PREPARATION AND PROPERTIES OF VESICLES ENCLOSED BY FATTY ACID MEMBRANES

J.M. GEBICKI and M. HICKS
School of Biological Sciences, Macquarie University, North Ryde N.S.W. 2113, Australia

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Stable preparations of microscopic particles were obtained from long-chain fatty acids by mechanical agitation of evaporated films in presence of buffer solutions. Oleic and linoleic acids were used. Studies of osmotic swelling and shrinking of the particles indicated that they are enclosed by semipermeable membranes. The particles, which were named ufasomes, are also capable of entrapping glucose in spaces inaccessible to enzymes. It was concluded that the ufasomes closely resemble phospholipid liposomes in their structure and properties.

I. Introduction

Studies of biological membranes are complicated by the chemical heterogeneity of their constituents. A fruitful approach to greater simplicity without drastic departure from natural conditions was provided in recent years by model membranes made from phospholipids [1–3]. The black lipid and the liposome membranes have yielded useful information on the probable assembly, stabilization, permeability and molecular motions of the lipid constituents of biological membranes. However, even natural phospholipids are chemically heterogeneous, and pure synthetic phospholipids are not yet available in reasonable quantities. Accordingly, we have recently produced stable membranes from pure long-chain fatty acids [4]. The membranes were formed around microscopic aqueous spaces, very much like the phospholipid liposomes. For convenience, the resultant particles were named ufasomes, because of their obligatory content of unsaturated fatty acids.

The advantage of ufasomes over liposomes is the ready availability of fatty acids. Their disadvantage is that while phospholipids constitute the predominant lipid of natural membranes, free fatty acids are seldom found there. However, it is becoming increasingly clear that it is the acyl fatty acids of phospholipids and not the polar head groups that determine the properties of lipid bilayers, so that a fatty acid model membrane is potentially capable of giving useful information even on natural membranes.

This paper describes conditions under which ufasomes can be prepared and sum-
marises evidence for the suggestion that they are enclosed by semipermeable membranes. Results of a study describing light and electron microscopic observations on ufasomes will be published separately.

II. Materials

Fatty acids were obtained from the Lipid Preparation Laboratory of the Hormel Institute, Minnesota. Their purity, stated to be over 99%, was checked after esterification with BF₃-methanol reagent [5] in a Pye Unicam Series 104 Gas Chromatograph on a polyethylene glycol adipate column. Both oleic and linoleic acid esters were at least 99.7% pure. Some linoleate samples showed a triple absorption peak with maxima at 259, 269 and 279 nm, which suggested the presence of eleostearic acid. The contamination was less that 0.3% as estimated with molar extinction coefficient of $6.12 \times 10^4$ [6]. Attempts to remove this contaminant by preparative GLC were not successful because peak overlap was almost total.

Yeast hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) each with a specific activity of 200–300 I.U./mg were obtained from Calbiochem (Australia) Pty. Ltd. This was also the source of ATP, NADP and "Triton X 100" used in glucose assays. The tris-hydroxymethyl aminomethane buffer was from the Sigma Chemical Co., St. Louis, Missouri. All other chemicals and solvents were of analytical grade. Water was triply distilled in a stream of oxygen from alkaline permanganate and acid dichromate.

III. Methods

A. Preparation of particle suspensions

Stock 10% solutions of oleic and linoleic acids in chloroform were stored at $-20^°C$. Stability tests showed that oleic acid remained uncontaminated by peroxides for at least 6 weeks while linoleic developed significant absorption at 234 nm after 2–3 weeks. Only unoxidized materials were used. For typical preparations, 0.02 ml of the stock solution was evaporated in a test tube on a water pump and finally dried with a stream of nitrogen. The fatty acid film was then broken up completely in 0.2 ml of 0.1 M tris buffer, pH 8–9, by vigorous shaking on a vortex mixer. The resultant suspensions of ufasomes were stable for at least 24 hr. In some experiments an ultrasonic generator (Branson Sonic Co., Dunburry, Connecticut) with a titanium micro tip was used to prepare the particles. Air was first removed from the buffer by a stream of nitrogen and the suspension was blanketed with the gas during irradiation. Constant temperature was maintained by an ice water bath.
B. Osmotic responses

Measurements of the effects of osmotic pressure changes on the amount of light scattered by ufasomes were made with an Aminco-Morrow stopped-flow apparatus. This had a quartz mixing and observation chamber with a 1 cm optical path and a dead time of less than 5 msec. It was operated in conjunction with an Aminco DW-2 spectrophotometer which was used in the absorbance mode. Fast initial signal changes were displayed on a Hewlett-Packard 1703A storage oscilloscope and photographed. Slow changes were recorded directly.

