STUDIES ON CHEMICALLY INDUCED
CELL FUSION

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SUMMARY

Hen erythrocytes that were fixed after treatment with lysolecithin in aqueous solution for 30 s at 37 °C showed evidence of bridge formation between adjacent lysed cells. Generally, the homokaryons that were produced using lysolecithin in this way contained large numbers of nuclei. These giant syncytia had damaged nuclear membranes and unstable plasma membranes; complete disintegration of the syncytia occurred within 1 min of adding lysolecithin to the erythrocytes.

In order to localize the action of lysolecithin, the fusing agent was incorporated into microdroplets of lipid. Cell fusion following the addition of lysolecithin in an aqueous glyceride-lecithin emulsion was slower than with lysolecithin in aqueous solution, taking 10–30 min, and it was accompanied by considerably less damage to the plasma and nuclear membranes. The fused erythrocytes, which usually contained only two or three nuclei, lysed slowly during the 45 min following fusion, and lysis could be arrested by cooling the fused cells. The plasma membranes of lysed, multinucleated cells remained intact at 37 °C for at least 90 h.

Mouse fibroblast-hen erythrocyte heterokaryons formed with the aid of the emulsion were more stable than those produced with lysolecithin in solution, but the hybrid cells nevertheless had damaged subcellular organelles. Viable clones of hybrid mouse–hamster fibroblast cells were obtained using the emulsion although, possibly owing to reduced viability of the lysolecithin-treated cells, only at twice the frequency of spontaneously produced hybrids.

INTRODUCTION

In addition to its well known haemolytic action, lysolecithin has been reported to induce phagocytosis (Burdzy, Munder, Fischer & Westphal, 1964), to cause elongation of surface microvilli (Wilkinson & Cater, 1969), to increase the permeability of cell membranes (Mehrishi & Butterworth, 1969), and to cause pre-lytic sphering of erythrocytes (Klibansky & DeVries, 1963). Lysolecithin has been implicated in various processes involving membrane fusion in vivo such as the release of catecholamines (Blaschko, Firemark, Smith & Winkler, 1967; Winkler, 1971) and histamine (Högberg & Uvnäs, 1957) from membrane-bound compartments within cells, and also in membrane fusion occurring in the lysosomal vacuolar system (Lucy, 1969). The calcium-dependent ATPase of fragmented sarcoplasmic reticulum requires small quantities of unsaturated acids or lysolecithin for its activity (Fiehn & Hasselbach, 1970), and lysolecithin-treatment of this membrane system gives rise to elongated, irregularly shaped, branching tubular structures which may arise, in part, from rearrangement of the vesicles with membrane fusion (Agostini & Hasselbach, 1971).
It has been found in this laboratory that lysolecithin can induce cells to fuse in vitro with the formation of both multinucleated homokaryons (Howell & Lucy, 1969) and heterokaryons (Poole, Howell & Lucy, 1970). Cell fusion was, however, accompanied by extensive degradation of the treated cells. In the present paper, interactions between fusing cells, and the degradative changes observed when hen erythrocytes are treated with a solution of lysolecithin are reported in more detail.

One aim of the studies described here was to see if cells could be fused by lysolecithin without extensive membrane damage, since this would provide circumstantial evidence for the possibility that lysolecithin may be concerned in some of the processes of membrane fusion occurring in living cells. In addition, in view of possible future applications of lysolecithin-induced cell fusion in investigations employing cell hybridization, it is important to know whether or not viable cell hybrids can be obtained with the aid of lysolecithin. If the effects of lysolecithin on the plasma membranes of cells can be localized, cell fusion might occur in the absence of widespread damage to membranes. One way of localizing lysolecithin is to include it in the lipid phase of an aqueous emulsion of fat. Since about 10% of the phospholipid of the nascent fat droplets of cow’s milk is lysolecithin and the secretion of milk fat has been suggested to depend on exocytosis (Keenan, Morre, Olson, Yungans & Patton, 1970), it seems possible that the presence of lysolecithin in the nascent fat droplets of milk may be at least partially responsible for membrane fusion involved in the secretion of milk fat by exocytosis. With this in mind, emulsions of fat containing lysolecithin have been prepared and used in experiments on cell fusion. These emulsions have been found to induce the formation of relatively stable polykaryocytes and heterokaryons.

