AN ABSTRACT OF THE THESIS OF

Gene F. Cota for the Master of Science

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Title: Lysolecithin Induction of Cell Fusion

Abstract approved: Gaylen Hufford

The phospholipid lysolecithin was tested in various concentrations, temperatures and environments for its effects on cell fusion, disintegration and mortality. Cholesterol and mineral oil were both assayed for their actions in reducing cell loss. Temperature extremes both high and low proved to be cytotoxic and cytolytic although higher temperatures increased cell fusion events. Cholesterol was found to be of little significance in decreasing lysolecithin's cytotoxic effects. Mineral oil solutions of lysolecithin did not significantly improve cell survival, but these mineral oil solutions proved to be significant in the fusion of cells without the presence of lysolecithin. A mechanism is proposed for this mineral oil induced cell fusion.

LYSOLECITHIN INDUCTION OF CELL FUSION

A Thesis
Submitted to
the Division of Biological Sciences
Emporia State University, Emporia, Kansas

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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ACKNOWLEDGEMENT

I would like to express my gratitude first and foremost to Dr. H. Michael LeFever for his infinite patience in awaiting the completion of this thesis. Thank-you Mr. Larry Brown for polishing my meager statistical analysis program so well that it would sit-up, roll-over and eat out of my hand! I would also like to thank Ms. Dee Ann Reagan for encouraging me to "blow the dust off" this paper after such a long time. Warmest thanks go to Ms. Joy Harrold who showed me the path to the finish line, cheering all the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

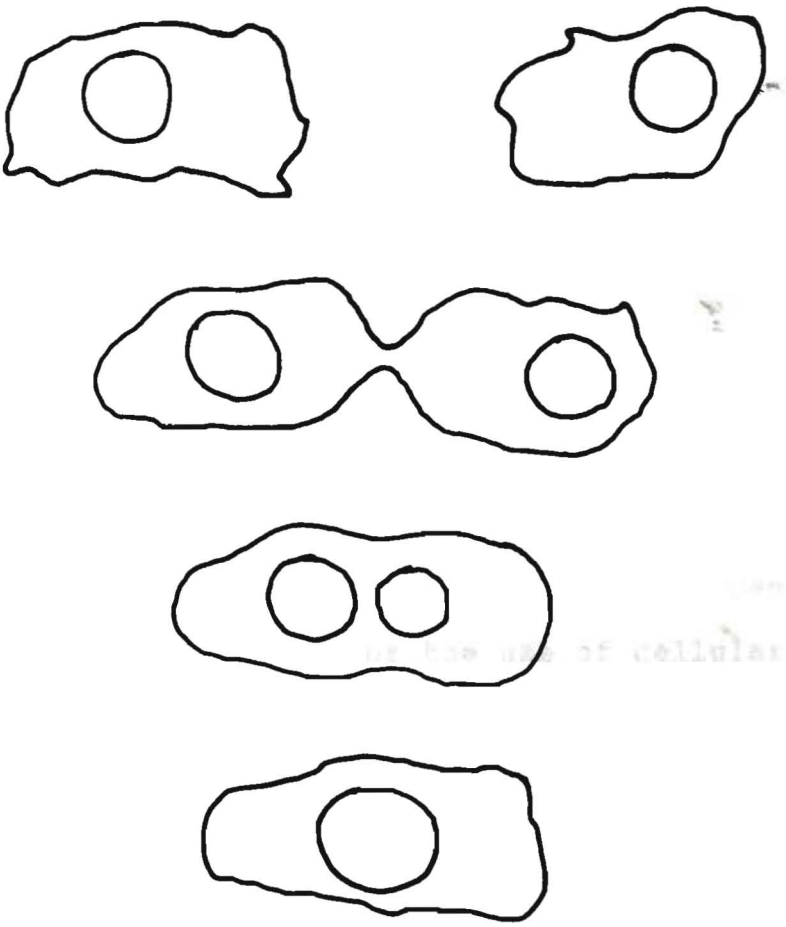
The mapping of genes on chromosomes is an activity of classical genetic study that has been restricted to either bacterial-phage systems or Mendelian techniques. Detailed study of crossover frequency for mammalian systems was the only practical method for localizing genes in relation to their relative chromosomal placement. For the human genome, mapping problems became obvious. Generation time and social-ethical restrictions made human gene mapping a virtual impossibility. Only the determination of sex-linkage and study of defective cases were fruitful in human gene study (Lindsey, 1932; White, 1940).