For osmotic shrinking measurements, a 2 ml suspension of ufasomes made from 20 mg of fatty acid was diluted to 20 ml with 0.1 M tris pH 8. This was placed in one storage reservoir of the stopped-flow machine. The other reservoir was filled with a similarly buffered sucrose solution. Because of the geometry of the apparatus, the ufasomes were exposed on mixing to sucrose concentration equal to a half of that in the storage reservoir. For swelling experiments, the ufasomes were prepared in buffered sucrose and diluted 1 : 1 with buffer in the mixing chamber during flow.

C. Solute retention

Retention of solute by ufasomes was measured by including 0.5 M glucose in the buffer in which the spheres were formed. The suspensions were then dialysed at room temperature with stirring against 0.01 M tris at pH 8 for 2–5 hr. About 500 ml of solution were used for every 0.2 ml of dense ufasome suspension. After dialysis the glucose was measured by a method based on the technique used by Kinsky et al. [7] for phospholipid liposomes. The main modification was omission of the KCl and NaCl, which rendered ufasomes unstable. Assays were carried out by adding 0.05 ml of the dialysed ufasome suspensions (with about 10 mg of the fatty acid per ml) to a spectrophotometer cuvette containing the following: 1.5 ml of 0.1 M tris buffer pH 8, 0.5 ml water, 0.5 ml 0.002 M MgCl₂, 0.1 ml NADP (8 mg/ml water), 0.1 ml ATP (13 mg/ml water), 25 I.U. of hexokinase and 10 I.U. of glucose-6-phosphate dehydrogenase. After mixing, the increase in absorbance at 340 nm due to formation of NADPH was recorded. When all glucose external to the spheres was exhausted, a constant slow increase of absorbance indicated that glucose was leaking from the ufasomes at a steady rate. Addition of 0.1 ml of 10% triton X-100 solution in water ruptured the sphere membranes, releasing trapped glucose and producing a rapid increase in absorbance.

IV. Results

A. General properties of ufasomes

Initial studies explored materials which might be suitable membrane formers. Analyses of natural membrane phospholipids [8] and information from the pressure-
area measurements on fatty acid surface films [9] suggest that the 12 to 22 carbon fatty acids would fall in this category. In fact, most of our studies were confined to the C-18 acids because they showed the greatest promise in early trials. A preparation was judged successful if a thin film of the substance tested resuspended completely on shaking in aqueous medium. The suspension had then to be stable for several hours, show spherical birefringent particles under a microscope, and possess ability to hold glucose in spaces inaccessible to enzymes. Only oleic and linoleic acids formed membranes which enabled the ufasomes to fulfill these criteria. Many other substances were incorporated into oleic and linoleic membranes but only a small number did not affect them adversely. Of other fatty acids, palmitic is tolerated up to 33% and stearic up to 5% by weight in an oleic acid membrane. Relatively large amounts of cholesterol can also be added, and its effect on membrane properties was studied more extensively. Charging of the membrane with small amounts of oleic, linoleic or stearic acid amides did not improve the preparations. This was also true for dicetyl phosphate which is used extensively in phospholipid liposomes [10].

Two parameters crucial to successful preparations are the pH and the buffer in which ufasomes are formed. The particles are only stable between pH 8 and 9; below this range the acids only form unstructured precipitates, while above, they are too soluble. The best buffer appears to be tris-hydroxymethyl aminomethane. However, spheres also form in borate, glycine—hydroxide and bicarbonate solutions. Of these, spheres prepared in bicarbonate did not hold glucose, while the borate preparations could not be tested for retention because of formation of glucose—buffer complex. With tris, the optimum weight of buffer has to equal the weight of fatty acid used to form membranes; thus, 0.1 ml of 0.1 M tris at pH 8 is needed to form ufasomes from 1 mg of fatty acid.

As oxidation of the fatty acids might have a deleterious effect on membrane stability, the degree of peroxidation of linoleic ufasomes was measured at different stages of their preparation. Oxidation was detected by comparing the absorption of samples at 234 nm and converting to peroxide concentrations using 30,000 for the molar extinction of conjugated oxidized derivatives [6].

