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Fusion of Photoreceptor Membrane Vesicles

W.T. Mason,* S.B. Hladky,** and D.A. Haydon

Physiological Laboratory, Downing Street, University of Cambridge, Cambridge, England CB2 3EG

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Summary. The *n*-alkyl bromides with 6 to 10 carbons induce formation of vesicles of 5 to 100 μm diameter from the small vesicles (0.1 μm average diameter) produced by disruption of the discs from frog rod photoreceptors. The *n*-alkanes, *n*-alkyl iodides and *n*-alkyl chlorides are relatively ineffective. The formation of large vesicles is independent of calcium concentration and is distinguished from fusion processes previously reported by the large number of vesicles involved. The results reported here together with others suggest the occurrence of multiple fusion (and/or rupture-resealing) events between vesicles, induced by the *n*-alkyl bromides.

It is well known that membrane fusion forms the basis for numerous events in the biology of cells. Among these events are fertilization, synaptic nerve transmission, endo- and exocytosis, and intercellular fusion to form multinucleate cells. These and other events have been reviewed by Poste and Allison [21].

A number of agents have been found to be capable of inducing membrane fusion. Among these agents are inactivated Sendai virus [3, 9, 14, 17, 19, 20, 26], lysolecithin [13], phospholipases [4, 22], aliphatic alcohols [1], polyethylene glycol [15], and calcium ions at appropriate concentrations [5, 6, 18, 19, 23–25]. Furthermore, it appears these agents promote fusion of natural and artificial membranes both with themselves and with each other.

Ahkong *et al.* [2] briefly reported earlier that hen erythrocytes fuse on incubation with tetradecane, hexadecane, and octadecane. Our investigation of alkanes began with the observations by Dagger [7] that the aliphatic hydrocarbon *n*-decane induced aggregation and small amounts of fusion in human red cell suspensions. Brooks and Haydon (*personal*

* *Present address:* Biophysics Unit, A.R.C. Institute of Animal Physiology, Babraham, Cambridge, CB2 4AT England.

** *Present address:* Department of Pharmacology, University of Cambridge, Cambridge, England.

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communication) later found that the halogenated derivatives of the alkanes are more effective in inducing fusion of red cells, such that the fusion products still contain a high concentration of hemoglobin.

This paper reports experiments which demonstrate that the *n*-alkyl bromides promote the formation of large single membrane vesicles from many (greater than 10^2) small vesicles derived from the disc membranes of photoreceptors isolated from frog retinae.

Materials and Methods

Preparation of Photoreceptor Disc Membrane Vesicles

For these experiments, retinae of the common frog (*Rana temporaria*) were used. Membranes of dark and light adapted animals were examined. In the case of dark adapted animals, manipulations were performed under dim red light using a 15 W light bulb through a Kodak Wratten 1 A filter. Animals were dark adapted for at least two hours prior to isolation of the retinae. The retinae were initially dissected into an isolation medium prepared from a modified high sodium Ringer solution (115 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM Tris chloride, 11 mM glucose, adjusted to pH 7.2 with HCl) containing 38 g sucrose per 100 ml final solution (38%, wt/vol). The density of this hypertonic sucrose isolation medium was 1.13 g/ml. Dissected retinae were shaken in the sucrose Ringer, using one retina per ml. Following agitation, 10 ml Ringer solution without sucrose were layered on top of the suspension to form a discontinuous gradient. After centrifugation for 20 min at $21{,}000 \times g$ in an MSE refrigerated ultracentrifuge, the rod outer segments (ROS) were removed from the interface. They were then resuspended in 40 ml isotonic Ringer solution and washed by gentle agitation and recentrifugation at the same speed. The membrane pellet from this final wash was resuspended in 10 µl per retina of high-potassium Ringer solution, containing reversed concentrations of sodium and potassium relative to the high-sodium Ringer. The rods were then mechanically disrupted either by drawing the suspension repeatedly (10 times) in and out of a small volume plastic syringe fitted with a needle of internal diameter of approximately 150 µm, or alternatively by syringing with a 50-µl capacity Hamilton glass syringe.

