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THE CORRELATION BETWEEN THE SATURATION OF MEMBRANE FATTY ACIDS AND THE PRESENCE OF MEMBRANE FRACTURE FACES AFTER OSMIUM FIXATION

ROBERT JAMES AND DANIEL BRANTON
Department of Botany, University of California, Berkeley, Calif. 94720 (U.S.A.)
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SUMMARY

In osmium-fixed membranes, there is a decrease in the membrane fracture faces concomitant with an increase in unsaturation of the membrane fatty acids. The data suggest that both the number of double bonds and their position in the fatty-acid chain are critical to the disappearance of membrane fracture faces.

INTRODUCTION

Membranes can be split and their inner hydrophobic face can be exposed by the fracture process of freeze-etching[1-3]. These fracture faces have been the object of many detailed electron microscopic examinations (for reviews see refs. 3–5). Because the fracture process depends upon the presence of membrane lipids[6,7], it may also depend upon the organization of these lipids within the membrane.

One approach relating the appearance of the fracture faces to the organization of lipids has utilized model systems containing lipids in known phases[8,9]. The results showed that lamellar phase lipids consistently produce fracture faces resembling extended, smooth sheets[9]. Another way of relating the fractures to the lipids involves purposeful perturbations of lipid components in well characterized artificial and biological membranes. In this paper we examine the effects of OsO_4 on the fracture properties of liposomes and *Mycoplasma laidlawii* cell membranes containing lipids of various degrees of saturation.

We chose *M. laidlawii* because X-ray diffraction[10,11], calorimetry[12,13], and electron paramagnetic resonance[14,15] show that most of the lipids are organized as a lamellar bilayer. Furthermore, the polar lipids of *M. laidlawii* reside almost exclusively in the cell membrane[16], and the fatty-acid composition of these polar lipids can be altered by supplementing the growth medium[17,18].

Previous studies of the effects of OsO_4 fixation on the freeze-etch fracture process have led to apparently contradictory observations. In *Bacillus subtilis*, OsO_4 had little effect[19,20], whereas in chloroplasts (R. B. PARK AND D. BRANTON, unpublished observation), mitochondria[21], and yeast cells (M. MOOR, personal communication), OsO_4 fixation resulted in a striking loss of membrane face fractures; only cross fractures occurred. It appeared to us that the fatty-acid composition of these

different membrane types could account for the differing effects of OsO_4. In *B. subtilis*, there are almost no unsaturated fatty acids[22,23], whereas in chloroplasts, for example, 95 % of the fatty acids are the unsaturated linolenic and linoleic acids[24].

MATERIALS AND METHODS

Culture conditions

M. laidlawii was grown statically at 37° in 10–500 ml of medium in glass tubes or Erlenmeyer flasks. The medium was modified only quantitatively from that of RAZIN *et al.*[18], and 1 l contained the following: 20 g acetone-extracted Difco Tryptose, 10 g glucose, 5 g NaCl, 3.7 g Tris, 4 g very fatty-acid-poor bovine albumin (Grade B, CalBiochem), and 100000 units potassium penicillin G. The unadjusted pH of this medium was 8.3. Fatty-acid supplements were added as ethanol solutions to give a final concentration of 120 μM. The ethanol in the medium did not exceed 0.5 %. Cells were harvested during late-log phase by centrifugation at 10000 $\times$ *g*. Cells were washed 3 times in β-buffer[18] diluted 1:4 and then were either (1) lipid-extracted, (2) dispersed in growth medium *minus* penicillin and fatty-acid supplement for use in OsO_4-fixation experiments, or (3) prepared for freeze-etching.

Lipid extraction

After washing and pelleting, the cells for lipid extraction were weighed wet, and 10 vol. methanol (vol. per cell wet wt.) was added to the cells in glass centrifuge tubes. The cells were macerated using a glass rod and then incubated at 65° for 5 min. 20 vol. chloroform (vol. per cell wet wt.) were added, and the suspension was incubated at 65° for 20 min with intermittant maceration[25]. The suspension was then filtered through lipid-extracted Whatman No. 1 filter paper. The filtrate was washed 3 times with chloroform–methanol (2:1, v/v).