No peroxidation occurred during the short periods required for hand vortexing. Under the more violent ultrasonic resuspension, linoleic acid oxidized at 0.1% per minute in air saturated buffers when exposed to 30 watt irradiations. Since 3 min was the longest exposure used, this method did not produce extensive oxidation of even this sensitive acid. In the 4 hr dialysis times usually employed, linoleic ufasomes oxidized at 0.05% per hour in contact with solution deaerated with a slow stream of nitrogen, and at 0.23% when oxygen was not removed. As no change in the capacity to retain glucose was detected for spheres oxidized to the extent of 2%, dialysis was usually carried out with air saturated stirred solutions.

Most electrolytes inhibit formation of ufasomes. However, once the spheres are stabilized in appropriate buffer, they can be exposed to solutions of phosphates or chlorides and still retain occluded glucose. Dialysis against dilute inorganic buffers can thus be used to remove tris buffer which may be undesirable for some experi-
ments. A more rapid means of changing the bathing solution is column chromatography. In a limited number of tests, coarse Sephadex G-25 appeared to offer best separation between ufasomes and glucose. The spheres eluted in the void volume and had normal appearance and light scattering properties, but they held less glucose per mg of fatty acid than dialysed ufasomes.

B. Osmotic response

Both swelling and shrinking of ufasomes were studied. Sucrose was employed as the slowly penetrating solute, because electrolytes made the spheres unstable. Initial attempts to measure light scattering changes by rapid addition of ufasomes to solutions of different osmotic pressures in spectrophotometric cuvettes were not satisfactory, because too much information was lost in the first part of the scan. This was corrected when the stopped-flow equipment became available. A similar instrument coupled to a Beckmann DU spectrophotometer was previously used by Schullery and Garzaniti [11] to study osmotic responses of phospholipid liposomes. This equipment measured turbidity values which depended only on stability of the baseline signal during the run for accuracy. In our work the Aminco spectrophotometer was used in the single cell mode, in which light pulses at two different wavelengths pass through the stopped-flow cell alternatively. Output depends on the difference in attenuation of the two pulse trains. Thus, although any baseline drifts are

Fig. 1. Absorbance of ufasome suspensions measured at 375 nm on a Varian Techtron Spectrophotometer and absorbance differences ($A_{375} - A_{800}$) measured on Aminco DW2 Spectrophotometer. • oleic ufasomes, o linoleic ufasomes.
Fig. 2. Absorbance changes of swelling linoleic ufasomes measured directly on DW2 Spectrophotometer. Original $A_{375}-A_{800}$ converted to absorbance at 375 nm. Ufasomes prepared in 0.1 M Sucrose buffered with 0.1 M tris pH 8 were mixed in the stopped-flow cell with 0.1 M tris pH 8. Insert: Oscilloscope scan of fast change — total absorbance fall 0.04 units, total time 1.8 sec. Graph: Slow changes recorded from same run.

corrected, absorbance differences rather than absolute values are obtained. Since absolute measurements are required in some analyses, we compared the absorbances of oleic and linoleic acid ufasomes at 375 and 800 nm on a Varian spectrophotometer with absorbance differences measured over the same interval on the Aminco instrument. The results are shown in fig. 1. Clearly, absorbance differences were proportional to absolute absorbance over the region in which all our measurements were subsequently made. It is possible to convert a $A_{375}-A_{800}$ reading of the Aminco instrument to absolute $A_{375}$ by multiplying the former by a simple factor: 1.28 for oleic and 1.25 for linoleic acid ufasomes. Use of the spectrophotometer in the absorbance mode meant that the usual conversion of turbidities was unnecessary.

Typical examples of absorbance changes accompanying swelling and shrinking of ufasomes are shown in figs. 2 and 3. The inserts show rapid changes photographed from the storage oscilloscope screen and the traces show slow changes recorded by the spectrophotometer on the same samples. Rapid initial swelling of linoleic ufasomes (fig. 2) was recorded at 200 msec/cm and the slow changes at 40 sec/cm. Evidently the fast initial fall of absorbance resulting from movement of water into
Fig. 3. Absorbance changes of shrinking linoleic ufasomes measured directly on DW2 Spectrophotometer. Original $A_{375} - A_{800}$ converted to absorbance at 375 nm. Ufasomes prepared in 0.1 M tri$\text{S}$ pH 8 were mixed with buffered sucrose in the stopped-flow cell to a final concentration of 0.1 M Sucrose in 0.1 M tri$\text{S}$ pH 8. Insert: Oscilloscope scan of fast change — total absorbance increase 0.09 units, total time 0.9 sec.

the particles continues at a reduced rate for at least 10 min. This process is irreversible in that absorbance of swollen ufasomes does not increase on subsequent addition of sucrose. Oleic acid spheres showed similar responses.

