respiration in Oriented Particles

	μg-atoms O/min/mg protein				
	Mitochondria		ETP/SMP		
Substrate	$-$cyt c	$+$cyt c	$-$cyt c	$+$cyt c	References/comments
NADH	0.02	0.22	6.9	7.2	Harmon and Crane, 1976; KCl-washed beef heart
	–	–	3.9	3.9	Crane *et al.*, 1956; beef heart
	–	–	0.352	0.375	Thayer and Rubin, 1979; rat liver + CCCP[a]
	0.2	0.47	0.86	0.88	Gautheron *et al.*, 1977; pig heart
	0.043	0.43	8.36	8.36	Harmon and Crane, 1974; KCl-washed beef heart
	0.7	1.7	2.8	2.8	Fleischer *et al.*, 1974
	–	–	0.66	0.705	Huang and Lee, 1975; X-particles
	–	–	0.68	0.75	Huang and Lee, 1975; KCl-washed X-particles
	–	–	0.407	0.75	Huang and Lee, 1975; Y-particles
			0.332	0.987	Huang and Lee, 1975; KCl-washed Y-particles
Succinate	–	–	0.92	1.0	Crane *et al.*, 1956; beef heart
	0.126	1.26	3.46	3.46	Harmon and Crane, 1974; beef heart
	–	–	0.018	0.019	Moury and Crane, 1964; rat liver
	0.08	1.02	1.83	1.94	Harmon and Basile, 1982; KCl-washed beef heart
	0.51	0.96	0.96	1.15	Fleischer *et al.*, 1974
	–	–	0.163	0.159	Lenaz and MacLennan, 1966; beef heart
	0.038	0.195	0.173	0.182	Lenaz and MacLennan, 1966; KCl-washed beef
Ferrocytochrome c (μmol cyt c oxidized/min/mg)					
	0	3.28	0.73	0.83	Eytan *et al.*, 1975; beef heart

[a] CCCP = carbonylcyanide-*m*-chlorophenylhydrazone.

that exhibit more than 9-fold stimulation of cytochrome oxidase activity by cytochrome addition in the presence of deoxycholate (to expose the latent cytochrome oxidase activity and cyt c binding site within the SMP vesicle).

Cyt *c* can be displaced from the membrane by basic high molecular weight (and therefore impermeant) proteins such as poly-L-lysine or protamine sulfate. We have observed (cf. Table IV) these proteins to inhibit respiration in those membranes with the cyt *c* site exposed; 90% or greater inhibition has been observed for ubiquinol-10 or duroquinol–cyt *c* reductase activities (Hare and Crane, 1971), NADH–cyt *c* reductase, NADH oxidase, and cytochrome oxidase activities (Smith and Minnaert, 1965), and succinate–cyt *c* reductase, succinoxidase, and cytochrome oxidase activities in beef heart mitochondria. Greater than 95% inhibition of succinoxidase in intact beef heart mitochondria that exhibit low NADH oxidase activity (Harmon *et al.*, 1974; Harmon and Crane, 1976) has also been observed. Ruzicka and Crane (1971) have demonstrated that high levels of poly-L-lysine act as an inhibitor of NADH-duroquinone activity in inverted vesicles; as a result, polylysine inhibitions can be observed in inverted as well as noninverted vesicles if NADH is used as substrate. For this reason, inhibition of respiration by polylysine/protamine should be measured only with succinate as substrate to avoid possible misinterpretation due to the inhibition of NADH-linked activities in inverted vesicles.

Smith *et al.* (1980) observed up to 7-fold stimulation of cytochrome oxidase activity by cyt *c* addition to SMP derived by sonication. Inhibition of cytochrome oxidase activity due to endogenous cyt *c* was not affected by added polylysine while activity due to exogenous cytochrome *c* was virtually inhibited by polylysine. Smith *et al.* (1980) interpreted these data to indicate that the SMP preparation contained approximately equal amounts of inverted and noninverted particles. Moore and Bonner (1981) observed 76% inverted orientation of SMP from mung bean mitochondria on the basis of cytochrome *c* stimulation and inhibition of respiration by 0.25 μM polylysine; they were able to study only inverted particles by inclusion of polylysine in the assay medium. Moore and Bonner (1981) used polylysine addition to "substrate select" activity of inverted particles in a mixture of inverted and noninverted vesicles to measure H^+/ATP, Δ pH, and membrane potentials. Polylysine was used to inhibit the NADH-driven respiration of noninverted vesicles from mung beans with cyt *c* exposed; thus only the activity of inverted vesicles was measured. While this method seems viable, Moore and Bonner (1981) note that errors in the estimate of sucrose-impermeable space and calculation of the phosphorylation potential may arise due to the presence of the (inhibited) mitochondria.