**MATERIALS AND METHODS**

Blood (approximately 2 ml) was removed from the brachial vein of an adult hen, and taken into a syringe already containing 1 ml of citrate anticoagulant solution (DeGowin, Hardin & Alsever, 1949). It was then transferred to a large volume of this solution and well mixed. The cells were washed twice in a large volume (approximately 20 ml) of the anticoagulant solution, and then twice in isotonic saline. The packed cell volume was measured and 1 ml of packed cells diluted to 8.5 ml with isotonic saline. After dilution, cells were counted in a haemocytometer: the population was generally $7-8 \times 10^8$ cells/ml.

Lysolecithin used in the initial experiments was a gift from Professor G. R. Webster. It was prepared from ovolecithin with snake venom (*Aghistrodon p. piscivorus*) and was chromatographically pure. Comparable preparations were obtained from Lipid Products, Epsom, and were also prepared in our laboratory. All preparations of lysolecithin behaved similarly in our experiments.

Ovolecithin was prepared according to the method of Papahadjopoulos & Miller (1967). It was stored in chloroform, under nitrogen, in small, sealed aliquots containing known quantities of phospholipid P (Perry, Tampion & Lucy, 1971). Glyceryl trioleate was obtained from B.D.H. and glyceryl dioleate from K. & K. Laboratories Inc.; thin-layer chromatography (Cater & Hallinan, 1971) revealed the presence of mono-, di- and tri-glycerides in both of these preparations. No significant differences were observed between the 2 preparations in our experiments with lysolecithin.
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Lysolecithin in aqueous solution

The techniques used have been described in a previous paper (Howell & Lucy, 1969): the final incubation mixture (1.7 ml) consisted of $4 \times 10^8$ avian erythrocytes, and 70-560 μg of lysolecithin in 160 mM NaCl and 44 mM sodium acetate at a final pH of 5.7. In early experiments, acetate buffer was occasionally used at pH 5.0. Cells were incubated at 37 °C.

Lysolecithin in lipid emulsions

A solution in chloroform of lysolecithin, lecithin and either glyceryl dioleate or glyceryl trioleate was prepared in a siliconized flask. All traces of chloroform were then removed by evaporation to dryness from an atmosphere of nitrogen in a rotary evaporator. The lipids were allowed to swell for 15-30 min in a 3:5 ratio mixture (v/v) of 0.9% NaCl and 0.15 M sodium acetate buffer at pH 7.2, and then sonicated under a stream of nitrogen at about 4 °C with a 60-W ultrasonic disintegrator tuned to maximum intensity (using a 19-cm diameter titanium probe, M.S.E. Ltd., London) for 1-2 min or until the bulk of the lipids was dispersed. All emulsions were used within 10 min of preparation, and contained 30 mg of glyceryl dioleate (or glyceryl trioleate), 270 μg lecithin and 30 μg lysolecithin per ml.

Washed hen erythrocytes were suspended in 0.9% NaCl, and 0.8 ml of the lipid emulsion in the buffered saline (pH 5.6) was added to 0.5 ml of red cell suspension containing approximately $8 \times 10^8$ cells/ml.

Mouse LS fibroblasts were derived from a culture kindly provided by Professor S. J. Pirt. The cells were grown as a monolayer in Eagle’s medium, supplemented with 10% foetal calf serum; they were harvested by shaking, washed 3 times with Hank’s saline and suspended in 0.9% NaCl just before use. 1.6 ml of lipid emulsion in the buffered saline (pH 5.6) was added at 4 °C to 10 ml of a mixed cell suspension in 0.9% NaCl (containing $7.3 \times 10^7$ hen erythrocytes and 8.5 \times 10^6 fibroblasts). The treated cells were centrifuged into a pellet to facilitate fusion. After 5 min at 4 °C the preparation was incubated at 37 °C for 15 min. Cells were fixed for electron microscopy by gently resuspending them in the glutaraldehyde fixative. A control experiment without the lipid emulsion was undertaken to determine the effect of the low pH on the ultrastructural appearance of the fibroblasts and on their ability to exclude trypan blue.