Rapid shrinkage of linoleic acid ufasomes mixed with 0.1 M sucrose (fig. 3) was completed in about 1 sec. This was followed by a slower fall of absorbance to the original level. Subsequently a complex change occurred; the second slow increase, followed by an even slower decrease of absorbance, was quite consistent and reproducible. Oleic acid ufasomes behaved similarly, but the second rise in absorbance was not so pronounced.

The fast kinetic curves were analysed by measuring positions of closely spaced points on the centre of photographed line with a travelling microscope and relating them to heights above a horizontal line. These heights were converted to voltages by reference to oscilloscope grid spacings measured similarly. Since voltages correspond directly to optical absorbance values in this system, initial readings and rates could be expressed in units of absorbance.

The initial changes in suspensions undergoing shrinkage obeyed first order kinetics
Table 1
Half lives for shrinking ($t_s$) and reswelling ($t_r$) of tris ufasomes mixed with buffered sucrose (final concentration given in first column).

<table>
<thead>
<tr>
<th>Sucrose (mole $1^{-1}$)</th>
<th>Oleic ufasomes</th>
<th>Linoleic ufasomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_s$ (msec)</td>
<td>$t_r$ (sec)</td>
</tr>
<tr>
<td>0.02</td>
<td>325</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>450</td>
<td>65</td>
</tr>
<tr>
<td>0.06</td>
<td>430</td>
<td>71</td>
</tr>
<tr>
<td>0.08</td>
<td>430</td>
<td>64</td>
</tr>
<tr>
<td>0.10</td>
<td>410</td>
<td>64</td>
</tr>
<tr>
<td>0.12</td>
<td>430</td>
<td>64</td>
</tr>
<tr>
<td>0.15</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>340</td>
<td>55</td>
</tr>
</tbody>
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for the first 100 msec. This simplified the measurement of zero-time absorbances and initial rates, since the semilogarithmic plots had a constant slope and could be accurately extrapolated to the start of measurement. In addition, half-lives for the shrinkage process were calculated. These, together with the half-lives for the subsequent slower changes, are listed in table 1. Sucrose concentrations above 0.4 M could not be used in the stopped-flow machine because of problems associated with rapid flow and mixing of viscous solutions.

![Graph](image)
These results indicate that neither oleic nor linoleic acid ufasomes are impermeable to sucrose. It is possible to observe light scattering changes in suspensions mixed rapidly with solutions of different osmolarities only because water is a much faster permeant than sucrose. This contrasts with suspensions of osmotically shrunk or swollen liposomes which acquire a new equilibrium absorbance, stable over several hours and related to the new average particle volume [10]. Reciprocal plots of this absorbance as a function of concentration of non-penetrating solute show a limiting absorbance at infinite solute concentration. This corresponds to the osmotically inactive volume of liposomes. Bangham and his coworkers calculated this volume by measuring the relationship between absorbance and volumes of centrifuged liposomes. Although a similar experiment cannot be performed with ufasomes because they do not concentrate on centrifugation, it can be shown that they behave qualitatively like the liposomes. Fig. 4 was plotted from maximum absorbance values achieved by suspensions shrunk with various concentrations of sucrose. It was assumed that the penetration by sucrose during the time required to achieve this absorbance was negli-

![Graph](image)

Fig. 5. Absorbance of ufasome suspensions prepared from oleic, linoleic and oleic:linoleic acid (1:1) in 0.1 M tris pH 8. • oleic, ○ linoleic, ■ oleic:linoleic (1:1) ufasomes.
Fig. 6. Glucose retention by oleic ufasomes as assayed by NADPH formation from the hexokinase – glucose-6-phosphate dehydrogenase reactions. Ufasomes were lysed with triton X-100 after 15 minutes (arrow). GE—glucose external to the ufasomes, GI—glucose sequestered by ufasomes. GT = GE + GI.

gible. Linearity of the plot for both oleic and linoleic ufasomes and the intercept on the A⁻¹ axis shows that the particles behave as osmometers over a range of sucrose concentrations and they possess an osmotically dead space.

