Acknowledgment

The authors are grateful to Mr. Alvernon Hayes for excellent technical assistance.

References

Balzer, H., Makinose, M., Fiehn, M. G., and Hasselbach, W. (1968b), *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 260*, 456.
Balzer, H., Makinose, M., and Hasselbach, W. (1968a) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 260*, 444.
Barber, A. A., Harris, W. W., and Padilla, G. M. (1965), *J. Cell Biol. 27*, 281.
Bartley, W., and Dean, B. (1968), *Anal. Biochem. 25*, 99.
Blum, J. J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 81.
Blum, J. J. (1968), *Mol. Pharmacol. 4*, 247.
Brizzi, G., and Blum, J. J. (1970), *J. Protozool. 17*, 553.
Cann, J. R. (1968), *C. R. Trav. Lab. Carlsberg, 36*, 319.
Conger, N. E., and Eichel, H. J. (1966), *J. Protozool. 13*, 13.
DeDuve, C. (1969), *Proc. Roy. Soc. Biochem. 173*, 71.
DeLisle, G., and Fritz, J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 790.
Folch, J., Lees, M., and Sloane-Stanley, C. H. (1957), *J. Biol. Chem. 226*, 497.
Heath, D. F. (1968), *Biochem. J. 110*, 313.
Hertting, G., Azelrod, J., and Whitby, L. G. (1961), *J. Pharmacol. Exp. Ther. 134*, 146.
Hogg, J. F., and Elliott, A. M. (1951), *J. Biol. Chem. 192*, 131.
Hogg, J. F., and Kornberg, H. L. (1963), *Biochem. J. 86*, 462.
Kidder, G. W. (1967), *in* Chemical Zoology, Vol. I, Kidder, G. W., Ed., New York, N. Y., Academic Press, p 93.
Kornberg, H. L. (1967), *in* Metabolic Pathways, Vol. I, Greenberg, D. M., Ed., New York, N. Y., Academic Press, p 209.
Landau, B., Bartsch, G., Katz, T., and Wood, H. (1964), *J. Biol. Chem. 239*, 68.
Levy, M. R. (1970a), *Biochem. Biophys. Res. Commun. 39*, 1.
Levy, M. R. (1970b), *J. Cell Biol. 47*, 121a.
Levy, M. R., and Scherbaum, O. H. (1965), *J. Gen. Microbiol. 38*, 221.
Levy, M. R., and Wasmuth, J. J. (1970), *Biochim. Biophys. Acta 201*, 205.
London, W. P. (1966), *J. Biol. Chem. 241*, 3008.
Lowenstein, J. M. (1967), *in* Metabolic Pathways, Vol. I, Greenberg, D. M., Ed., New York, N. Y., Academic Press, p 180.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maxwell, R. A., Eckhardt, S. B., and Te, G. H. (1970), *J. Pharmacol. Exp. Ther. 171*, 62.
Müller, M., Hogg, J. F., and DeDuve, C. (1968), *J. Biol. Chem. 243*, 5385.
Munk, N., and Rosenberg, H. (1969), *Biochim. Biophys. Acta 177*, 629.
Reich, J. (1968), *Eur. J. Biochem. 6*, 395.
Reynolds, H. (1970), *J. Bacteriol. 104*, 719.
Rognstadt, R. (1969), *Arch. Biochem. Biophys. 129*, 13.
Ryley, J. F. (1952), *Biochem. J. 52*, 483.
Schrago, E., Brech, W., and Templeton, K. (1967), *J. Biol. Chem. 242*, 4060.
Threlfall, C. J., and Heath, D. F. (1968), *Biochem. J. 110*, 303.
Van Niel, C. B., Thomas, J. O., Ruben, S., and Kamen, M. D. (1942), *Proc. Nat. Acad. Sci. U. S. 28*, 157.
Warnock, L. G., and Van Eys, J. (1962), *J. Cell. Comp. Physiol 60*, 53.