Electron microscopy

Cells were fixed in suspension immediately following incubation with lysolecithin by the addition of ice-cold cacodylate-buffered glutaraldehyde (Glauert, 1965). They were postfixed in osmium tetroxide containing ruthenium red (Pate & Ordal, 1967), stained in block with uranyl acetate (Farquhar & Palade, 1965) and embedded in Araldite (Glauert, Rogers & Glauert, 1956). Sections were cut on an LKB Ultratome III and stained with lead citrate (Venable & Coggeshall, 1965) when necessary. An AEI EM6 B electron microscope was used at an accelerating voltage of 80 kV and micrographs were taken at an initial magnification of $x 5000-15000$.

Cell hybridization

Cells of the mouse fibroblast line, 3T3 TK-; and of the hamster fibroblast line, Wg 3 IMP-,” were kindly provided by Professor G. Pontecorvo. Both cell lines received 2 passages in Dulbecco’s medium supplemented with 10% foetal calf serum before experimental use. The cells were grown to confluent monolayers and were seeded in 90-mm Petri dishes 20 h prior to hybridization. Suspensions of hamster Wg 3 IMP- cells were prepared by treating monolayers of the cells with a solution of trypsin (0.04%)-EDTA (0.02%) for a total of 2.5 min. The trypsinized cells were bathed in serum-containing Dulbecco’s medium, and then suspended in Dulbecco’s medium, free from serum (10^6 cells in 2.0 ml of medium). Monolayers comprising $5 \times 10^5$ 3T3 TK- mouse cells were treated for 5 min at room temperature, while being rocked gently, with 0.8 ml of lipid emulsion (prepared as described above) in serum-free Dulbecco’s medium. The monolayer was washed twice with serum-free Dulbecco’s medium
before the addition of the suspension of hamster cells. After 7 min at room temperature during which the dishes were gently rocked, hamster cells that were not adhering to the monolayer of mouse cells were removed. The monolayer was then washed twice with 2·5 ml of foetal calf serum before the addition of HAT selective medium (Littlefield, 1964). A control experiment without the lipid emulsion was done simultaneously with each lysolecithin experiment. Clones of hybrid cells began to appear by the 5th day after hybridization. The clones were counted on the seventh day, and the counts checked after a further 10 days to ensure that the cells remained viable. Coverslip cultures were prepared from the clones on the 17th day, and karyograms were then obtained using the procedure of Rothfels & Siminovitch (1958).

RESULTS

Erythrocytes with lysolecithin in solution

Since vertebrate cells carry a net negative charge (Weiss, 1970), a low pH was used in experiments with erythrocytes in order to reduce the electrostatic repulsion between cells. It was thought that this might facilitate cell fusion: Franklin (1958) has reported that the formation of giant cells in tissue cultures of chicken macrophages occurs most readily between pH 5·5 and 6·0. Hen erythrocytes did not clump or lyse when they were incubated for 15 min with buffered saline, and the cells had a normal appearance despite being subjected to the relatively low pH of 5·6. Figs. 1 and 2 illustrate the typical appearance of a cell that was incubated for 3 min under these conditions.

In the hope that cell fusion could be induced without extensive membrane damage, erythrocytes were treated with relatively low concentrations of lysolecithin in solution (70 µg in 1·7 ml of incubation medium). At this concentration, the cells that had lysed were irregularly shaped. In some of these experiments, cells with bilobed nuclei were frequently observed by light and electron microscopy within 5-15 min of adding lysolecithin, both at pH 5·0 and 5·6 (Fig. 3). Although these cells could possibly have resulted from cell fusion followed by nuclear fusion, no cells in the process of fusion were detected in these experiments. It is likely therefore, especially in view of the detergent-like effects of higher concentrations of lysolecithin on hen erythrocyte nuclei, which are described below, that the bilobed nature of the nuclei resulted from damage to the nuclear envelope. This interpretation is supported by the fact that a cell with a similar, so-called double nucleus has been observed in blood from a hen that had previously received 2·322 x 10^-3 C kg^-1 (900 R) total body irradiation (Lucas & Jamroz, 1961).