C. Glucose retention

As the determinations of glucose concentrations were carried out at 340 nm, effect of the amount of fatty acid dispersed to form ufasomes on absorbance was checked at that wavelength. It can be seen from fig. 5 that within the useful range of the spectrophotometer the relationship between ufasome concentration and the amount of light scattered is linear. Evidently, the particles do not clump as their concentration increases. Spheres prepared from linoleic acid always gave less turbid suspensions than oleic ufasomes. This may be due to a difference in particle size or intrinsic properties of membranes formed from these compounds.

Results of typical measurements of glucose retention by hand-vortexed preparations are shown in fig. 6. Amounts of glucose are expressed in n moles per μ mole of fatty acid used. GE is the glucose external to ufasomes after dialysis and GI is the amount trapped by the spheres and released by the detergent. The sum of these is GT. The ratio GI/GT is a measure of the integrity and stability of the particles. This
Figure 8. Effect of linoleic acid on percent of total glucose segregated by oleic isomers.

Figure 7. Effect of sonication time on the amount of glucose trapped by linoleic isomers.
Fig. 9. Effect of cholesterol on the percent of total glucose sequestered by ufasomes. • oleic ufasomes, ○ linoleic ufasomes.

Fig. 10. Leakage of glucose from ufasomes of different composition. • oleic acid, ▲ oleic acid + 17% cholesterol, ○ linoleic acid, △ linoleic acid + 17% cholesterol.
ratio is quite reproducible and depends mainly on the degree of dispersion achieved, which can be controlled by the method used to form the ufasomes. Spheres prepared by sonication retain less solute per unit weight of fatty acid. This is probably due to the much smaller size of spheres prepared by the more drastic treatment. The effect is illustrated in fig. 7.

Tests with various molar ratios of the fatty acids and cholesterol showed only minor systematic trends in the spheres' capacity to retain glucose. For most preparations spontaneous leakage rates at the end of dialysis fell between 7 and 30 μ mole glucose released per min per m mole acid. This compares well with values published by Demel et al. [12] for lecithin liposomes, known to be enclosed by tight, stable membranes. The one generalization that can be made about ufasomes prepared from different materials is that as unsaturation of their membranes increases, so does the permeability to glucose. This effect is shown in fig. 8, where the glucose held by various mixtures of oleic and linoleic acids is shown as a percentage of total glucose in the system. Results of similar experiments designed to test the effect of cholesterol on oleic and linoleic acid ufasomes are summarized in fig. 9. Although the additive is tolerated by either type of particle up to 30 mol%, there is a rapid decrease in the ability to hold glucose in presence of higher proportions of cholesterol. Also, there is no enhancement of membrane impermeability at any cholesterol concentration. This is confirmed by results shown in fig. 10 where the leakage of glucose from oleic and linoleic ufasomes is compared with leakage from spheres containing 17% of incorporated cholesterol by weight. Besides confirming the deleterious role of cholesterol on membrane permeability to this solute, the results underline the relatively leaky nature of the more unsaturated membranes.

V. Discussion

Long chain fatty acids can form homogeneous or heterogeneous systems with water. At near neutral pH, dilute homogeneous solutions contain soap ions and various acid–soap complexes, which are converted to soap as the pH increases. In more concentrated solutions the ions associate to form micelles [13]. This renders the system heterogeneous. Other well known heterogeneous water–fatty acid systems are emulsions, separate bulk phases or surface monolayers.

This paper elaborates our earlier report [4] that, under certain conditions, another stable heterogeneous system also exists, in which the fatty acid molecules are organised to form membranes enclosing sealed aqueous spaces. We have called the resultant vesicles ufasomes. Evidence for their existence rests mainly on their ability to respond to changes in osmotic pressure and to trap glucose. Further evidence that they are capable of mimicking in many ways the properties of phospholipid liposomes comes from polarization and freeze-etch microscopy. This material will be published separately.