Cytogenetics was limited to assessment of gross chromosomal abnormalities. Mammalian chromosomes could only be differentiated by general morphology, chromosome number, and abnormal meiotic configurations. The accuracy and applicability of mammalian cytogenetics was given an enormous boost with the discovery of methods to differentially stain the chromosomes to yield banding patterns not unlike those found in the polytene chromosomes of dipterans (Caspersson et al., 1970; Drets and Shaw, 1971; Seabright, 1971; Arrighi and Hsu, 1971). This enabled cytogeneticists to determine the extent of chromosomal abnormality and to separate non-homologous chromosomes of identical morphology with significant accuracy. The greatest boost to the cytogenetic field of study came with the discovery of cellular hybridization (Barski et al.,

1961). Barski discovered the hybridization phenomenon while attempting to demonstrate a pneumococcus-like transformation in somatic cell culture between two mouse cell lines. A fusion of the two cells had occurred spontaneously and, retaining viability, the resultant cell had the combined genome of the two parental lines (Fig. 1).

The formation of viable hybrids due to spontaneous fusion occurs approximately once in every 500,000 cells (Croce et al., 1971). Isolation of hybrids from spontaneous fusion was facilitated by the use of selective culture conditions (Littlefield, 1964; Yoshida and Ephrussi 1967). These methods involved temperature alterations for those systems where hybrids outgrew parentals in lower temperatures, and media selection in those systems using auxotrophic cell lines. The most commonly used selection method is that described by Littlefield (1964) where only hybrid cells possess the proper enzymes for growth in the selective medium.

Because of the low hybridization frequency due to spontaneous fusion, methods were sought to increase the rate of cell fusion <u>in vitro</u>. Harris and Watkins (1965) found that treatment of cells with an ultraviolet light inactivated virus could induce cell fusion at a relatively high rate. This discovery made the hybridization process quite efficient and more applicable for a variety of studies (Ephrussi, 1972).



The application of somatic cell hybridization to formal genetics became most apparent with the discovery of Weiss and Green (1967) that hybrids between human and mouse cells lost chromosomes preferentially from the human genome. Most human-mouse hybrids stabilize after several generations with a genome consisting of mostly mouse chromosomes and very few human chromosomes. With electrophoretic analysis of hybrid proteins, human and mouse enzymes can be isolated and identified. Identification of the human chromosomes and the human gene products in the hybrid allows the assignment of specific genes to specific chromosomes (Tan et al., 1973; Bruns and Gerald, 1976; D'Eustachio and Ruddle, 1983). With the use of human cells carrying known translocations or rearrangements, human genes can be further mapped to specific regions of chromosomes (Ruddle, 1973). Gene mapping is a frequent reason for the use of cellular hybridization. By the late seventies there were about 210 human genes that had been assigned chromosome locations (McKusick and Ruddle 1977). Most of the autosomal assignments were made with the aid of somatic cell hybridization. The technique is also of value in studies of cellular differentiation and cancer (Ephrussi, 1972). Most recently, cell hybridization has gained widespread use in the formation of hybridomas for the production of monoclonal antibodies (Srikumaran et al., 1983; Taggart and Samloff, 1983). Hybridomas present great medical and commercial

value for hybridization studies, as well as legal and ethical problems (Sun, 1983).

For any particular cell hybrid study, it is most desirable to fuse the cells with the highest possible efficiency and the lowest possible cell loss. Fusion efficiency varies greatly with the particular technique used to induce fusion. Cell loss is primarily due to toxic factors introduced with the fusion medium and cell lysis as a result of membrane interactions. The original investigations of cell hybridization (Barski et al., 1961) involved spontaneous cell fusion. This technique was inefficient as far as mating rate was concerned, but absolutely no abnormal toxic factors were introduced into the culture system.