Liposome preparation

Dipalmitoyl lecithin, $\geqslant$ 96 % pure, (Applied Science Laboratories, State College Pa). was used as the saturated lipid in the liposome experiments. Asolectin, $\geqslant$ 95 % phosphatides, containing approx. 3 % linolenic acid and 37 % linoleic acid by wt., (Associated Concentrates, Woodside, N.Y.) was used as the unsaturated lipid.

The methods for preparation of liposomes were adapted from those of BANGHAM, *et al.*[26] and REEVES AND DOWBEN[27]. For the extracted lipids, portions of the chloroform–methanol extract were placed in Erlenmeyer flasks, the solvent was evaporated by a moist stream of N_2, and the remaining lipid was weighed. For the commercial lipids, weighed portions were dissolved in 2 vol. of chloroform (v/w) and dried under a moist stream of N_2.

To the dried preparation, distilled water was added to give a dispersion containing 0.1 % lipid (w/v). These dispersions were incubated for 1 h at 50° under N_2 and with frequent shaking. The liposomes produced in this way were either harvested by centrifugation at 15000 $\times$ *g* and then frozen or resuspended and fixed with OsO_4.

Fixation, freeze-etching and electron microscopy

Resuspended cells and liposomes were combined 1:1 (v/v) with 2 % OsO_4 in a 0.1 M phosphate buffer (pH 6.8) and fixed for 2 h at 4°. The fixed cells and liposomes

were then pelleted at 10000 × *g* and 15000 × *g*, respectively. Cells were washed 2 times in 0.05 M phosphate buffer and pelleted. Liposomes were not rinsed.

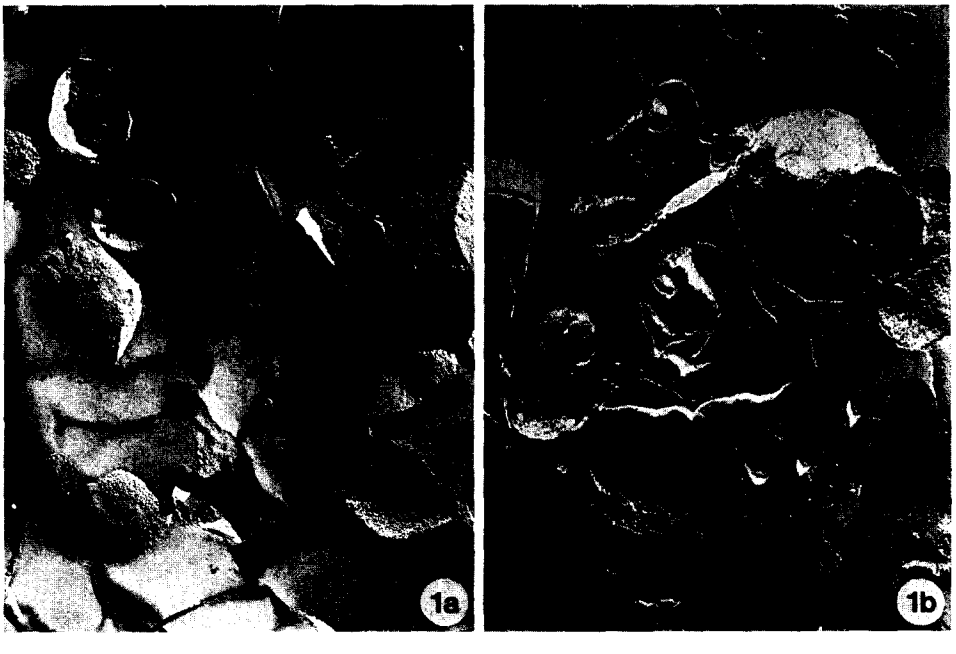
Samples from the various pellets were pipetted into 3-mm cardboard discs and frozen in liquid freon cooled by liquid N_2. Samples were freeze-fractured at —110° with no etching[1,28].