Erythrocytes that were treated with the highest concentration of lysolecithin used (360 µg in 1·7 ml) were consistently found to be lysed within 30 s of coming into contact with lysolecithin. Cell fusion was also observed, but not all cells participated in the fusion process. What appears to be an early stage in cell fusion is shown in Fig. 4, in which 2 cytoplasmic bridges are present between the main body of a syncytium and the cytoplasm of a single cell that is apparently in the process of fusing with the giant cell. Fig. 5 is an electron micrograph of part of a large syncytium in which the apparent remains of plasma membranes can be seen: the 3 nuclei are contained within a common boundary membrane. Palade & Bruns (1968) have observed similar weakly staining residues of membranes during fusion of coated vesicles with the plasmalemma membrane in the endothelium of blood capillaries of the tongue in rats, and Cornell
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(1970) has reported comparable findings during micropinocytosis in cultured murine embryo cells.

Limited numbers of giant cells containing two or three nuclei were observed on treating the hen erythrocytes with lysolecithin. Generally, the homokaryons were composed of large numbers of nuclei: 50 or more nuclei were sometimes visible in a single section through a syncytium. Part of a large homokaryon is shown in Fig. 6. The cytoplasmic material between the nuclei in these large homokaryons has a fibrillar appearance and may represent the remnants of the haemoglobin of the original erythrocytes. It is significant, however, that unfused cells were always completely lysed at this stage, and it seems more likely that the cytoplasmic contents of the giant cells are damaged plasma, or possibly nuclear, membranes. The nuclei of the homokaryons were sometimes irregularly shaped, and in extreme cases they had a scalloped outline. In addition, it may be seen by comparing Figs. 2 and 7 that the outer nuclear membrane had disappeared from the nuclei present within homokaryons. The loss of the outer nuclear membrane resulting from the treatment with lysolecithin resembles similar effects that have been observed with citric acid (Gurr, Finean & Hawthorne, 1963) and with detergents, e.g. with Triton X-100 on the nuclei of a plasma cell tumour (Hymer & Kuff, 1964).

A ruthenium red-staining layer, possibly representing glycoprotein, was often still present at the periphery of homokaryons fixed within 30 s of adding lysolecithin (Fig. 7). However, in many other instances the plasma membrane was extremely indistinct. The lack of a clearly defined plasma membrane in these preparations presumably reflects the instability of lysolecithin-treated membranes. Seeman (1967) has reported discontinuities that were 30-40 nm wide, which he interpreted as holes, in thin sections of lysolecithin-treated human red cells. In our experiments parts of the boundary membranes of some syncytia disappeared within 30 s of the addition of lysolecithin, and after 1 min of incubation at 37 °C with lysolecithin most of the homokaryons had completely disintegrated and could not be visualized by electron microscopy.

Erythrocytes with lysolecithin in lipid emulsions

With the light microscope, hen erythrocytes were seen to clump after treatment for 5 min with an aqueous emulsion of lipid containing lysolecithin and after 10-15 min they began to fuse, generally in pairs or groups of 3. The cells rounded-up when complete fusion had taken place, then gradually lysed over a period of 45-60 min. Lysis could be postponed by keeping the cells in ice after an initial 15-min incubation period.

Electron microscopy of erythrocytes fixed after 15 min incubation at 37 °C revealed that small lipid droplets of various sizes, averaging 0.5 µm in diameter, were associated with the aggregated groups of cells. The droplets were only lightly stained, and they were similar in appearance to the droplets of glyceryl trioleate seen by Carr (1970) in studies on phagocytosis by peritoneal macrophages. In many instances, it appeared that adjacent cells were held together by a lipid droplet lying between, and in contact with, both plasma membranes (Fig. 8). At this time, little lysis had occurred; the
plasma membrane, and in favourable areas the outer nuclear membrane also, remained intact (Fig. 9).