Hybridization of Membranes by Sonic Irradiation*

Norihiro Tsukagoshi and C. Fred Fox†

ABSTRACT: Mixtures of membranes labeled with a density-labeled fatty acid and unlabeled membranes can be resolved by equilibrium centrifugation in sucrose density gradients. Subjecting the density-labeled and unlabeled membranes individually to sonic irradiation does not interfere with subsequent resolution by density gradient centrifugation. When a mixture of density-labeled and unlabeled membranes is treated by sonic irradiation, however, a new membrane species of intermediate density is formed. If a mixture of density-labeled and unlabeled membranes is sonicated in the presence of sucrose, only partial hybrid formation is observed, and the hybridized species is the lowest in particle weight of the membranes in the population. Controlled sonic treatment in the presence of sucrose allows a significant fragmentation of membranes with only limited hybrid formation.

Recently, Morrison and Morowitz (1970) have published radioautographic evidence which suggests that lipids are incorporated into the membrane of *Bacillus megaterium* KM at one or a few foci. Donachie and Begg (1970) have shown that *Escherichia coli* grow by an apparent extension from one pole of the organism and have extrapolated from their data to claim that the membrane of *E. coli* grows from some fixed focus with accompanying conservation of old and newly synthesized membrane during cellular growth and division.

* From the Department of Biochemistry, University of Chicago, Chicago, Illinois (N. T.), and from the Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California 90024 (C. F. F.). *Received May 3, 1971.* Supported in part by Public Health Research Grants AM-10987 and GM-18233 and by Grant N69-31 from the Chicago Heart Association.

† Recipient of Public Health Service research career development award 42359 from the National Institute of General Medical Sciences; to whom to address correspondence.

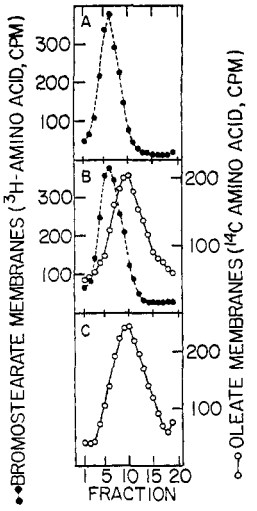

FIGURE 1: Banding of light and density-labeled membranes in a linear sucrose density gradient. The details of membrane purification and equilibrium density gradient centrifugation in a 60–35% linear sucrose gradient are given in Methods. Fractions were collected from the bottom of the centrifuge tubes with fraction 1 being that with the highest density. (A) Intact [^{3}H]isoleucine-labeled bromostearate membranes, 45 μg of membrane protein. (B) Intact [^{3}H]isoleucine-labeled bromostearate membranes, 60 μg of membrane protein, and intact [^{14}C]isoleucine-labeled oleate membranes, 115 μg of membrane protein, were mixed together and centrifuged to equilibrium in the same density gradient. (C) Intact [^{14}C]-isoleucine-labeled oleate membranes, 58 μg of membrane protein.

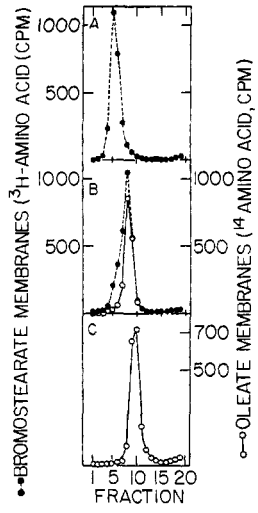

FIGURE 2: The effect of magnesium ion on the banding profile of light and density-labeled membranes. The experimental conditions are those described for Figure 1 except that the membrane suspensions applied to the sucrose density gradients contained 5 mM magnesium chloride. (A) Intact [^{3}H]isoleucine-labeled bromostearate membranes, 85 μg of protein. (B) Intact [^{3}H]isoleucine-labeled bromostearate membranes, 85 μg of protein, and intact [^{14}C]isoleucine-labeled oleate membranes, 147 μg of protein, were mixed together and centrifuged to equilibrium in the same density gradient. (C) Intact [^{14}C]isoleucine-labeled oleate membranes, 147 μg of protein.

The chromosomes of *E. coli* and *B. subtilis* are apparently attached to membrane at both the replicating point and at the origin (terminus) of replication (Fielding and Fox, 1970; Ganesan and Lederberg, 1967; Smith and Hanawalt, 1967; Snyder and Young 1969; Sueoka and Quinn, 1968), and the possible relationship between DNA–membrane attachment and membrane assembly at a fixed focus has been the subject of an attractive hypothesis to explain chromosome segregation in prokaryotes (Jacob *et al.*, 1963).