It seems profitable to discuss ufasomes by comparing them with the thoroughly
studied liposomes. There are many similarities. Starting with the method of preparation, virtually identical techniques can be used for either type of vesicle. The one interesting difference is that intensive sonication of fatty acid dispersions does not lead to uniformly-sized particles. Instead, there is some evidence to suggest that oleic and linoleic acids can be forced into solution to produce a clear supersaturated system which becomes turbid after standing for a few minutes. The particles which form in that time consist of spherical and cylindrical vesicles showing weak light scattering. Inability to produce a suspension of particles of constant size by this method is a considerable disadvantage, since it renders quantitative studies of ufasome permeabilities unreliable.

Compared to liposomes, ufasomes are much more sensitive to pH and ionic strength of medium. While the phospholipid vesicles tolerate a range of conditions [2], fatty acid membranes fail to form, except at slightly alkaline pH and at low ionic strengths.

Comparison of the light scattering properties of ufasomes and liposomes shows that the phospholipid vesicles are stronger scatterers per mole of material. It is not easy to make an exact comparison; Bangham et al. [10] measured the absorbance at 450 and not 340 nm (fig. 1) and their particles showed a non-linear response with concentration. Roughly, a $10^{-3}$ molar liposome suspension has absorbance of 0.7 while a similar preparation of ufasomes reads about 0.2. Part of this difference may lie in the relatively large cross-sectional area of phospholipids. If it is assumed that the fatty acids are oriented in membranes like the phospholipids i.e. with hydrocarbon chains not far from perpendicular to the surface, the molecular areas can give a clue to the relative size of a bilayer formed from each compound. This requires a further assumption that membrane molecules are closely packed by a force equivalent to a compression in a surface monolayer. Reasonable cross-sectional areas at 10–20 dyne cm$^{-1}$ are 0.8 nm$^2$ for lecithin [14] and 0.4 for oleic and linoleic acids [15]. It appears likely, therefore that a mole of lecithin forms a membrane twice as large as that formed from a mole of either of these acids. Since the size distribution of liposomes and ufasomes in the preparations considered appears to be similar there may be twice as many particles in the phospholipid suspension for a given number of molecules. This would go some way towards explaining the differences in absorbance. However, a similar argument cannot explain the consistent differences in amount of light scattered by oleic and linoleic particles (fig. 5). As there is no evidence that oleic ufasomes are smaller, there may be some intrinsic difference in the membranes formed which would account for this observation.

One type of evidence in support of the view that ufasomes are surrounded by a semipermeable membrane comes from measurements of their osmotic properties. Such measurements have been used extensively for membranous particles such as mitochondria, chloroplasts and liposomes [10,16,17]. An extensive study of charged and uncharged phosphatidyl choline liposomes by Bangham et al. [10] showed that the particles behave as good osmometers over a range of osmotic pressures. The authors calculated permeability coefficients from measurements of
particle volumes and surface areas. These were later shown to be unreliable because of the presence of several compartments in the particles [18] but ultrasonic preparation of small liposomes overcame this problem in subsequent experiments [19].

The interpretation of our absorbance data in terms of swelling and shrinking assumes that ufasome volume changes can be measured by the amount of light scattered. An analysis based on the theory of scattering by suspensions of particles with dimensions comparable to the wavelength of analysing light shows that absorbance is inversely proportional to the $\frac{4}{3}$ power of particle volume [20]. In practice the simple empirical relationship $A^{-1} = KV + B$, where $A$ is absorbance, $V$ volume of particles and $K$ and $B$ are constants, holds for liposomes and mitochondria [10,16]. Since the parameters which determine light scattering properties of these particles and of ufasomes are similar, we have assumed that absorbance measurements are a valid guide to volume changes of the fatty acid membranes.