The volume concentration of membrane was determined by placing 40 µl final membrane suspension in a 1.0 mm diameter capillary tube and spinning for 15 min in an MSE microhaematocrit centrifuge. Volume concentrations are expressed as the percentage of membrane volume compared to the total suspension volume.

Microscopic Techniques

Membrane preparations were studied by light and electron microscopy. Unfused preparations were examined by light microscopy by using a Zeiss microscope fitted with either Nomarski or phase interference optics. For conventional electron microscopy, membranes were prepared in 2 ml volume plastic centrifuge tubes. The final membrane suspension

was fixed in glutaraldehyde in isotonic buffer. After two hours the membranes were washed three times in isotonic saline without fixative and resuspended in 1% osmium tetroxide in saline for a further two hours. Following this, the solution was changed to osmium-free buffer and the membrane pellet dehydrated in a series of graded alcohol washes up to absolute ethanol. They were then transferred to toluene and embedded in araldite resin.

Thick and thin sections were cut with glass knives on a Reichert ultramicrotome. For light microscopy, thick sections were examined by staining with 1% toluidine blue and 1% sodium borate, filtered prior to use. For electron microscopy, thin sections were placed on Formvar coated copper grids (50 or 100 mesh), and stained with 1% uranyl acetate followed by 1% lead citrate under moisture-free conditions. Examination of thin sections was carried out on a Phillips 300 electron microscope in the Department of Anatomy, University of Cambridge.

Initial Studies of Large Vesicle Formation

The compounds studied in this work were the alkanes of chain length C_6–C_{10}, and some of the corresponding brominated, iodinated and chlorinated derivatives. These compounds were prepared by saturating solutions of the simplified disc membrane suspension medium (containing only 120 mM KCl and 10 mM Tris chloride buffered to pH 7.2) with the appropriate substance. An excess of the substance under study was added to the saline in a separating funnel and allowed to equilibrate for 48 hr. An aliquot of the oil-saturated solution was used to resuspend the membrane pellet from the final wash to a volume concentration of 2–10%. The suspension volume was now about 100 µl. At this point, 1 µl or less of pure oil, an amount in excess of its aqueous solubility, was added to the suspension and the mixture agitated by either shaking or vortexing. This treatment was adequate to effect membrane fusion by some of the oils. In later experiments the membranes were suspended as described above (greater than 3% vol/vol) in saline which did not contain oil, excess oil ($< 1\%$ vol/vol) added directly to the membrane suspension, and the suspension then shaken or vortexed. This concentration of *n*-hexyl bromide is far in excess of the amount required to completely saturate the aqueous phase in the absence of membranes.

Results

ROS from the frog retina are cylindrical structures when isolated in isotonic medium or when examined in the intact frog retina. However, when they are isolated on hypertonic gradients such as the sucrose Ringer medium used in these experiments, their return to isotonic media disrupts the normal disc-like structure of the outer segment and small spherical vesicles of photoreceptor membrane are formed. When mechanically disrupted by syringing, these vesicles are released into the suspension medium. Most of these vesicles are too small to be seen by conventional light microscopy. However, when fixed and viewed in the electron microscope, it can be seen they generally have a typical diameter of about 0.1 µm, as in Fig. 1.