A report from this laboratory describes the incorporation of a density-labeled fatty acid into the membrane lipids of an unsaturated fatty acid auxotroph of *E. coli* (Fox *et al.*, 1970). If mixtures of density-labeled and unlabeled (light) membranes can be fragmented without the introduction of artifact, the use of the lipid density label in a density shift experiment allows a direct test of the extent to which membranes may grow at discrete foci with concomitant conservation of old and newly synthesized membrane. This report describes a procedure which permits the fragmentation of membrane mixtures without the artifact of membrane hybridization which accompanies uncontrolled membrane fragmentation.

Methods

Growth of Bacteria. Strain 30E (Fox *et al.*, 1970; Wilson and Fox, 1971), an unsaturated fatty acid auxotroph of *E. coli*, was grown in medium A (Davis and Mingioli, 1950) supplemented with 1% Difco Casamino Acids, 5 μg/ml of thiamine·HCl, 0.25% of the nonionic detergent Triton X-100 (Rohm and Haas), and 0.02% of either oleic or bromostearic acids. Oleic acid (99%+ purity) was purchased from the Hormel Institute, Austin, Minn., and bromostearic acid, a mixture of 9-bromo- and 10-bromostearic acids, was that synthesized previously (Fox *et al.*, 1970). For amino acid labeling of cells, [^{14}C]isoleucine (247 mCi/mmole) or [^{3}H]isoleucine (2.85 Ci/mmole) was included in the growth medium at 0.2 and 0.4 μCi per ml, respectively. For lipid labeling, [^{14}C]linoleic acid (56 mCi/mmole) or [9,10-^{3}H]oleic acid (2.29 Ci/mmole) was included in the growth medium at 0.4 and 1.0 μCi per ml, respectively. All isotopes were purchased from New England Nuclear.

Cultures of 500 ml were grown with rotary shaking at 37° in 2-l. flasks. At a density of 10^9 cells/ml, the cells were collected by centrifugation and washed once by suspension in 200 ml of 0.01 M Tris·HCl buffer (pH 7.6) followed by centrifugation.

Preparation of Membranes. The procedure for preparation of the "inner" or "cytoplasmic" membrane fraction from spheroplasts of *E. coli* is essentially that described previously (Fox *et al.*, 1970) with one minor modification. Prior to the step where washed membranes were applied to discontinuous gradients containing 35 and 65% sucrose, the membranes had been centrifuged, and the pellet was suspended in a solution of Tris·HCl and 2-mercaptoethanol. This centrifugation step has been eliminated, and the dialyzed membranes from the previous step are applied directly to the discontinuous gradient containing 35 and 65% sucrose.

The membrane fraction designated band I in the previous report is that used exclusively in the work described here. This membrane fraction was suspended in 0.01 M Tris·HCl buffer of pH 7.6 containing 0.01 M 2-mercaptoethanol and stored refrigerated at 4°.

Fragmentation of Membranes. For fragmentation by sonication, the membrane samples were suspended in 3 ml of 0.01 M Tris·HCl buffer of pH 7.6 containing 0.01 M 2-mercaptoethanol in a glass 10-ml beaker. Temperature throughout

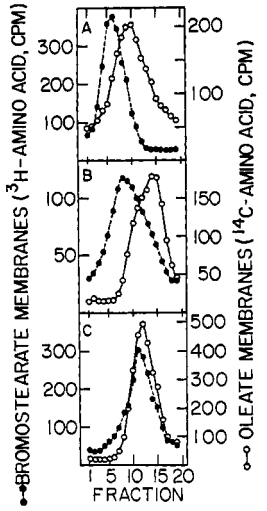

FIGURE 3: The effect of sonic treatment of light and density-labeled membrane fractions fragmented singly or together where both membrane fractions are protein labeled. (A) Mixture of intact [^{3}H]isoleucine-labeled bromostearate membranes, 60 μg of protein, and intact [^{14}C]isoleucine-labeled oleate membranes, 115 μg of protein. (B) Bromostearate and oleate membranes were sonicated individually for 2 min at protein concentrations of 500 and 1020 μg per ml, respectively, and 0.1 ml of each sonicate was applied to the same gradient. (C) Bromostearate and oleate membranes were mixed at protein concentrations of 400 and 1280 μg per ml, respectively, before the 2-min sonication, and 0.15 ml of the sonicated mixture was applied to the gradient.