Viral induced cell fusion has the advantage of a very high mating efficiency (Harris and Watkins, 1965). The U.V. inactivated Sendai virus (hemagglutinating virus of Japan) has an agglutinating effect on the cells and the result is a fusion of the membranes of neighboring cells to form binucleate or multinucleate homokaryons or heterokaryons. Toxicity and cell loss resulting from treatment with Sendai virus is very low and the fusion rate is high. As a result, many studies of hybrids make use of viral induction (Tan et al., 1973; Bruns and Gerald, 1976; McGrath and Solter, 1983). The disadvantages of viral induction are found in preparation of the virus itself. Cultivation, inactivation,

and titering of the virus are time consuming and uneconomical for small laboratories. Titering of the virus is not always an accurate representation of its cell fusing capabilities (Croce et al., 1971). Quite a number of other chemical and physical techniques have been developed in an attempt to avoid these problems.

One physical method of inducing cell fusion is the technique of microsurgery (Diacumakos, 1973). This involves the physical joining of two cells with a microsurgical apparatus. This method has two distinct advantages. The cells to be mated can be chosen and fused without any question to the parental identity, and the fused binucleate cell may be cultured without the necessity of a selective culture system. The disadvantages of such a system are found in the low success rate of viable hybrids for each fusion event, and in the acquisition and use of micromanipulation devices. Microsurgery of this type requires skill and experience with the micromanipulator (Diacumakos et al., 1971).

Another physical method of fusion induction is high voltage electrical pulsing (Teissie et al., 1982). They produced a high yield of multinucleated cells by exposure to short duration electrical pulses. Five 1000 volt pulses with a duration of 50 microseconds produced up to twenty percent fusion in mouse fibroblasts. This particular method shows great practicality for hybrid studies in that it is

non-specific, relatively easy, and highly efficient.

Treatment with freeze-thaw cycling also produces membrane fusion. Here the mechanism is thought to be due to point defects in lipid bilayers coupled with close membrane contact. The point defects are thought to be the result of mechanical alterations caused by ice crystalization. The point defects allow close contact reorganization of lipid bilayers with resultant fusion of the cell membranes (Hui et al., 1981).

There are numerous chemical methods for fusion induction. One of these involves the use of polyethylene glycol (PEG). This has been an effective agent in fusing plant protoplasts, mammalian cells, plant-animal heterokaryons, and hybridomas (Norwood et al., 1976; Davidson and Gerald, 1976; Jones et al, 1976; Srikumaran et al., 1983). The most effective PEG concentration for fusion is a minimum of 50% (w/w). Toxicity to the cells remains low during short exposure and the fusion rate is fairly high. The fusion procedure is carried out very similarly to the viral induction technique. Although the fusion efficiency is not as high as with viral induction, this method promises to be an economical way to avoid the problems found with viral-induced fusion.

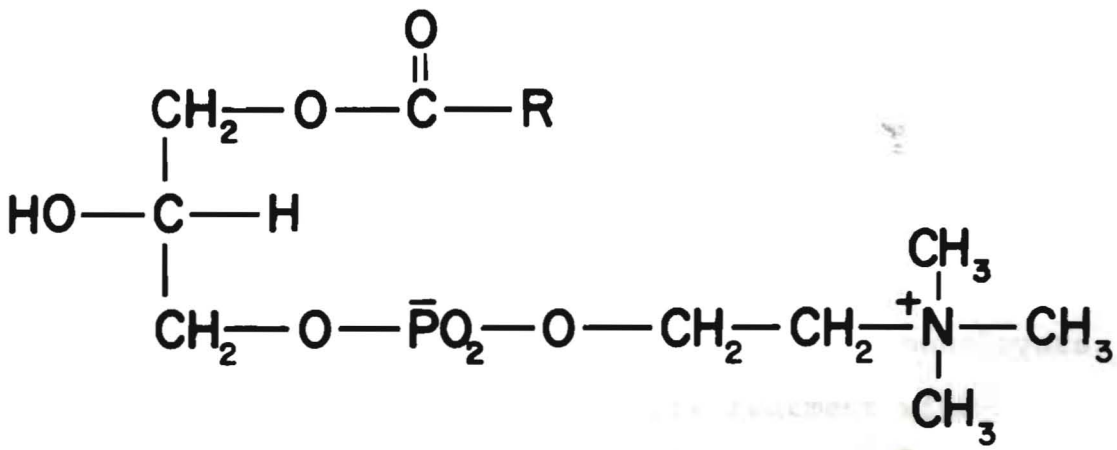
One of the few chemical techniques having the quality of low cell toxicity is that of induction with lipid vesicles. Cell toxicity resulting from treatment with lipid