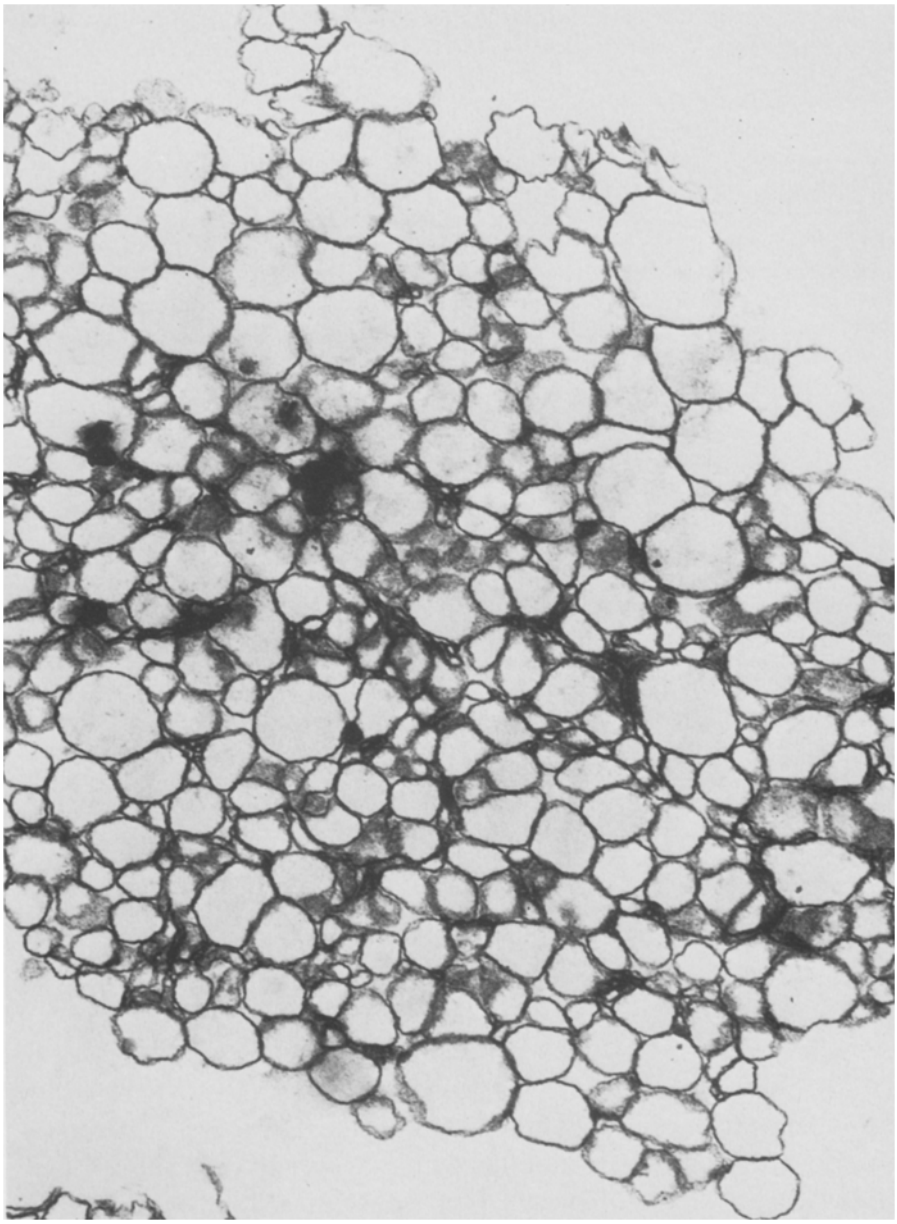


Fig. 1. Electron micrograph of a thin section through a pellet of frog photoreceptor-derived disc membrane vesicles, following isolation on a hyperosmotic sucrose gradient as described in the text. The material shown is the vesicular material used for subsequent studies of membrane fusion by the *n*-alkyl bromides. Lead and osmium staining. Magnification scale: 1 cm = 0.17 μm

When photoreceptor membrane vesicles are isolated and resuspended in small volumes of either high-potassium or high-sodium Ringer solutions, it is found that addition of solutions previously saturated with small amounts of alkyl bromides results in aggregation of membrane material, though without any clear formation of large vesicles. However, if quantities of the alkyl bromides are added in slight excess such that small droplets of bulk oil are present, the formation of large vesicles is rapid. These have various shapes and sizes ranging from several micrometers in diameter to larger than 100 μm. The appearance is independent of whether the photoreceptor membrane material is dark or light adapted. Light micrographs of two such vesicles are shown in Fig. 2. Observations of these vesicles by phase or Nomarski interference light microscopy, when they are unfixed and unstained, show that the shape and dimensions of the vesicles vary widely. Smaller vesicles (5–40 μM) are roughly spherical, whereas the larger vesicles (>40 μm) are increasingly asymmetrical.