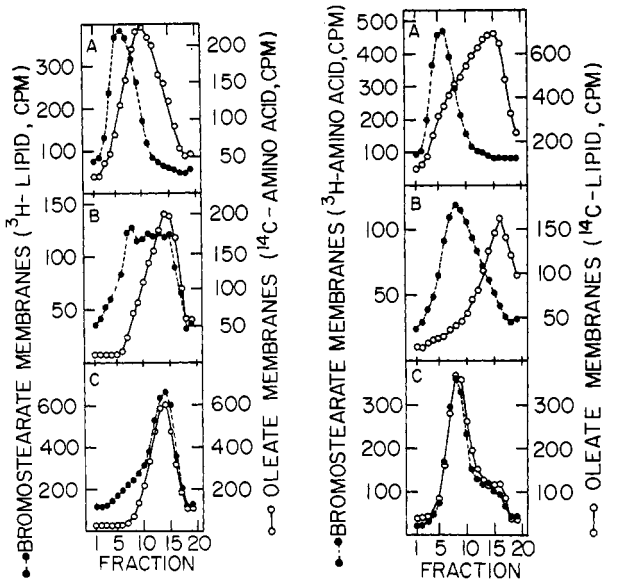

FIGURE 4: The effect of sonic treatment of light and density-labeled membranes fragmented singly or together where one membrane fraction is amino acid labeled and the other, lipid labeled. (a, left) Lipid-labeled, density-labeled membranes and amino acid labeled, light membranes. (A) Mixture of intact [^{3}H]fatty acid labeled bromostearate membranes, 0.4 μg of protein, and intact [^{14}C]isoleucine-labeled oleate membranes, 116 μg of protein. (B) Bromostearate and oleate membranes were sonicated individually for 2 min at protein concentrations of 3.0 and 1020 μg per ml, respectively, and 0.1 ml of each sonicate was applied to the same gradient. (C) Bromostearate and oleate membranes were mixed at protein concentrations of 6.0 and 1200 μg per ml, respectively, before the 2-min sonication, and 0.15 ml of the sonicated mixture was applied to the gradient. (b, right) Amino acid labeled, density-labeled membranes, and lipid-labeled, light membranes. (A) Mixture of intact [^{3}H]isoleucine-labeled bromostearate membranes, 98 μg of protein, and intact [^{14}C]fatty acid labeled oleate membranes, 36 μg of protein. (B) Bromostearate and oleate membranes were sonicated individually for 2 min at protein concentrations of 500 and 60 μg per ml, respectively, and 0.1 ml of each sonicate was applied to the same gradient. (C) Bromostearate and oleate membranes were mixed at protein concentrations of 400 and 60 μg per ml, respectively, before the 2-min sonication, and 0.15 ml of the sonicated mixture was applied to the gradient.

sonication was maintained below 10°. Sonication was performed using the lowest power setting of the Model W140 Sonifier cell disruptor, Branson Instruments, Inc., employing a "step horn."

Sucrose Density Gradient Centrifugation. Linear 60–35% sucrose density gradients of 5 ml containing 0.01 M Tris·HCl of pH 7.6 and 0.01 M 2-mercaptoethanol were generated with a Beckman density gradient former. Samples of 0.1–0.2 ml were layered on top of the gradients, and the tubes were centrifuged for 20 hr at 45,000 rpm in the Spinco SW 50.1 rotor. The contents of the tubes were fractionated by puncturing the tubes on the bottom and collecting drops.

Assay of Protein and Radioactivity. Protein was determined as described by Lowry *et al.* (1951). Radioactivity was measured by combining the sample and water in a total volume of 0.75 ml and by mixing this with 10 ml of the solution described by Patterson and Greene (1965) containing toluene–Triton X-100 (3:1, v/v) for liquid scintillation counting.