vesicles is not significantly greater than that resulting from viral-induced fusion. The vesicles, at a dose of approximately 100 per cell are composed of lecithin, lysolecithin, and stearylamine. They can fuse up to one per every three cells, a frequency comparable to that found with viral induction (Papahadjopoulos et al., 1973; Martin and McDonald, 1976). Martin and McDonald (1976) found that the fusion event increased linearly with stearylamine concentration and sublinearly with lysolecithin concentration.

Lysolecithin (Fig. 2) was first suggested as a possible agent for the induction of cell fusion by Lucy (1969). His suggestion was prompted by the fact that lysolecithin produces micelles in lipid bilayers (Bangham and Horne, 1964). Soon after, Howell and Lucy (1969) showed lysolecithin to be a potent chemical for fusing hen erythrocytes. A low pH was optimal for fusion in a sodium acetate buffer, with fusion occurring within 30 seconds after exposure to lysolecithin.

Croce et al. (1971) published a comprehensive study in which they attempted to define the optimal conditions for the induction of cell fusion using lysolecithin. They used a variety of esablished mammalian cell lines rather than hen erythrocytes. Fusion was enhanced when carried out at a pH of 7.2 rather than the acid pH recommended by Lucy (1970). Also, different species of cells had different

Figure 2. The structure of lysolecithin (lysophosphatidyl choline). In most samples the R-group is 67% palmitic acid and 33% stearic acid.



susceptibilities to lysolecithin toxicity. He recommended that in studies using lysolecithin, the toxicity for the particular cell type in use should be determined prior to fusion procedures. This would facilitate the choice of the proper lysolecithin concentration. In a later publication, Croce et al. (1972) determined that a pH of 7.6 to 8.0 was optimal for both virus and lysolecithin fusion. Also, maintenance of a pH of 8.0 for the first three days following treatment enhanced hybrid formation.

Keay et al. (1972), contrary to other reports, observed extensive clumping and complete loss of viability when cells were treated in suspension at any lysolecithin concentration. Lysolecithin was less toxic and less troublesome when cells were treated in confluent monolayers. Gledhill et al. (1972) found that a pretreatment with lysolecithin increased the fusion efficiency when fusing rabbit spermatozoa with hamster somatic cells. The fusion technique, identical to that used by Croce et al. (1972), yielded a 15-25% fusion efficiency for spermatozoa-hamster and hamster-hamster hybrids. In these experiments, lysolecithin held a distinct advantage over Sendai virus. The spermatozoa underwent nuclear disaggregation and DNA synthesis in the lysolecithin fused products. This activity was not observed in Sendai virus fused products.

Papahadjopoulos et al. (1973) found lysolecithin fusion efficiency to be roughly half that of Sendai virus. He also

found the efficiency of phospholipid vesicles to be variable between lysolecithin and virus efficiencies, depending on the component lipids of the vesicles. The most effective vesicle composition contained ten percent lysolecithin. He also noted a stabilizing effect in the presence of cholesterol. Cholesterol in equimolar amounts seemed to reduce the fusion efficiency. Toyoshima and Osawa (1976) showed that cholesterol was inhibitory to the temporary increase in membrane fluidity of lymphocytes as induced by mitogenic lectins. Kwak et al. (1975) showed that cholesterol had a stabilizing effect on lysosomes. When lysolecithin and cholesterol were used in 1:2 molar mixtures, the strong lytic effect of lysolecithin was greatly reduced although membrane instability did increase with increased lysolecithin:cholesterol concentration.