The volume occupied by the vesicles is the same, within 10%, before and after treatment with the oil. If the pellet of *n*-alkyl bromide-treated

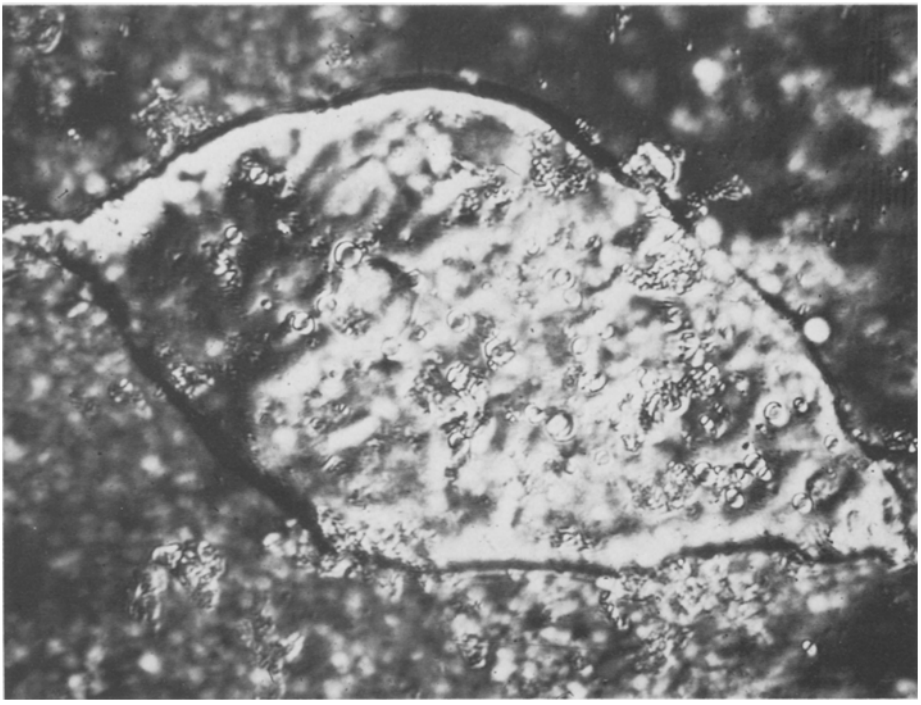
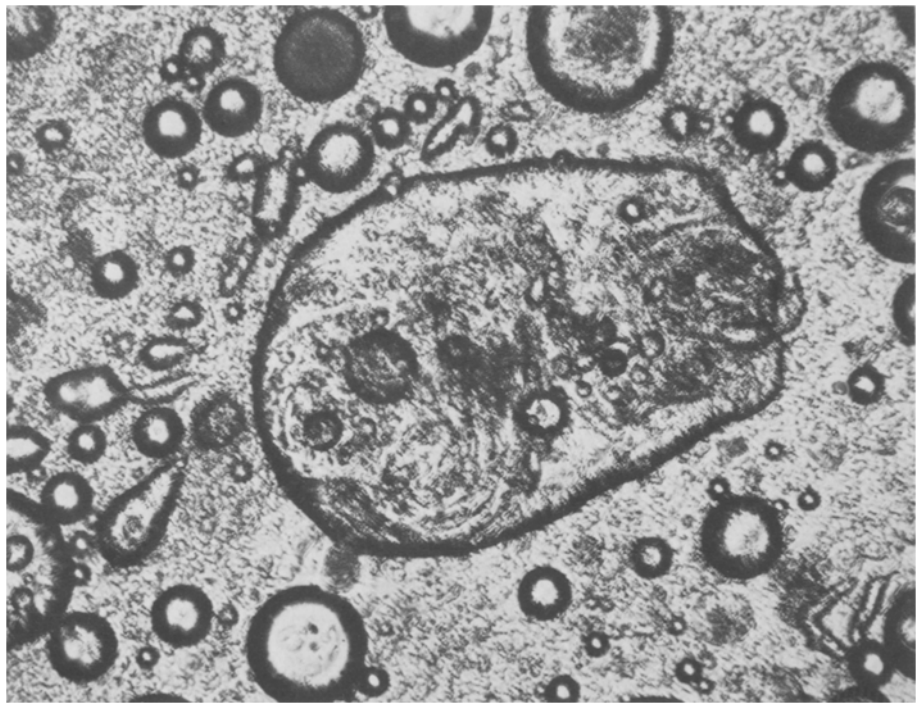