Conversely, addition of polylysine or protamine to inverted vesicles inhibits respiration less than 6% (Harmon *et al.*, 1974; Harmon and Basile,

Table IV. Effect of Polycationic Proteins on Respiration in Oriented Vesicles

Assay	% Inhibition of Control Activity		References/comments
	Mitochondria	Inverted vesicles	
NADH → O_2	80	< 10	Smith and Conrad, 1956; Keilin–Hartree particles + polylysine
NADH → O_2	80	< 10	Smith and Minnaert, 1965; Keilin–Hartree
NADH → cyt *c*	> 90	> 90	Smith and Minnaert, 1965; not oriented
cyt *c* → O_2	> 90	> 90	Smith and Minnaert, 1965; not oriented
Ascorbate + PMS → O_2	> 95	0	Harmon, unpublished
Ascorbate + PMS → O_2	–	0	Smith *et al.*, 1980; endogenous cyt *c*
Ascorbate + PMS → O_2	–	80–100	Smith, *et al.*, 1980; exogenous cyt *c*
Succinate → O_2	78	0	Harmon *et al.*, 1974; beef ETP; polylysine
Succinate → O_2	88	0	Harmon, unpublished; beef ETP; protamine sulfate
Glutamate → O_2	> 80	–	Schwartz, 1974; rat liver
Ferrocyanide → O_2	85	0	Harmon, unpublished
NADH → CoQ	17	35	Harmon, unpublished
NADH → duroquinone	20	53	Harmon, unpublished

1982) and has no effect in inverted vesicles from mung bean mitochondria (Moore and Bonner, 1981). Open sheets will show extensive inhibition while "scrambled" (if they exist) or mixed preparations will show intermediate effects.

The presence of ascorbate-driven cytochrome oxidase activity in the absence of a mediator such as phenazine methosulfate has been used as an indication of exposure of cyt *c* and its binding site (Tsou, 1952; Lee, 1971; Hackenbrock and Hammon, 1975). Ascorbate cannot act as substrate for cytochrome oxidase (Hackenbrock and Hammon, 1975; Lee, 1971, 1979); ascorbate oxidation proceeds via exposed cyt *c*. Ascorbate-driven activity will be observed in those preparations with cyt *c* present in its exposed binding site (mitochondria or sheets). Smith and Ragan (1980) observed rapid reduction of soluble cyt *c* by ascorbate in the presence and absence of mitochondria as did Quintanilha and Packer (1977); in contrast, the reduction of cyt *c* in SMP was 1–2 orders of magnitude slower.

Just as disruption of the membrane permeability barrier to NADH in intact mitochondria stimulates NADH activity, disruption of the permeability barrier of inverted vesicles allows cyt *c* access to its binding site. Mackler and Green (1956) first showed a 5- to 6-fold increase in cyt *c* oxidase and NADH–cyt *c* reductase activities in ETP by the addition of deoxycholate. D'Souza and Lindsay (1981) observed a 10-fold stimulation of cytochrome oxidase by opening the membrane with deoxycholate. Lenaz and MacLennan (1966) isolated cyt *c*-deficient particles and observed a 6-fold increase in succinoxidase by cyt *c* only if deoxycholate were present. Under similar conditions, Crane *et al.* (1956) observed a 12-fold stimulation of succinoxidase. Gautheron *et al.* (1977) and Huang *et al.* (1973) demonstrated similar findings using Lubrol as detergent. Huang *et al.* (1973) showed that cyt *c* oxidation by cytochrome oxidase did not readily occur in inverted "X-particles" in contrast to noninverted "Y-particles"; Lubrol stimulated cyt *c* oxidase activity 8-fold in "X-particles" (cyt *c* gained access to its binding site) but only 2-fold in "Y-particles." Harmon (1982) induced cyt *c* stimulation of beef heart ETP by brief sonication. These citations all illustrate the concept that inverted vesicles sequester cyt *c* and lack the ability to reduce or oxidize external cyt *c*. Cyt *c* reactivity can be restored if the impermeability barrier of the vesicle is altered.