Results

Separation of Density-Labeled and Unlabeled Membranes by Equilibrium Density Gradient Centrifugation. When a mixture of density-labeled and light membranes is centrifuged to equilibrium in a sucrose density gradient the two membrane species band at positions identical with those of the same species banded singly (Figure 1). If the two fractions are first mixed with $MgCl_2$ at 5 mM final concentration before the centrifugation, two pronounced changes from the banding patterns of Figure 1 are observed (Figure 2). First, the banding profile is much sharper, and second, the light and density-labeled membranes have aggregated and band at a density intermediate between the densities of the light and density-labeled membranes centrifuged individually. Though the reason for this aggregation phenomenon is not obvious, it provides a rationale for one of the critical steps in the separation of the cytoplasmic and outer membranes of *E. coli*, the prolonged dialysis in the presence of EDTA (Fox, *et al.*, 1970).

Fragmentation of Membranes by Sonication. The design of experiments being currently undertaken in this laboratory demanded that techniques be developed which would allow the fragmentation of mixtures of membranes without disturbing the identities of the membrane species in the mixture. Extensive fragmentation by sonic irradiation leads to an apparent decrease in the density of both light and density-labeled membranes, but these membrane species are still easily separable when mixed after being subjected to sonication individually (Figure 3A,B). On the other hand, when a mixture of the two membrane species is exposed to the standard sonication procedure (Methods), a hybrid density membrane band with an average density intermediate between those of the individual sonicated membrane species is produced (Figure 3C). The membranes used for the experiment in Figure 3 are almost exclusively labeled in material not extractable by lipid solvents. Less than 1% of the radioactivity of the [^{14}C]isoleucine and [^{3}H]isoleucine labeled membranes could be extracted with

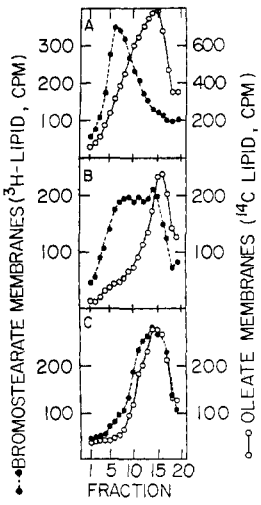

FIGURE 5: The effect of sonic treatment on light and density-labeled membranes fragmented singly or together where both membrane fractions are lipid labeled. (A) Mixture of [^{3}H]fatty acid labeled bromostearate membranes, 0.5 μg of protein, and intact [^{14}C]fatty acid labeled oleate membranes, 36 μg of protein. (B) Bromostearate and oleate membranes were sonicated individually for 2 min at protein concentrations of 5.6 and 90 μg per ml, respectively, and 0.1 ml of each sonicate was applied to the same gradient. (C) Bromostearate and oleate membranes were mixed at protein concentrations of 4.0 and 60 μg per ml, respectively, before the 2-min sonication, and 0.15 ml of the sonicated mixture was applied to the gradient.

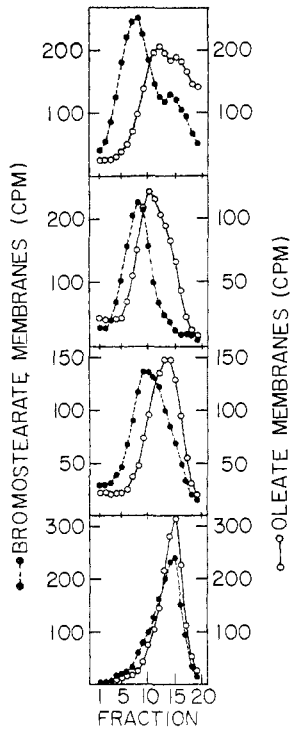

FIGURE 7: Density gradient analysis of regions of the gel filtration column eluates shown in Figure 6. The gradients contained, from top to bottom: (1) 0.2 ml of the sonicated membrane mixture applied to the column, (2) 0.3 ml of fractions 19–23 concentrated to 0.4 ml by pressure filtration, (3) 0.3 ml of fractions 25–34 concentrated to 0.4 ml by pressure filtration, and (4) 0.3 ml of fractions 35–44 concentrated to 0.4 ml by pressure filtration.

chloroform–methanol (2:1, v/v). In order to test the possibility that much of the apparent hybridization is the result of exchange of proteins between the two membrane species of the population, the experiment described in Figure 3 was repeated with mixtures of lipid and amino acid labeled membranes, and with a mixture of membranes labeled almost exclusively in lipid with different radioisotopes (Figures 4a,b and 5). In all cases, a hybrid membrane band was observed when mixtures of light and density-labeled membranes were sonicated, and the resultant hybrid density was that predicted by the relative quantities of the species of membranes in the mixture. When 6 μg of [^{3}H]lipid-labeled density-labeled membranes was sonicated together with 1200 μg of [^{14}C]amino acid labeled light membranes, for example, the density of the resultant hybrid band was essentially that of the species present at greater mass, the light membranes. When the more dense species of membranes predominated in the population (Figure 4b), its density was the major determinant of the density of the hybrid band observed after sonication.