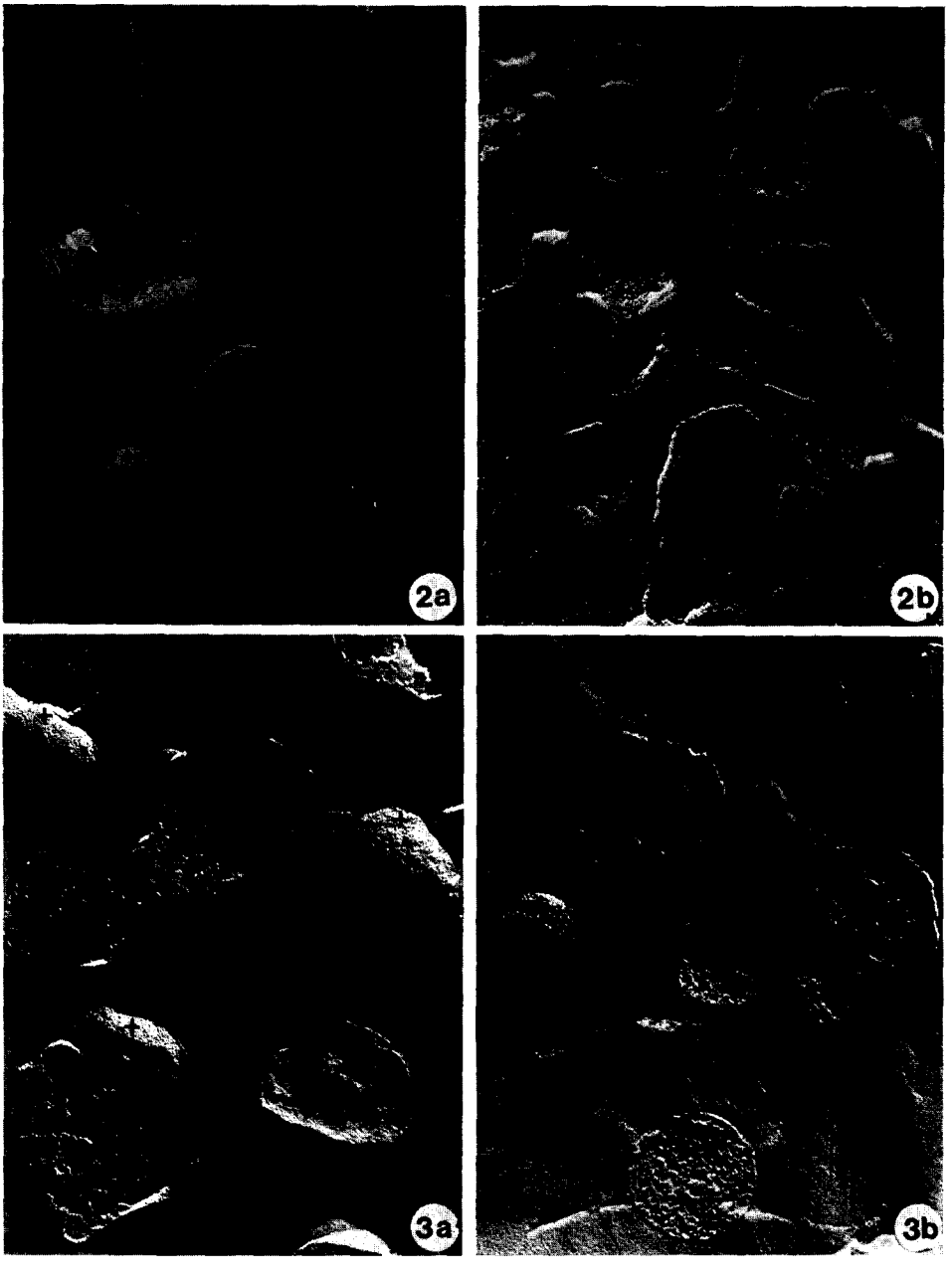
Replicas were viewed in a Siemens Elmiskop 1 using direct magnifications of 4000–20000. Replicas were scanned and areas to be photographed were selected solely on the basis of membrane concentration and replica quality. Prints of these photographs were used to determine the number of cells or liposomes showing membrane face fractures, as opposed to those showing only cross fractures. Cells or liposomes having less than 1 mm of membrane face exposed at 16000 final magnification were scored as cross fractures. Any cells or liposomes about which there was any doubt were not scored.