Other factors affecting the activity of lysolecithin on membranes include temperature and the after effects of proteolytic enzyme treatment. Breisblatt and Ohki (1975) showed temperature to have a direct effect on the stability of phospholipid spherical membranes in the presence of lysolecithin. Unfortunately, this study was never extended to biological membranes. Weltzien et al. (1967) found the sensitivity of cells to lysolecithin was greatly increased by pretreatment with trypsin, or with neuraminidase in the case of human erythrocytes.

The most generally accepted mechanism for the action of

lysolecithin on biomembranes is that proposed by Lucy (1970). He proposed that the wedge shaped lysolecithin molecule causes micelle formation in the membrane lipid bilayer. As the micellar areas of two membranes are brought in close contact, the micelles intermingle. When the lysolecithin is neutralized, the membranes restabilize as bilayers (Fig. 3 and 4).

The purpose of this investigation was to test the cholesterol and temperature effects described by Kwak et al. (1975) and Breisblatt and Ohki (1975) in lysolecithin induced fusion of biological membranes. In addition, this investigation tested a new approach to decreasing the toxic effects of lysolecithin in an attempt to elucidate the optimal conditions for cell fusion induced by lysolecithin.

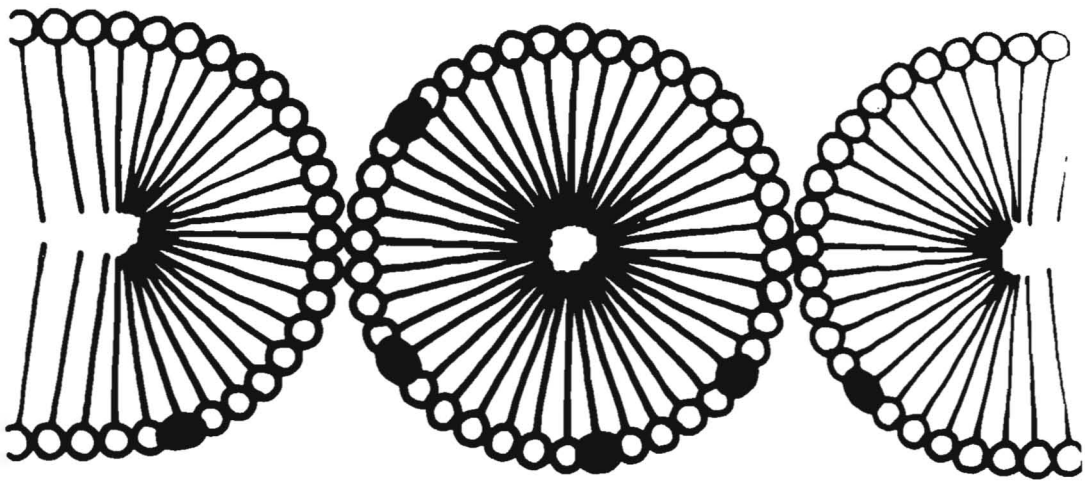
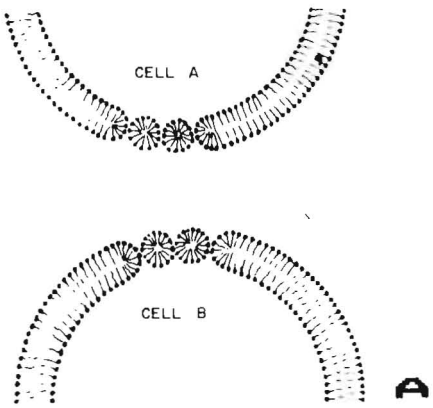
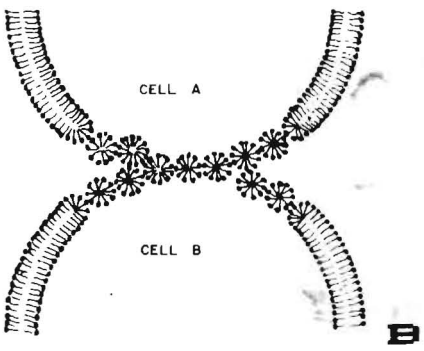
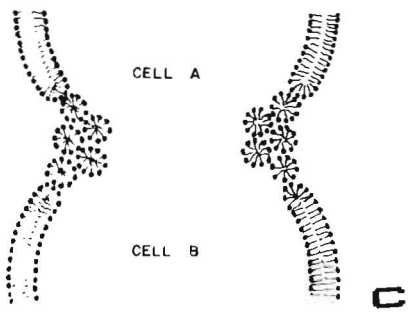
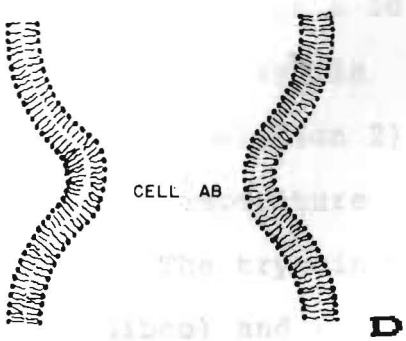