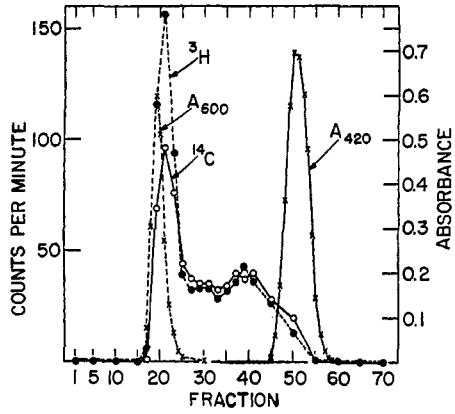

FIGURE 6: Gel filtration of a mixture of light and density-labeled membranes fragmented together in the presence of sucrose. A mixture of [^{3}H]isoleucine-labeled bromostearate membranes (850 μg of protein) and [^{14}C]isoleucine-labeled oleate membranes (2200 μg of protein) was sonicated for the standard 2-min period except that the mixture also contained 20% w/v of sucrose. The sonicate was concentrated to 1 ml by pressure filtration and applied to a 15 × 260 mm column of Bio-Gel A-150m (Calbiochem, Los Angeles, Calif.). The column was eluted at a flow rate of 6 ml/hr with a solution containing 0.1 M Tris·HCl of pH 7.6 and 0.01 M 2-mercaptoethanol. Fractions of 1 ml were collected and 0.1 ml of each fraction was assayed for radioactivity. The elution profiles of intact *E. coli* and of *p*-nitrophenol were established in a similar, but separate experiment by measuring the absorbance at 600 and 420 nm, respectively. Bromostearate membranes (●---●), oleate membranes (○—○), intact *E. coli* (×---×), and *p*-nitrophenol (×—×).

When taken together with preliminary electron microscopic evidence (obtained in collaboration with F. Eiserling), the data in Figures 3–5 indicate that the hybrid bands result from sonic fragmentation by an intermixing of both the lipids and proteins of the two species of membranes in the population, rather than by a simple aggregation of heavy and light membranes. Electron micrographs of the hybrid membrane band after equilibrium sucrose density gradient centrifugation show only individual membrane fragments with no evidence of aggregation. With the standard conditions of sonication (Methods), the hybridization produced by a 15-sec sonication is equivalent to that obtained after 2 min. Though a similar hybridization was obtained with the use of the Aminco French press operating at 10,000 psi, no hybridization was detected after mixtures of membranes were homogenized extensively with a motor-driven glass–Teflon mortar and

pestle or after a 15-min blender treatment in the presence of glass beads using the microhomogenizer attachment of the Sorvall Omni-Mixer at maximal speed. The latter treatments, however, do not produce the small fragments which result either from sonic irradiation or from the use of the French press.

Conditions for Avoiding Hybrid Formation during Sonic Irradiation. Mixtures of light and density labeled membranes were sonicated under the standard conditions in the presence of a number of solutes such as EDTA, urea, potassium chloride, and sucrose. Of these, only sucrose reduced the extent of hybridization. The material which has undergone the most extensive hybridization as a result of sonic treatment is also that which yields the smallest fragments (Figures 6 and 7). A mixture of amino acid labeled light and heavy membranes was sonicated in the presence of 20% sucrose and then subjected to gel filtration by Bio-Gel A-150m chromatography (Figure 6). The unfractionated sample showed evidence of a hybrid region in addition to bands composed largely of light and heavy membranes upon equilibrium density gradient centrifugation (Figure 7). Though the hybrid peak is less dense than the band of exclusively light membranes, it should be recalled that extensive sonic fragmentation leads to a marked reduction of the sucrose buoyant density of these membranes (Figures 3–5). It is therefore not surprising that the hybrid density membranes are the last to elute from the Bio-Gel column, and are thus the lowest in particle size of the sonic treated membranes applied to the column. Since membrane fragments not subjected to sonic irradiation eluted from Bio-Gel A-150m at the void volume (data not shown), these data indicate that a significant fragmentation of membrane mixtures can be accomplished without the accompanying production of artifact by hybrid formation. An electron microscopic study is currently in progress to ascertain the extent to which membranes can be fragmented without hybrid formation.