Fatty-acid analysis

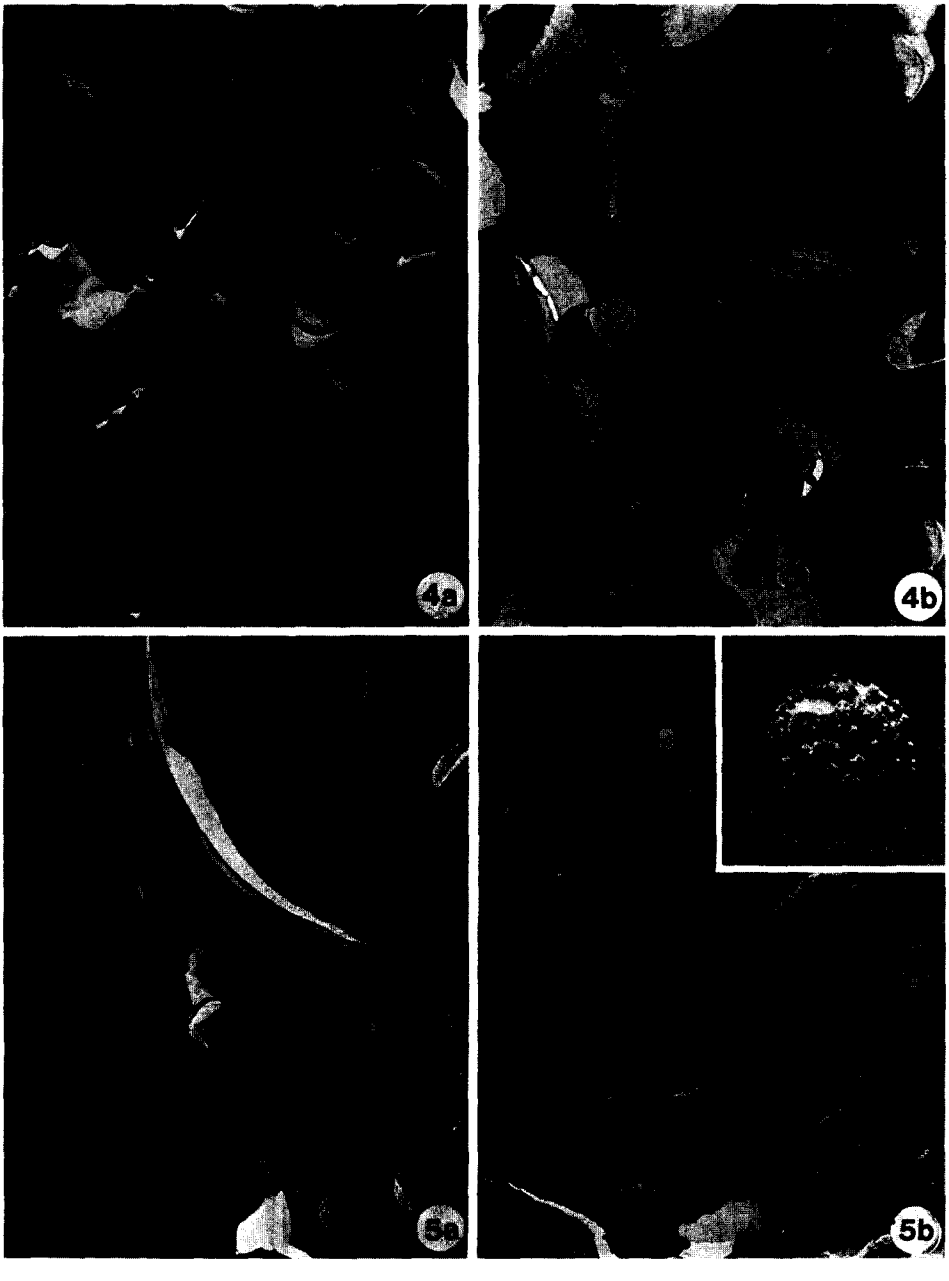
Lipid extracts were evaporated to 1 ml, 10 ml chloroform added and then evaporated again to 1 ml. Lipids in 1 ml chloroform were separated from the non-lipid contaminants on a Sephadex column, according to the methods of RADIN[25]. The lipids in the "Folch lower phase" were then evaporated to approx. 5 ml, and the phospholipids separated from neutral lipids in a Silicic acid column (Bio-Sil A, Bio-Rad Laboratories, Richmond, Calif.) by eluting the neutral lipids with chloroform–methanol (99:1, v/v) and then the phospholipids with 100% methanol[18,29].