After 15 min, cells in all stages of fusion could also be seen. Some were attached to each other by one or two narrow cytoplasmic bridges (Fig. 10), similar to those observed between the fused erythrocyte ghosts in experiments in which lysolecithin was used in solution (Fig. 4). The cytoplasms of other cells were joined via several such bridges, extending over a wider area of membrane contact (Fig. 9). Occasionally after 15 min and more often after 30 min of incubation, multinucleated cells showed virtually complete cytoplasmic continuity (Fig. 11). Such fused cells still contained much of their haemoglobin.

The homokaryons obtained in this way, which generally contained only 2 or 3 nuclei, finally rounded-up into spherical cells. In an experiment designed to determine the subsequent stability of these multinucleated cells, they were washed in Hanks's saline and resuspended in Eagle's medium supplemented with 10% foetal calf serum. Multinucleated erythrocyte ghosts, some containing up to 10 nuclei, were still present after 90 h at 37 °C. Unlysed, single erythrocytes were also seen in these preparations. These several experiments clearly show that the use of lysolecithin in microdroplets of lipid to fuse hen erythrocytes is much less damaging than lysolecithin in aqueous solution.

It is interesting to consider the interaction of the lipid droplets with the cells in more detail. Figs. 8 and 9 indicate that droplets of varying sizes enter the cells, but these micrographs provide no clear evidence for the presence of invaginated plasma membrane between the haemoglobin and the smooth convex surfaces of the lipid droplets. That this apparent absence of membrane is due to a failure of stain to penetrate the cells would seem unlikely, since staining of individual sections with lead citrate did not alter the appearance of the interface between lipid droplets and haemoglobin. By contrast, positively staining material is present at the outer surface of lipid droplets that become incorporated into cellular cytoplasm and subsequently lie at the cell periphery (Fig. 9). While this staining may be due to the lipid constituents of the droplets themselves, the thickness of the stained layer suggests that cell membrane material may be present at the surface of the lipid droplets. The dimensions of the initial bridges between the 2 cells of Fig. 10 indicate that microdroplets that have a diameter of less than 100 nm are probably more important in fusion than the relatively large droplets of Fig. 8. For example, a small protrusion at the surface of one of the cells in Fig. 10 may represent the site of incorporation into the cell of a minute lipid droplet, while the vesicle adjacent to the other cell may be a similar protrusion in cross-section, or an as yet unincorporated droplet. Membrane fusion induced by droplets containing lysolecithin may require contact between such points on adjacent cells.

Small numbers of fused erythrocytes were seen on some occasions when cells were incubated for 15 min at 37 °C with emulsions of the commercial preparations of glyceryl trioleate, or dioleate, without added lysolecithin and lecithin. Fusion was never seen, however, with cells incubated in buffer/saline alone. In recent experiments it has been found that glyceryl monooleate and oleic acid are able to cause fusion of
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hen erythrocytes during incubation at 37 °C for 1–2 h (Lucy, Ahkong, Cramp, Fisher & Howell, 1971). Since thin-layer chromatography showed that glyceryl monooleate and traces of oleic acid were present in the commercial samples of glyceryl trioleate and glyceryl dioleate used in the present work, it seems likely that the contaminating monooleate was responsible for fusion observed with emulsions prepared from these materials without added lysolecithin. Many more fused cells were observed after 15 min when lysolecithin was present than when it was absent, however, and it is therefore considered that lysolecithin was primarily responsible for the fusion observed when this substance was incorporated into the microdroplets of lipid.

Formation of cell hybrids using lysolecithin

The possibility that lysolecithin or other purified chemicals might be used for the formation of cell hybrids, instead of using viruses, is of considerable interest particularly as a chemical method would avoid the time-consuming cultivation of virus. As pointed out previously (Poole et al. 1970), there would also be no possibility, with hybrids produced by a chemical treatment, of attributing the behaviour of the hybrid cells to the effects of viral components.