Discussion

Sonic treatment of a mixture of membranes of different densities leads to the production of material of intermediate density. Sonic treatment under controlled conditions, *i.e.*, in the presence of sucrose, reduces the extent to which the material of intermediate density is produced. Two explanations can be advanced to account for the apparent hybrid formation: (1) the hybrid density material represents truly hybridized membrane and (2) the hybrid density material is the result of nonspecific clumping of particles of the light and heavy membrane species in the population. The second explanation is unlikely for two reasons. First, after sonication under controlled conditions where both hybrid density and nonhybrid density materials coexist in the population, the hybrid density material is the lowest in particle size. Were it composed of clumps of membrane particles, the inverse might be expected. Second, preliminary electron microscopic studies of the hybrid density material give no evidence for nonspecific clumping, and the hybrid density membrane band appears to consist primarily of lamellar sheets which exhibit a normal bilayer dimension when viewed on end.

Our data suggest that membranes may fragment in two ways in response to sonic irradiation. One mechanism of fragmentation would be by fission perpendicular to the plane of the membrane giving rise to a stepwise reduction in particle size without hybrid formation, as observed in the case of sonication in the presence of sucrose. The second mechanism would be fission giving rise to smaller particles than those actually observed, these particles aggregating to form larger particles which exhibit normal structure by electron microscopy. These particles could be produced either by fission perpendicular to the plane of the membrane, and/or by fission parallel to the plane of the membrane, giving rise to a cleavage of the bilayer analogous to that obtained with freeze etching for electron microscopy (Branton, 1966). In order to explain the effects of sucrose which permit the production of membrane fragments without hybridization, it is then only necessary to postulate that sucrose, perhaps by increasing the viscosity of the medium exposed to sonication, inhibits fragmentation by the second mechanism preferentially, or retards the rate of all fragmentation processes allowing the isolation of nonhybrid fragments.

Our study gives some indication of the care which should be taken in the choice of methods for cell disruption where isolation of a given membrane species in a purified state is desired. Cell rupture by either sonic irradiation or by use of the French press may give rise to gross artifacts, whereas rupture by homogenization or by blender treatment does not result in the intermixing of different membrane species. One apparent exception to this statement is the study by Schnaitman (1970) where rupture of intact *E. coli* using the French press with conditions similar to those used by us nevertheless allowed separation of the cytoplasmic and outer membrane species with little evidence of hybrid formation between them. In Schnaitman's study, however, the outer membrane was isolated still bound to the cell wall, and this attachment could have prevented a hybridization of the cytoplasmic and outer membranes.

References

Branton, D. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1048.

Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60*, 17.

Donachie, W. D., and Begg, K. J. (1970), *Nature (London) 227*, 1220.

Fielding, P., and Fox, C. F. (1970), *Biochem. Biophys. Res. Commun. 41*, 157.

Fox, C. F., Law, J. H., Tsukagoshi, N., and Wilson, G. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 598.

Ganesan, A. T., and Lederberg, J. (1967), *Biochem. Biophys. Res. Commun. 18*, 824.

Jacob, F., Brenner, S., and Cuzin, F. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 239.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Morrison, D. C., and Morowitz, H. J. (1970), *J. Mol. Biol. 49*, 441.

Patterson, M. S., and Green, R. C. (1965), *Anal. Chem. 37*, 854.

Schnaitman, C. (1970), *J. Bacteriol. 104*, 890.

Smith, D. W., and Hanawalt, P. C. (1967), *Biochim. Biophys. Acta 149*, 519.

Snyder, D. W., and Young, P. C. (1969), *Biochem. Biophys. Res. Commun. 35*, 354.

Sueoka, N., and Quinn, W. G. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 695.

Wilson, G., and Fox, C. F. (1971), *J. Mol. Biol. 55*, 49.