The phospholipids were saponified and methyl esters of the fatty acids produced according to BOTTCHER *et al.*[30]. Analysis of the methyl esters was performed on a Varian Model 600 gas chromatograph fitted with a polar column (DEGS), flame-





Figs. 1–3. Freeze-etch views of *M. laidlawii* cells enriched with stearate (Fig. 1), oleate (Fig. 2), and linoleate (Fig. 3). (a) unfixed cells; (b) fixed cells. Examples of cells scored as showing no fracture face (cross fractured) are indicated by (−) and those showing fracture faces by (+). All × 20000.



Figs. 4 and 5. Freeze-etch views of liposomes prepared from lipid extracts of *M. laidlawii* cells enriched with oleate (Fig. 4) and linoleate (Fig. 5). (a) unfixed liposomes; (b) fixed liposomes. × 20000. Inset × 60000.

ionization detector, and disc integrator. Identification of unsaturated methyl esters was confirmed by hydrogenation of the double bonds.

RESULTS

Figs. 1a, 2a, and 3a show freeze-etched *M. laidlawii* cells grown in media supplemented with stearic, oleic, or linoleic acid. In each of these, the plasmalemma fractures so as to reveal the membrane face characteristic of the freeze-etch technique[1,2]. The fracture face of stearate-supplemented cells (Fig. 1a) shows large smooth areas and particulate areas, while that of oleate-supplemented cells (Fig. 2a) is more uniformly particulate. Similar observations have been made by TOURTELLOTTE *et al.*[14]. The fracture face of linoleate-supplemented cells (Fig. 3a) appears similar to that of oleate-supplemented cells (Fig. 2a). Figs. 1b, 2b, and 3b show cells grown in medium supplemented with stearic, oleic, and linoleic acids but fixed in 1% OsO_4 before freeze-etching. When compared with unfixed cells, fixed cells show fewer fracture faces. The reduction in the number of fracture faces increases with an increase in the number of double bonds per fatty acid (Table I). Thus, OsO_4 fixation had its greatest effect in linoleate-supplemented cells where fracture faces extending over the entire surface of an OsO_4-fixed cell are extremely rare. These results explain the observations of NANNINGA[19,20] that OsO_4 fixation of *B. subtilis* (no unsaturated fatty acids) had little effect on the membrane fracture plane and those of PARK AND BRANTON (unpublished observations) that OsO_4 fixation of chloroplasts (70% linoleic acid) obliterated the fracture plane.

Freeze-etched liposomes made of lipids extracted from oleate- and linoleate-supplemented cells are shown in Figs. 4a and 5a with their fixed counterparts in Figs. 4b and 5b. The morphology of unfixed and fixed liposomes made from stearate-

TABLE I

UNSATURATION OF MEMBRANE FATTY ACIDS AND PERCENTAGE OF CELLS AND LIPOSOMES SHOWING A SIGNIFICANT FRACTURE FACE

Membrane fatty acid	*Double bonds per fatty acid*	*Percent fracture faces**		
		Unfixed	*Fixed*	*Difference (unfixed−fixed)*
M. laidlawii intact cells				
stearic acid	0.25	77	49	28
oleic acid	0.47	79	43	36
linoleic acid	0.62	71	3	68
M. laidlawii liposomes				
stearic acid	0.25	90	94	−4
oleic acid	0.47	89	84	5
linoleic acid	0.62	93	33	60
Commercial lipid liposomes				
dipalmitoyl lecithin	0.00	91	91	0
Asolectin	1.42	88	48	40

* Each percentage represents counts of more than 300 cells or liposomes from at least 2 experiments and 3 freeze-etch replicas.