The observance of 90 Å knobs, spheres, or "lollipops" on the surface of negatively stained membranes using electron microscopy was instrumental in determining the locus of ATPase on the M-side (see review by Harmon *et al.*, 1974). This technique can be used in the determination of orientation but is time-consuming and frequently not reliable (Malviya *et al.*, 1968) since a smooth-appearing membrane may be inverted but lacking ATPase (Kagawa and Racker, 1966).

Measurement of ATP hydrolysis activity cannot be used as an indicator of membrane orientation since both inverted and noninverted mitochondria are capable of oligomycin-sensitive uncoupler-stimulated ATPase activity (Huang and Lee, 1975; Kagawa and Racker, 1966; Harmon, 1982); mitochondria, even with their ATPase sequestered within the membrane, are capable of ATP hydrolysis with the normally impermeant ATP transported into the mitochondria by the ATP/ADP translocator.

Atractylate (or carboxyatractylate) is an impermeant inhibitor of the ADP/ATP translocator in the inner membrane. In intact mitochondria (coupled or uncoupled), exogenous ATP is transported across the membrane to the matrix where hydrolysis via the ATPase on the M-side occurs. Atractylate displaces ADP and ATP from the carrier and binds to the carrier on the C-side; alternatively, permeant bongkrekic acid will bind at the M-side (in the presence of ADP) and fix the carrier in that state on the M-side (Klingenberg, 1981). The addition of either inhibitor to mitochondrially oriented vesicles will inhibit ATP hydrolysis and ATP-driven reactions such as proton efflux driven by ATPase. In ETP/SMP or sheet systems where the ATPase is exposed and activity is not dependent on the carrier-mediated ATP (in contrast to mitochondria), these inhibitors are without effect (Wehrle *et al.*, 1978; Huang *et al.*, 1973). Astle and Cooper (1974) demonstrated up to 98.7% inhibition of P_i uptake and 86% inhibition of 6-*p*-toluidinonaphthalene-2-sulfonate fluorescence (driven by ATP hydrolysis) by atractylate in intact rat liver mitochondria, but only 5 and 8% inhibition of these activities in sonicated inverted vesicles (all activities were over 98% oligolmycin-sensitive in both membrane types), illustrating the utility of this assay in determining orientation. Rosier *et al.* (1980) determined that atractyloside inhibits 95% of phosphorylation in noninverted particles but only 7% in submitochondrial particles.

Mitochondrial ATPase contains a small polar inhibitor polypeptide (Horstman and Racker, 1970) readily isolated and soluble in water; as such it is expected to be unable to cross the membrane. Presence of the inhibitor will inhibit ATPase/synthase-mediated reactions. Hydrolysis of ATP does not readily occur unless the inhibitor subunit can be removed from the ATPase complex as occurs during respiration or ADP phosphorylation (Harris *et al.*, 1979; Schwerzmann and Pedersen, 1981) or during ATP hydrolysis (Husain and Harris, 1983). In SMP, ATP hydrolysis or respiration leads to a release of bound inhibitor into the solution (Husain and Harris, 1983); ATP synthesis also causes inhibitor release in SMP (Schwerzmann and Pedersen, 1981).

Wehrle *et al.* (1978) added the inhibitor protein to inverted mitochondrial vesicles from rat liver and observed 94% inhibition of ATPase activity; the same membrane fraction was not affected by atractyloside, indicating the

inverted nature of the vesicles. Unfortunately, the experiment was not repeated with intact mitochondria. On the positive side, the atractyloside insensitivity correlates well with the inhibition of ATPase by the inhibitor protein and the presence of 90 Å knobs on the surface of negative-stained SMP. Furthermore, these vesicles exhibit respiration-driven H^+ uptake. Harmon (1982) was able to demonstrate 97 and 98% inhibition of ATPase activity in ETP and SMP, respectively, by the inhibitor peptide and only 4 and 3% availability of cyt *c* in those same particles. In contrast, addition of the peptide inhibitor to intact mitochondria resulted in only 8% inhibition of ATPase activity. Intact mitochondria show 12-fold stimulation of respiration by cyt *c*. SMP isolated by the sonication procedure of Huang *et al.* (1973) show 25% stimulation of respiration by exogenous cyt *c* and only 80% inhibition of ATPase (Harmon, 1982). Thus, the sensitivity of ATPase to its inhibitor correlates well to the lack of cyt *c* reactivity. The two assays demonstrate the extent to which the membrane surface is exposed.