In view of the destructive effects of lysolecithin when it is used in aqueous solution at a sufficiently high concentration to induce fusion of hen erythrocytes (see p. 773), it seemed improbable that this procedure could be developed per se as a reliable method of obtaining viable cell hybrids. Since lysolecithin in microdroplets of lipid was, however, found to induce cell fusion and also to be less damaging, these microdroplets were used in experiments on interspecific cell fusion with mouse fibroblasts (3T3 TK−) and hamster fibroblasts (wg 3 IMP−). Hybrids formed by the fusion of these cells can give rise to viable clones of cells in the selective HAT medium of Littlefield (1964) but neither of the parent cell lines is able to grow in this medium (Pontecorvo, 1971).

Hybrid cells were selected in HAT medium after hamster wg 3 IMP− cells were added to a monolayer of mouse 3T3 TK− cells that had been treated with a lipid emulsion containing lysolecithin. An average of 2 viable clones was produced per 104 mouse cells in 5 experiments, in each of which 106 wg 3 IMP− cells were added to a monolayer of 5 × 105 3T3 TK− cells in Dulbecco’s medium. That the cells in these clones were hybrids and had not arisen by back mutation was confirmed by the fact that they contained both mouse and hamster chromosomes (compare Pontecorvo, 1971). However, approximately half of this number of viable clones was obtained with these cell numbers in control experiments with untreated 3T3 TK− cells.

Cellular damage by lysolecithin may possibly have been responsible for the limited increase in the number of viable clones of hybrid cells in the treated cultures, as compared with the spontaneous formation of cell hybrids in the controls. This view is supported by the results of investigations on mixed populations of hen erythrocytes and mouse LS fibroblasts, in which interspecific fusion occurred when the cells were treated at pH 5.6 with lipid droplets containing lysolecithin. The fused cells were considerably more stable than the corresponding heterokaryons obtained with lysolecithin in solution (Poole et al. 1970). However, they showed quite extensive
damage to the structure of the membranous, subcellular organelles derived from the fibroblasts shortly after fusion (Fig. 12), while fibroblasts exposed to acetate buffer at pH 5-6 without lysolecithin were undamaged and exhibited no loss of ability to exclude trypan blue. The degree of damage induced by treatment with the lipid emulsion was similar to that observed by Negroni & Tilly (1970) in polyoma virus-transformed mouse cells after treatment with specific immune serum and complement which resulted in a reduction of cell viability in vitro.

DISCUSSION

Hen erythrocytes are useful for studies on chemically induced cell fusion since, being nucleated, they make fusion easy to detect. In addition, because mature erythrocytes do not divide, there can be no confusion between fusing and dividing cells. A further important factor concerns the virtual absence from mature hen erythrocytes of organelles other than the nucleus. Thus it has recently been estimated by Zentgraf, Deumling, Jarasch & Franke (1971) that 54-2% of the total cellular membrane material of the hen erythrocyte is composed of plasma membrane, with nuclear membrane representing 45-6%. Other membranes such as those from mitochondria, lysosomes, mitochondrial extrusion bodies and endoplasmic reticulum-type cisternae were found by these workers never to exceed 1% of the total membrane material. As a result, it can be concluded with reasonable confidence, even with lysolecithin in solution, that the fusion of erythrocyte plasma membranes observed in our experiments was due to a direct effect of the added lysolecithin on the plasma membrane, and that it did not result indirectly from the release of hydrolytic enzymes from lysosomes which had been damaged by the lysolecithin.