It is clear from figs. 2 and 3 that, unlike liposomes, the ufasomes are permeable to sucrose. The swelling curve shows that although over 90% of the volume change occurs in the first two minutes following mixing, the process continues at a decreasing rate. Bangham et al. [10] showed that addition of KCl to suspensions undergoing swelling resulted in contraction of the spheres. The opposite effect, re-swelling of liposomes shrunk by sucrose, was reported by Rendi [21]. We have found that swelling of ufasomes cannot be reversed by addition of sucrose. It is important to note that in Bangham's experiments swelling was not completed at the time of addition of KCl. It is possible that reversal of swelling cannot be induced in suspensions showing minimum absorbance because at that stage the membranes may be ruptured. Haydon [3] has pointed out the limited ability of phospholipid leaflets to extend through thinning and predicted rupture after only a small extension. Absorbance measurements cannot distinguish between greatly swollen and ruptured particles, as has been shown with mitochondria [22]. For liposomes, Kinsky [23] reported that particles exposed to hypotonic conditions leaked glucose, indicating lysis. Our results suggest therefore that for ufasomes swelling is quickly followed by membrane fracture. A second indication of the fragility of ufasome membranes is given by the behaviour of the particles after shrinking in sucrose (fig. 3). The initial rapid increase in absorbance caused by movement of water from ufasomes is followed by a much slower fall resulting from the flow of sucrose into particles (table 1). This sequence resembles changes which liposomes undergo when exposed to solutes with permeability coefficients of the order of $10^{-6}$ cm/sec [24]. Equations used to derive these constants from absorbance measurements cannot be applied to ufasomes because they require knowledge of particle volume and surface area [25]. It is possible to derive relative coefficients for permeating solute without this information, provided the size parameters of the particles and the amount of light scattered by their suspensions are constant. Conversion to absolute permeability coefficients requires that at least one of them is known in an absolute sense. There are several possible sources of error in these measurements: Schullery and Garzaniti [11] have shown that the dependence of $d(A^{-1})/dt$ on shrinking rate and per-
meability also varies with total mass, number and size distribution of liposomes in suspension. These findings are also likely to hold true for ufasomes, but it is clear that their membranes exhibit the properties of semipermeable barriers in the early stages of swelling or shrinking (figs. 2, 3).

Ufasomes and liposomes have a similar capacity to entrap glucose. The very useful technique for measuring this capacity was pioneered by Kinsky and his collaborators [7]. Their preparations made up from lecithin with added cholesterol and dicetyl phosphate held about 1200 n mole glucose per µ mole lipid. When lecithin was replaced by sphingomyelin, this amount was nearly doubled [23]. Compared to this, ufasomes entrap about 450 n mole of glucose per µ mole of fatty acid (fig. 6). This may again be due to a smaller number of spheres forming per mole of fatty acid.

The measurement of amount of trapped glucose provides a sensitive test for the quality of ufasome preparation. Suspensions showing abnormally high spontaneous leakage rates or entrapping low quantities of glucose were always discarded. The two common sources of high permeability were incomplete removal of solvent from fatty acids and accidental oxidation during preparation of particles. Presence of residual solvent is often held responsible for the high permeability of black lipid membranes (BLM) when compared with liposomes. For example, the permeability of coefficients for glucose are $10^{-10}$ cm/sec (upper limit) for BLM [26] and $6.8 \times 10^{-12}$ for soy bean lecithin liposomes [27]. Oxidation is considered a major hazard in preparation of liposomes and many workers go to considerable trouble to prevent it. Although we have not found it necessary to work under an inert atmosphere, high permeability in some ufasome preparations was traced to the presence of hydroperoxides. We are currently studying this phenomenon.

Results summarised in fig. 4 show that increase in permeability of oleic acid membranes containing linoleic acid is linear with the amount of additive. This suggests that the acids mix completely in membranes at room temperature, as would be expected from their chemistry and melting points (16.3°C oleic, −5°C linoleic). Were it not so, a lateral phase separation could occur. A discontinuity in the amount of glucose held would then become apparent at the mole fraction of linoleic acid at which it began to dominate glucose efflux. Clearly this does not occur with ufasomes made from oleic and linoleic acids.

The experimental data reported above suggest that oleic and linoleic acids can, under suitable conditions, be induced to form semipermeable membranes, capable of enclosing aqueous spaces. There can be little doubt that the membrane fatty acids are oriented in a bilayer form with their hydrocarbon tails towards the membrane interior and the carboxyl groups in contact with water. Direct evidence for this consists at present of electron microscopic pictures of freeze-etched preparations and of photomicrographs taken under conditions designed to detect birefringence. Although these will be described in detail elsewhere, it can be noted here that the former studies show that many of the spheres consist of concentric layers of non-aqueous material, while the presence of birefringence provides evidence for molecular radial symmetry. This, together with the results given in this paper, renders any other orientation of the fatty acids unlikely.
The long-term stability of ufasome membranes means that their formation is accompanied by a decrease in free energy of the fatty acid—water system. Membrane formation is not spontaneous, because the acids form a separate phase at pH 8. However, even mild mechanical agitation is sufficient to induce bilayer formation under the right conditions. Clearly, much of the energy liberated in this process comes from the increased entropy of water which accompanies the hydrophobic interactions of the oriented hydrocarbon chains.