Fig. 2*a*



(*b*)

Fig. 2. Light micrographs of two unfixed, unstained membrane vesicles fused from photoreceptor disc vesicle membranes, viewed by Nomarski (*a*) and Phase (*B*) interference optics. The vesicle in *a* was fused with excess *n*-decyl bromide and that in *B* with excess *n*-hexyl bromide. The highly refractile spheres in *b* are droplets of excess oil as frequently seen in the membrane suspension following fusion under these conditions. Note the distinct differences in shape and symmetry of the fused membranes and the oil droplets. Magnification scale in *a* is 1 cm = 11 μm and in *B* is 1 cm = 9.4 μm

material is examined by light microscopy of thick sections of fixed material, it is found that approximately 95% of the pellet volume consists of large vesicles. The variety of vesicle sizes seen in Fig. 3 is an indication of the heterogeneous population of vesicle sizes and shapes obtained by this technique. Numerous small vesicles are observed as heavily stained material external to the larger vesicles. In electron micrographs (Fig. 4), the large vesicles are surrounded by a single membrane, and their internal space is devoid of electron dense or stained material. The membrane is of dimensions consistent with a single unit cell membrane viewed under the electron microscope.

The oils that were examined in greatest detail were *n*-hexylbromide, *n*-octylbromide and *n*-decylbromide. Qualitatively, it appears that the average diameter of the resulting large vesicles decreased with increasing chain length, from *n*-hexylbromide upwards. Under similar conditions,