enriched lipids is similar to that of their oleate-enriched counterparts (Figs. 4a and 4b) and is therefore not shown. The unfixed liposomes are all similar in appearance, and the percentage of unfixed liposomes showing fracture planes is about the same for each of these enrichments (Table I). None show the particulate areas seen in cells, but all show a marked increase over cells in their tendency to reveal membrane faces (Table I). The fixed stearate-supplemented and oleate-supplemented liposomes are similar to their unfixed counterparts (Figs. 4a and 4b), but very few of the fixed linoleate-supplemented liposomes show extended fracture faces (Fig. 5b). In those that do, the fracture face has irregular raised areas (arrows, inset Fig. 5b). As can be seen in Table I, fixation with OsO_4 has a greater effect on the fracture faces of linoleate liposomes then it does on oleate liposomes. Fixed and unfixed liposomes of dipalmitoyl lecithin (not shown) are similar in morphology to unfixed dispersions of the same lipid reported by FLUCK *et al.*[31]. Table I shows that the number of fracture faces is unaffected by OsO_4 fixation. The unfixed and fixed liposomes of Asolectin (not shown) are similar in appearance to their linoleic-enriched liposome counterparts, and OsO_4 fixation causes a substantial reduction in the number of fracture planes (Table I).

DISCUSSION

The results of our experiments explain the observations of NANNINGA[19,20] and others (ref. 21; R. B. PARK AND D. BRANTON (unpublished observation) and H. MOOR (personal communication)) and indicate that the effect of OsO_4 on the freeze-etch fracture plane in frozen membranes is dependent upon the degree of unsaturation of the fatty acids in the membrane phospholipids. This result is consistent with the demonstration of a chemical reaction between OsO_4 and the double bonds of free fatty acids and their esters[32–34], fatty acids in lipid monolayers [35],and fatty acids in cells[36]. These experiments show that the presence of double bonds is essential for the reaction of OsO_4 with lipids or free fatty acids. KORN[36] demonstrated that the products of the reaction in amoebae are osmate double bond diesters formed by the reaction of one OsO_4 molecule with two double bonds. Oleic acid is the unsaturated fatty acid, and the reaction product is bis-(methyl 9,10-dihydroxystearate) osmate. It has been suggested that fatty acids with multiple double bonds polymerize, accounting for an insoluble fraction among the reaction products[33,35,36].

These reactions of OsO_4 together with what is known about the physical-chemical structure of membranes should explain why unfixed, frozen membranes readily fracture in their inner, hydrophobic regions, whereas fixed, frozen membranes resist fracture. It is generally accepted that two of the major stabilizing forces in unfixed membranes in an aqueous environment are hydrophobic (entropic) bonding and van der Waals interactions[37–40]. The tendency of unfixed membranes to split is explicable since hydrophobic bonding would not be important after freezing, and only relatively weak van der Waals forces would stabilize membranes in hydrophobic regions[6,41]. In OsO_4-fixed membranes, the great reduction of face fractures in membranes containing unsaturated fatty acids could be accounted for by the formation of strong, covalently bonded diesters or polymers across the bilayer. Bonding across the bilayer is also suggested by the irregular features observed on the fracture faces of fixed linoleate-enriched liposomes (arrows Fig. 5b). The spatial relationships of the fatty acid tails would be critical to bond formation and therefore to the absence

of face fractures. The data showing that the introduction of linoleic acid causes a disproportionate decrease in the percentage of fracture faces when compared with oleic acid supports this hypothesis.

Another explanation of the decrease in face fractures is based on the observation that when monolayers of unsaturated phospholipids are reacted with OsO_4, the resulting osmate tails move from the hydrophobic to the hydrophilic region of the monolayers[35]. If osmate tails in a lipid bilayer or biological membrane also move to the hydrophilic region of the membrane, the normal fracture plane could be altered without any bonding across the bilayer. These two alternatives are not mutually exclusive, and the role of each may be dependent on the number and position of double bonds present.

The difference in the percentage of unfixed cells *versus* unfixed liposomes showing significant fracture faces is small but consistent (Table I). There are at least two possible explanations: (1) the liposomes are spherical and the cells irregular, and (2) the presence of particles in the cell membranes represent bonding sites in the hydrophobic matrix not affected by low temperature. Stronger bonds may be introduced at these sites when cells are fixed with OsO_4. This is suggested by an even greater decrease in the percentage of fixed stearate and oleate cells *versus* fixed liposomes showing fracture faces (Table I).

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