The use of experimental protocols to "select" the function of one orientation in a mixture of oppositely oriented vesicles is not without drawbacks. NADH is oxidized slowly by mitochondria (Lehninger, 1955; Rasmussen, 1969) (but not energy-linked) as well as by ETP/SMP; succinate is used by both vesicles and cannot be used to select functional populations. Specific antibody reactions involving cyt *c* provide opportunities where populations can be selected by inhibiting the activity of one vesicle population. Measurements of membrane potential or pH gradient may be adversly affected by substrate selection procedures depending upon conditions.

The reported values of the internal volume of SMP range between 0.3 (Berry and Hinkle, 1983) and 2.5 μl/mg protein (Papa *et al.*, 1973b), while the values for intact mitochondria range between 0.25 and 1.0 μl/mg protein (Harris and van Dam, 1968; Mitchell and Moyle, 1969; Bentzel and Solomon, 1967; Papa *et al.*, 1973a, b; Branca *et al.*, 1981).

Berry and Hinkle (1983) concluded that they may be underestimating the value of $\Delta\mu H^+$ in phosphorylating rat liver SMP, possibly due to the presence of vesicles incapable of generating membrane potentials or pH gradients. They state that these non-energy-linked vesicles (of either orientation) would have to contribute 40% of the total internal volume of the sample to explain the possible underestimation of $\Delta\mu H^+$. If 20% of the SMP were uncoupled mitochondria, then the total internal volume would be 88% of that expected if the preparation were 100% SMP (based on the values of 2.5 and 1.0 μl/mg protein for mitochondria and SMP, respectively; the difference is even less if similar values for internal volumes are used). Berry and Hinkle used particles of over 90% orientation (as determined by electron microsopic examination for ATPase molecules) and thus did not encounter this error.

Measurements involving the determination of internal volume, such as measurement of membrane potentials by thiocyanate or perchlorate uptake or measurement of pH gradients by ethylamine uptake, are apparently not affected by possible changes in internal volume resulting from the presence of oppositely oriented vesicles as shown in the previous example. The number of ions taken up is likely to be affected, however, if the uptake is unidirectional or if the uptake occurs in only one vesicle type. Thus a 20% contamination of mitochondria in an SMP preparation could result in a 40% decrease in ion uptake (compared to 100% SMP) and a corresponding underestimation in membrane potential or ΔpH.

pH electrode measurements (Reynafarje *et al.*, 1979) are particularly susceptible to error introduced by the presence of contaminating vesicles. In the case of SMP using succinate as substrate, 20% contamination by coupled mitochondria could lead to an observed rate of change of pH or H^+/O ratio only 60% of that observed with 100% SMP (assuming that both particles have the same buffering and coupling capacities). Even a 20% contamination with uncoupled mitochondria that are capable of respiration but not H^+ translocation would still give an H^+/O ratio 60% or less than the maximum value obtained with 100% SMP for the following reasons:

1. The uncoupled mitochondria still consume O but do not translocate protons; hence H^+/O decreases.
2. At pH 8.0 the total buffer capacity of uncoupled mitochondria is twice the buffer capacity of coupled mitochondria, while at pH 7.0 the total buffer capacity of uncoupled mitochondria is three times that of coupled mitochondria (Mitchell and Moyle, 1967). Protons transported by the SMP will be buffered by the uncoupled mitochondria; H^+ becomes a small and H^+/O decreases even more! Even if the buffer capacities of the SMP and coupled mitochondria are similar, uncoupled mitochondria are three times more effective in suppressing pH changes than are SMP; this could lead to large errors in the calculation of H^+/O or $H^+/2e^-$ ratios.