In their ultrastructural study of interspecific cell fusion induced by inactivated Sendai virus, Schneeberger & Harris (1966) found that cells which had large numbers of microvilli on their surfaces fused together more readily than those which had not. They concluded, however, that the presence of microvilli was not essential for virus-induced fusion to occur as they occasionally saw haemolysed, or partly haemolysed, erythrocytes that had fused with each other. Our experiments similarly indicate that the presence of microvilli is not necessary for lysolecithin-induced fusion. Fig. 10 in particular indicates that fusion occurs between quite flat areas of adjacent cells. It must nevertheless be borne in mind that these erythrocytes were treated with lysolecithin at pH 5-6 in an attempt to reduce the overall electrostatic repulsion between cells. It also remains possible that very small microvilli or protrusions in the plasma membrane (Fig. 10) are formed transiently during the fusion of cells by lysolecithin, and that it is only at these sites that intermolecular repulsion is small enough to allow contact to be made between adjacent cells (compare Poste & Allison, 1971).

Hosaka & Koshi (1968) reported that the plasma membranes of Ehrlich ascites tumour cells in contact with the envelope of an adsorbed Sendai virion often disappeared along a limited portion of the virion surface. This was interpreted as indicating that the virus has some degrading effect on the plasma membrane at 37 °C. Communication of the cytoplasm of adjacent cells then occurred through the breaks in the mem-
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branes. These findings are paralleled in our observations by the partial or apparently complete disappearance of plasma membrane at the sites of small intercellular bridges between cells treated with lysolecithin (Figs. 4, 9, 10). This would indicate that whereas a phase change in the organization of the constituents of the treated membranes may occur at the precise points of membrane fusion (cf. Lucy, 1970), the structures of the membranes between those points – i.e. within the initial bridge between 2 fusing cells – is altered even more drastically by the presence of the lysolecithin so that the membrane effectively disintegrates.

Glyceryl trioleate has been used in many investigations on phagocytosis (Carr, 1970). Even with a phagocytic cell like the peritoneal macrophage it is not entirely clear, however, how the lipid actually enters the cell; Williams & Carr (1968) considered that unhydrolysed fat could be absorbed in 3 ways: by phagocytosis of large particles; by micropinocytosis of small particles or micelles; and in molecular form. Since the hen erythrocyte is not a phagocytic cell, and has also given no indication of micropinocytosis in our studies, it seems that passive diffusion of lipid molecules through the membrane is the most likely of these 3 alternatives in the present context. In early studies on naked Arbacia eggs that were treated with oil droplets, Chambers & Kopac (1937) found that the droplets penetrated in less than one thirty-second of a second. The boundary of the oil drop remained sharply defined inside the egg, and the penetration of relatively small drops (10-25 μm in diameter) did not result in rupture of the egg. These workers regarded an oil drop together with the egg as a system of 2 drops that are immersed in an aqueous phase but are immiscible with it, and they concluded that the oil drop enters the egg spontaneously since this results in a decrease in surface tension (Kopac & Chambers, 1937). While considerations of this kind may apply to the present studies, it is to be expected that the interfacial tension at the surface of the microdroplets that we have used will be relatively low as a result of the presence of lecithin and lysolecithin. It is also relevant to note here the recent report by Korn (1970) on the uptake by the amoeba, Acanthamoeba castellanii, of positively charged liposomes prepared from 90% lecithin and 10% stearyl amine or stearyltrimethylammonium salts. Korn has observed osmiophilic microdroplets (about 60 nm in diameter) in the cytoplasm and nucleoplasm of amoebae following incubation with these liposomes. The droplets were not membrane-bound, and Korn suggests that neither pinocytosis nor phagocytosis is involved in the uptake process.

Even though we have found that the cytotoxic effects of lysolecithin can be reduced and localized by presenting the fusing agent to the cells in lipid droplets rather than in aqueous solution, it is apparent from the ultrastructural appearance of the hen erythrocyte-mouse fibroblast heterokaryons that membrane damage is not entirely eliminated by this procedure. It is therefore likely that other purified, membrane-active substances such as glyceryl monooleate – which has recently been observed to cause cell fusion and, in preliminary experiments, to induce the formation of cell hybrids when it is used without added lysolecithin (Lucy et al. 1971) – may prove to be more important than lysolecithin itself for the hybridization of cells without viruses. Nevertheless, it is still possible that lysolecithin may be quite useful for this purpose especially if it could be removed sufficiently rapidly from the site of action to obviate damage to
membranes, for example by enzymic conversion to lecithin, immediately after membrane fusion has occurred.