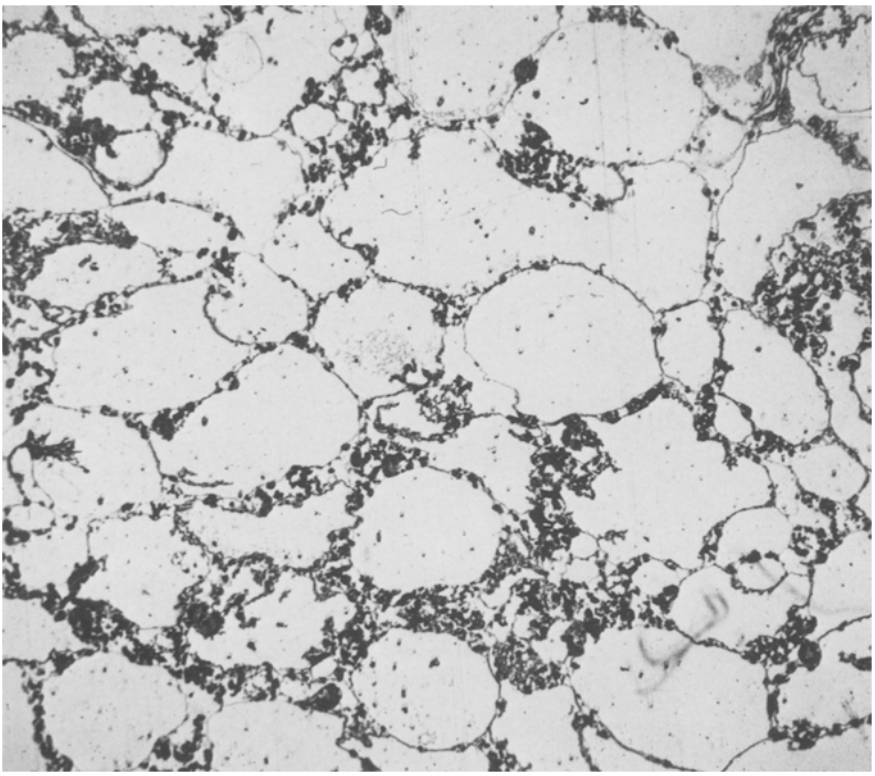


Fig. 3. Light micrograph of a thick section through a pellet of disc vesicle membranes fused with excess *n*-hexyl bromide. The membrane surrounding the fused vesicles can be noted, and large numbers of unfused vesicular membranes can be seen outside the area of the fused vesicles, even though their contribution to the pellet volume is small. Stained with 1% toluidine blue and 1% sodium borate. Magnification scale is 1 cm = 70 μm

the alkanes and their chlorinated or iodinated derivatives also promote formation of large vesicles, although the number of vesicles that combine, and the size of the resulting vesicles, is greatly diminished.

A final feature of the formation of large vesicles induced by the brominated alkanes is that no change in the process was observed when the free calcium concentration was adjusted between 10^{-9} and 10^{-2} M by additions of EGTA and $CaCl_2$.

Discussion

These initial observations indicate that alkylbromides in quantities more than sufficient to saturate the aqueous solutions are capable of inducing the formation of large vesicles (>20 μm diameter) from the small membrane vesicles (~0.1 to 1 μm diameter) isolated from frog