At pH 6, the buffer capacities of the vesicle interior and vesicle-suspending medium of ASU particles (inverted particles isolated following sonication in the presence of ammonia and their subsequent exposure to urea and Sephadex) (Racker and Horstman, 1967) are 10 and 43 ng H^+ pH unit^{-1} mg protein^{-1}, respectively (Hinkle and Horstman, 1971), while at pH 6 the inner and outer buffer capacities of rat liver mitochondria are approximately 30 and 43 ng H^+ pH unit^{-1} mg protein^{-1}, respectively (Mitchell and Moyle, 1967). Thus the total buffer capacity of uncoupled rat liver mitochondria is 38% greater than that of uncoupled inverted vesicles (73 vs. 53 ng H^+ pH unit^{-1} mg protein^{-1}). It is therefore conceivable that a 20% contamination by uncoupled

mitochondria could result in a 52% decrease in theoretical maximum value of $\Delta\mu H^+$ (compared to when only 100% inverted vesicles are used).

The underlying concept of this discussion is that a population of desired orientation can be functionally "selected." If the orientation of the preparation and other parameters (buffer capacity, internal volume, etc.) of the two vesicle types are known, the data can be corrected to reflect the activity for only one vesicle type.

Membrane Isolation

Intact Mitochondria

Intact mitochondria in this work is interpreted to mean vesicular physiologically oriented mitochondria and need not indicate the presence of "coupling"; it is indeed possible to have uncoupled mitochondria that are perfectly oriented. For studies of topography or respiration, for example, these uncoupled mitochondria are desirable since enzyme activity is maximal and ΔpH and membrane potential are minimal.

There are two basic procedures used to isolate mitochondria: limited proteolysis and/or homogenization sufficient to disrupt the tissue and cell structure but not the mitochondria. Techniques to isolate coupled mitochondria from rat liver, kidney, brain, skeletal muscle, heart, and other organs are well documented (for a review see Nedergaard and Cannon, 1979). For topographical studies or proton translocation studies where large quantities of mitochondria are needed for each assay, isolation of large amounts of material from heart is often desirable. For example, gram quantities of beef heart mitochondria (cytochrome *c*-depleted or containing) can quickly be isolated by the procedure of Harmon and Crane (1976). Coupled beef heart mitochondria can easily be isolated by procedures similar to that of Smith (1967). Coupled mitochondria can be isolated from rat heart or liver, pigeon breast, dog, rabbit or chicken heart, smooth muscle, and pigeon heart as well as other sources too numerous to mention. Coupled mitochondria exhibit ADP-stimulated respiration (Chance and Hagihara, 1963) as well as other energy-linked functions such as proton translocation (Mitchell and Moyle, 1969). That these mitochondria are able to establish ΔpH and membrane potentials is an indication of their intactness and impermeability toward molecules as small as a proton.

Inverted Particles

Inverted mitochondrial membranes are usually isolated by centrifugation following one of two basic procedures.

1. Homogenization or mechanical disruption of heart mince is utilized to isolate Keilin–Hartree particles (Keilin and Hartree, 1940). As shown

Table V. Parameters of SMP before and after Cytochrome *c* Affinity Chromatography Treatment

	Succinate oxidase activity (nmol O_2/min/mg)		Percent inversion	Cytochrome ratio[b]	
	−cyt *c*	+cyt *c*		$c + c_1/a$	$c + c_1/b$
Before treatment	612	840[a]	73	0.86	1.26
After treatment	621	665[a]	94	1.11	1.58
Before/after[c]	0.99	1.27	0.78	0.78	0.80
Other particles SMP (Harmon, 1982)	1340	1380[a]	97	1.0	1.17
X-particles (Huang and Lee, 1975)[d]	660	700[e]	94	0.82	0.91
X-particles, KCl-washed	680	750[e]	91	0.84	0.90
Y-particles (Huang and Lee, 1975)[d]	407	750[e]	54	0.87	0.94
Y-particles, KCl-washed	332	987[e]	34	0.59	0.60

[a] 200 μg or more cyt *c* added.
[b] Molar ratios.
[c] Ratio of data on SMP loaded on column to that of eluted SMP.
[d] NAOH oxidase.
[e] 3.3 μM cyt *c* added.

previously (Harmon *et al.*, 1974; Vinogradov and King, 1979), these particles are not homogeneously inverted but useful for protein isolation procedures.