In conclusion, the investigations described here indicate that similar sequences of ultrastructural changes are involved when cells are fused under the influence of Sendai virus, of lysolecithin in lipid droplets, and of lysolecithin in solution. It has also been shown that the destructive effects of lysolecithin on the ultrastructural organization of hen erythrocytes can be much diminished by modifying the physical manner in which lysolecithin is presented to the cells. This lends indirect support to the possibility that lysolecithin may be concerned in some of the known instances of membrane fusion occurring in vivo, particularly if the formation and removal of lysolecithin by endogenous enzymes is closely controlled as has been suggested earlier in this context by Poole et al. (1970). In particular, processes that involve membrane fusion during the movement of lipid droplets across membraneous barriers by exocytosis or endocytosis may depend on changes induced by the presence of lysolecithin that are similar to those reported here.

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REFERENCES


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Fig. 1. An electron micrograph showing a hen erythrocyte incubated for 3 min at 37 °C in a solution containing 106 mM NaCl and 44 mM sodium acetate, final pH 5.7. Sections were stained with lead citrate. × 15,000.

Fig. 2. Detail of a hen erythrocyte treated as in Fig. 1, showing the normal appearance of the nuclear and plasma membranes. × 54,000.

Fig. 3. Electron micrograph of an erythrocyte ghost with a 'bilobed' nucleus (n). Cells were incubated for 15 min at 37 °C in 1.7 ml of a medium similar to that of Fig. 1 but which contained, in addition, 70 μg of lysolecithin. The pH of the buffer was 5.0. Sections were stained with lead citrate. × 20,000.

Fig. 4. Electron micrograph showing a cell attached to part of a syncytium by 2 small cytoplasmic bridges (arrowed). Erythrocytes were incubated for 30 s in 1.7 ml of the medium of Fig. 1, with the addition of 360 μg of lysolecithin. Sections were stained with lead citrate. × 34,500.
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Fig. 5. Section through a chain of partially fused red cells. Membranous material (arrowed) is still present between adjacent nuclei (n), but the nuclei are all contained within a continuous boundary plasma membrane. Cells treated as in Fig. 4. × 18000.

Fig. 6. Section through part of a large homokaryon produced by lysolecithin. Cells treated as in Fig. 4. × 16000.

Fig. 7. Detail of Fig. 6 showing the absence of the outer nuclear membrane. A ruthenium red-staining layer is still, however, present on the plasma membranes (arrows). × 33750.
Fig. 8. Electron micrograph showing hen erythrocytes adhering together, some with emulsion particles (p) interposed. Little or no haemolysis has occurred, but the cells show various degrees of swelling. In 2 regions (arrowed), cells are apparently in the process of fusing. Cells in 0-9% NaCl were treated with the lipid emulsion containing lysolecithin (in buffered saline, pH 5-6) and incubated for 15 min at 37 °C as described in Materials and Methods. The cell suspension was placed in an ice bath for a further 30 min before fixation. × 9500.

Fig. 9. Detail of Fig. 8 showing lipid droplets (a) and (d) apparently inside the cells. In favourable regions (b), the outer nuclear membrane remains intact. At (c) the cytoplasms of 2 adjacent cells are in direct contact over quite a wide region. × 20000.
Fig. 10. Section through 2 red cells fusing via narrow bridges (a). A small protrusion at c may be due to a very small lipid droplet already incorporated into the cell cytoplasm; b may be similar, or a droplet still outside the cell. Cells treated as in Fig. 8. x 33750.

Fig. 11. Electron micrograph illustrating virtually complete cytoplasmic continuity between 2 erythrocyte nuclei. Cells treated as in Fig. 8. x 9250.

Fig. 12. Section through a heterokaryon containing a fibroblast nucleus (f) and an erythrocyte nucleus (e) showing that the cytoplasm is granular and vesiculated. An emulsion particle (p) is attached to the plasma membrane. The cells were treated as described in Materials and Methods. x 15000.