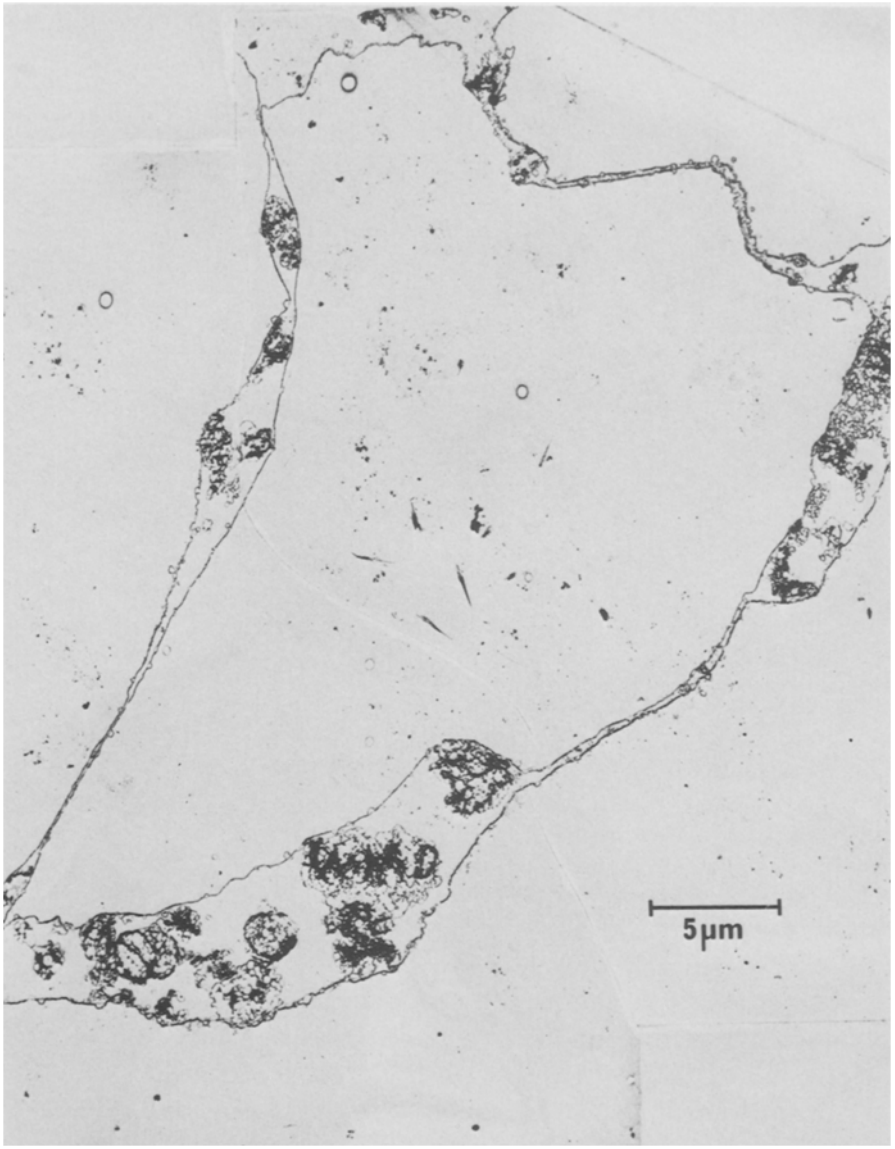


Fig. 4. Montage of an electron micrograph of a thin section through a pellet of disc vesicle membranes fused with excess *n*-hexyl bromide. The single membrane surrounding the vesicle can be noted, and the internal contents appear to be devoid of any membrane material. Three other fused vesicles protrude into the field of view in the lower right-hand, upper right-hand and middle left-hand areas of the montage. Unfused vesicle material can be seen in the spaces between the fused vesicles. Lead and osmium staining. Magnification scale is 1 cm = 2.9 μm

photoreceptors of outer segment origin. Both light and electron microscopic observations of the membrane following treatment with alkylbromides indicates formation of large vesicles bounded by a single membrane rather than simple aggregation.

From electron microscopic observation of photoreceptor membrane material not exposed to oil and subsequent observations of treated vesicle populations, it appears that the large vesicles are derived from 10^2 to more than 10^5 membrane vesicles. While the result of this calculation differs somewhat depending on whether it is assumed that the vesicles have fused at constant volume or the membranes have joined at constant area, it appears that a large number of membrane vesicles have formed the observed structures. The mechanism is not at present understood, but it is clear that the large number of vesicles involved is dissimilar to fusion processes previously studied.

Since excess oil is present, it was thought possible that the large vesicles might contain oil rather than electrolyte solution. However, the irregular shape of the larger vesicles and the difference in appearance between the vesicles and oil drops of similar size, using either Nomarski or phase interference optics, argues against this possibility. Furthermore, as discussed in detail elsewhere [16], on the basis of microspectrophotometric measurement of vesicles fused with *n*-alkylbromides containing an oil-soluble dye, the amount of dye associated with the large vesicles is much less than expected if they contained oil instead of electrolyte.

The evidence presented here does not distinguish conclusively between fusion of intact small vesicles and a process in which the small vesicles are ruptured and the membranes from a fraction of these reseal into the large vesicles observed.

However, the observations that the large vesicles (i) do not contain either small vesicles or membrane fragments, and (ii) that the large vesicle formation induced by *n*-hexyl bromide which occurs in suspension produces no change in total pellet volume, are suggestive of a mechanism of large vesicle formation arising from membrane vesicle fusion rather than rupture and resealing.

Until the recent work of Haydon and coworkers [10–12], little was known regarding the effect of alkanes on membrane properties. Earlier, however, Drabikowski *et al.* [8] found that the aliphatic hydrocarbon, heptane, at a stated concentration exceeding saturation of the aqueous phase, has no detectable effect on normal calcium transport and calcium-ATPase activities in sarcoplasmic reticulum, although its use as a fusogenic agent was not recognized.

Alkyl bromide-induced membrane fusion holds promise for future investigations of cells and subcellular organelles. The large size of the vesicles produced from the subcellular membrane fraction studied here makes particularly attractive the application of this technique to investigations of the properties of these membranes. One of us (WTM) will discuss elsewhere the application of microelectrode techniques to this preparation.

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