Homogenization of isolated beef heart mitochondria in the presence of either KCl or KOH (Crane *et al.*, 1956) has been used to isolate electron transfer particles. In the hands of the author, the KOH (alkaline) treatment yields excellent ETP that are greater than 94% inverted as shown previously (Harmon *et al.*, 1974; Harmon and Crane, 1976; cf. Tables II–V).

2. Sonication of isolated mitochondria was introduced in the early 1960's to rapidly yield preparations of submitochondrial particles (SMP). As shown previously by Harmon *et al.* (1974) and others (Huang and Lee, 1975; Smith *et al.*, 1980; D'Souza and Lindsay, 1981), the orientation of sonicated SMP can be extremely variable, and the preparations may contain vesicles of different composition, enzymatic function, and orientations (Malviya *et al.*, 1968; Huang *et al.*, 1973; Huang and Lee, 1975). Some preparations may contain outer membrane proteins as well (Albracht and Heidrich, 1975). Other procedures in use also are effective in isolating coupled SMP from various types of mitochondria (Thayer and Rubin, 1979; Wehrle *et al.*, 1978; Berry and Hinkle, 1983; Harmon, 1982), including French pressure isolation of SMP from mung bean mitochondria (Moore and Bonner, 1981).

Nonhomogeneously Oriented Vesicles

An experienced worker can isolate well-oriented preparations of mitochondria with almost certain regularity, while homogeneously inverted

vesicles are isolated in practice with less regularity. As shown in Fig. 1 and explained earlier, multiple subclasses of nonhomogeneous vesicles exist that putatively include mixtures of inverted, noninverted, or nonvesicular membranes as well as "scrambled hybrids" (if they exist). Such particles can be detected by routine verification using the rapid simple assays described in this paper.

If the percent homogeneity (orientation) is not at the level desired, the preparation can be "cleaned up" by a number of procedures used by this author and other investigators.

1. Specific antibodies can be prepared against mitochondrial proteins such as ATPase or cytochrome *c*. Addition of anti-ATPase to a preparation will precipitate membranes with exposed ATPase. Use of specific antibodies to isolate membrane fractions originated with the work of Hare *et al.* (1974) who used anti-ATPase to isolate inverted vesicles of *E. coli.* An effective modification of this procedure was presented by D'Souza and Lindsay (1981) who found that wheatgerm agglutinin binds to carbohydrate on the cytoplasmic surface; removal of noninverted membranes resulted following addition of anti-agglutinin antibodies. Eytan *et al.* (1975) were able to isolate highly inverted ETP (no reactivity with exogenous cyt *c*) by immunoprecipitation of noninverted membranes in their preparation with specific antibodies against subunits II, V, and VI of cytochrome oxidase on the C-side of the membrane.

Addition of anti-cyt *c* antibodies to a preparation containing noninverted membranes, fragments, or scrambled hybrids will precipitate those membranes with the cyt *c* site exposed. Sonication of beef heart mitochondria yielded a preparation of vesicles that were only 73% inverted (unpublished data) on the basis of cyt *c* stimulation and protamine inhibition of succinoxidase as performed by the procedure of Harmon (1982). Incubation of this preparation with polystyrene beads coated with anti-cyt *c* antibodies followed by low-speed centrifugation resulted in the binding of non-inverted vesicles to the beads; the vesicles in the supernatant exhibited 92% inversion, indicating that the preparation could be enriched in inverted vesicles by this procedure.

2. Affinity chromatography with cyt *c* immobilized on CNBr-activated Sepharose to separate noninverted membranes from submitochondrial particle preparations has been utilized by Godinot and Gautheron (1979) and Lotscher *et al.* (1979) with considerable success. Gautheron *et al.* (1977) used the column to determine if a mitoplast preparation contained inverted membranes. Those membranes with cyt *c* binding sites exposed (mitochondria, open vesicles, sheets, or "scrambled" membranes) bind to the immobilized cyt *c* and are retarded in their flow while inverted vesicles with cyt *c* sequestered in the vesicle are rapidly eluted. This procedure requires KCl-washed membranes with cyt *c* removed from noninverted membranes to allow

maximum retention on the column and is an effective means of enriching the inverted vesicle content in a nonhomogeneously inverted preparation.