This attractive interaction is opposed in the bilayer by mutual repulsions of the ionized carboxyl head groups. Adam [28] has shown that electrolytic dissociation decreases fatty acid film stability and may cause its disruption. Charge repulsion can be lessened by a decrease of the degree of head group dissociation, by formation of stable complexes between protonated and ionized carboxyl head groups or by presence of screening counterions. All these processes may operate in stabilization of ufasome membranes.

As the $pK_a$ of long chain fatty acids is 4.76 in solutions of ionic strengths around 0.1 [29], the carboxyl groups of membrane molecules should be virtually fully ionized in the pH range which allows formation of stable ufasomes. Fortunately for the stability of membranes, lateral charge repulsions are decreased by the lowering of pH which occurs at particle surface. This phenomenon was first investigated by Peters [30] who showed that stearic and oleic acids at a water—benzene interface were only 50% ionized when the bulk pH was 3 units above their $pK$ values. The effect was explained by the Gouy [31] theory of counterion distribution near a charged surface. Calculation showed that a negatively charged surface of 200 mV potential attracts enough protons to lower the surface pH by 3 units [32].

The ufasome surface is analogous to a charged monolayer of fatty acids in contact with aqueous medium. Since in our preparation the only available counterion is $H^+$, we can expect the surface pH to be also about 3 units below the bulk pH of 8. This brings the carboxyl groups right into their $pK$ region, so that the fatty acids in ufasomes are only about 50% ionized.

This decrease in ionization enhances membrane stability in several ways. First, the protonated molecules are virtually insoluble in water by comparison to the anions. Secondly, there is a reduction in lateral head group repulsion; in a film of closely packed head groups the average distance between charges increases by about 40% on the removal of every second charge, resulting in a halving of coulombic repulsions. Thirdly, protonated acid molecules ($AH$) and anions ($A^-$) form series of strongly bound complexes, with a $1:1$ complex the predominant species. Dimeric $AH:A^-$ acid soaps of many long-chain acids were first found in conductometric experiments [33] and more recently studied by differential thermal, X-ray, infrared and chemical composition analyses [34]. Other stable complexes of this type are also known: Eagland and Franks [35] have suggested the existence of $A^2_2$ and $AH:A^2_2$ species in order to explain the hydrolysis of C-14 and C-16 acids. Smith and Tanford [29] have shown recently that in solution $AH:A^-$ complexes of C-18 acids do not dissociate until pH 9 is reached.
The energy for this binding is made up of three contributions: free energy changes arising from hydrophobic interactions, entropy of demixing associated with formation of dimers and a free energy lowering brought about by the formation of hydrogen bonds between the protonated and ionized carboxyl groups. It is the last of these that distinguishes the acid soap dimers from simple hydrophobic association in ionic micelles. Studies of interactions in dicarboxylic acids have shown that exceptionally strong hydrogen bonds form between \(-\text{COOH}\) and \(-\text{COO}^-\) groups due to the presence of a negative charge close to the hydrogen involved in bonding [36].

The mechanism of counterion attraction can also account for the instability of ufasome membranes in presence of electrolytes. When protons are the only counterions, surface pH falls to a value which allows the several membrane stabilising factors to come into operation. If other cations are present, some of the surface protons are displaced, so that pH lowering is insufficient for maximum stability, unless bulk pH is also lowered. In practice a stable membrane formed in presence of protons only can tolerate low concentrations of salts which are apparently unable to displace the surface protons with ease.

Because of the more exacting conditions of pH, ionic strength and nature of stabilizing buffers, ufasomes do not offer many advantages for general membrane studies over phospholipid liposomes. However, they are chemically very simple and their membrane composition can easily be manipulated by alteration of the fatty acid composition or by addition of lipid soluble compounds. This offers an opportunity for studies of the behaviour of many substances in various membrane situations with virtual independence of the nature of the surface hydrophilic groups. Physical investigations by NMR, ESR, DTA and other techniques are all feasible.

In addition to such studies, ufasomes can be used as prototypes of simple cells with membranes made up of materials formed on primitive earth in the absence of life. Our previous communication [4] pointed out the conditions under which such primitive membranes could form. Other unsaturated hydrocarbons can be employed as possible membrane formers and the ufasomes or some related membranous particles can be used to study conditions under which essential biochemicals are synthesised in partial isolation from their environment.

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References

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