3. Recentrifugation of ETP derived by either sonication or KOH homogenization that exhibit only 73% inversion followed by resuspension of the top 1/2 to 2/3 of the pelleted membranes has been shown to yield a membrane preparation of over 95% inversion (Harmon, unpublished data). This simple procedure suggests that the smaller inverted vesicles (Malviya *et al.*, 1968) sediment slower than the noninverted or nonvesicular membranes.

The Nature of Nonhomogeneously Oriented Particles

That nonhomogeneously oriented preparations can be "cleaned up" to yield highly oriented preparations indicates that these preparations are a mixture of completely inverted and effectively noninverted vesicles (sheets, etc). If this were not the case, highly inverted vesicles could not be isolated from these preparations.

This point bears closer investigation since numerous reports (Chance *et al.*, 1970; Rosier *et al.*, 1980; Storey *et al.*, 1980) have indicated the presence of scrambled hybrids. Data in Table V indicate the exposure of cytochrome *c*-binding sites in KCl-washed SMP following sonication; exogenous cyt *c* results in 37% stimulation of succinoxidase. Passage of these SMP down a cytochrome *c* affinity column according to the method of Godinot and Gautheron (1979) results in membranes with less than 7% stimulation by exogenous cyt *c*. The ratio of cytochrome *c* and *c*l to cytochromes aa_3 and the $c + c_1/b$ ratio increase to the same extent as the percent inversion of the preparation (cf. Table V). Affinity column treatment causes an increase in 552 nm absorbance relative to the 562 or 604 nm absorbances, indicative of removal of membranes not containing cytochrome *c* (spectra not shown). These cytochrome ratios are similar to published values. SMP after treatment have ratios similar to those of well oriented (94%) SMP (Harmon, 1982) while SMP before treatment have ratios similar to particles derived by sonication in medium containing EDTA (Huang and Lee, 1975). Table V also lists data from Huang and Lee (1975); cytochrome ratios were calculated by this author from low-temperature (77 K) spectra of X- and Y-particles isolated by density gradient centrifugation following sonication. For comparison, NADH oxidase activities and cytochrome ratios for X- and Y-particles washed with KCl to remove any exposed cyt *c* are also provided. X-particles of almost (> 90%) total inversion show no decrease in cytochrome ratios or changes in cyt *c* stimulation of NADH oxidase by KCl treatment while Y-particles, of nonhomogeneously oriented nature, show a decrease in NADH oxidase activity, cyt *c* stimulation

of respiration, and changes in cytochrome ratios following the salt treatment. Huang and Lee (1975) agree that the X-particles are well oriented and that the Y-particles are not. KCl-washed membranes with the cyt *c* site exposed (but not occupied) have low cyt *c*/*a* and *c*/*b* ratios. If these sites are filled (by exogenous cyt *c*) or removed (by removal of the membranes with those sites), the *c*/*a* and *c*/*b* ratios will increase.

The findings of D'Souza and Lindsay (1981) and Moore and Bonner (1981) also substantiate the concept that nonhomogeneous preparations are mixtures of the two vesicle types. The former authors were able to separate the two fractions by immunoprecipitation (as was this author) while Moore and Bonner were able to "substrate select" or "selectively deactivate" the vesicles not desired. The affinity column procedure of Gautheron and co-workers also gives results consistent with the idea of mixtures of oppositely oriented vesicles or vesicles and opened membranes (sheets) and does not support the idea of "scrambled hybrid" vesicles.

Concluding Remarks

The importance of correctly defining the orientation of membrane vesicle preparations cannot be overstated. It is hoped that this work will allow more accurate determination of vesicle orientation to elucidate the structural and functional aspects of membrane-bound enzyme systems. While this work has focused on mitochondrial membranes, the general concepts of utilizing multiple markers on opposite membrane faces can be extended to other membrane systems.

The data presented clearly substantiate the ability to determine membrane vesicle orientation by using enzymatic assays or respiratory rates, exposure of cytochrome *c* sites, and ATPase exposure. Using these criteria, the orientation of some vesicles has been examined; space limitation prohibits evaluation of all particles.

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