Figure 4. The mechanism of lysolecithin mediated membrane fusion. Micelles are formed in the lipid bilayer (A) which interact (B) and rearrange (C) to combine into a single membrane upon restabilization (D).


CELL A
CELL B
A


CELL A
CELL B
B


CELL A
CELL B
C


CELL AB
D

MATERIALS AND METHODS

Cells. White blood cells were obtained by cardiac tap of mildly etherized adult rats. A 10 ml plastic syringe with a 1.0 in 21 ga. needle preloaded with 0.5 ml ammonium heparin (1000 i.u./ml) and 2.0 ml 6% (w/w) dextran (m.w. 200,000) was used for the tap.

Approximately eight ml of blood were drawn per tap. The syringe was then inverted and erythrocytes were allowed to settle for 30 min to one h. The supernatant plasma-leukocyte suspension was extruded into a 15 ml conical centrifuge tube. Cells were centrifuged at 800 RPM for five min and washed twice in Hank's balanced salt solution (BSS) (Appendix: Solution 1).

Liver cells were obtained from adult rats. Livers were removed, cut by crossed scalpel, and forced through a 10 ml plastic syringe into a solution of 0.25% (w/w) trypsin (Gibco 1:250 Hog pancreas) in GKN (Appendix: Solution 2). Cells were trypsinized for 30 min. at room temperature in a homogenizing flask on a magnetic stirrer. The trypsin was then neutralized with fetal calf serum (Gibco) and the suspension centrifuged at 800 rpm for five min. The cells were then washed twice with Hank's BSS.

Spleen cells were obtained from adult rats by excision and crossed scalpel dicing in Hank's BSS. Cells were then placed in a 16 X 125 mm culture tube containing approximately 5.0 g homogenizing glass beads (VirTis Co.

#16-220). The sample was vigorously stirred on a Vortex Jr. Mixer (Scientific Industries Inc. Model # K-500-J). After settling for 30 sec, the supernatant was aspirated with a pasteur pipette. The cells were washed twice in Hank's BSS.

Evaluation and Quantitation of Cell Dispersions. Samples of each cell isolate were evaluated for viability and dispersion. Viability was measured using the trypan blue exclusion method. Cell samples were incubated 10 min at 37 C in trypan blue solution (4.0% in NaCl 8.0% aqueous w/w), rinsed briefly in Hank's BSS and counted on a hemacytometer. Cell dispersion was evaluated by recording the relative frequency of single and multiple cell units present when counting on the hemacytometer. Cells left unstained by trypan blue were determined to be viable. All light microscope evaluations were performed on a Wild M-20 microscope.

Solutions used in Fusion Procedure. Lysolecithin (lysophosphatidyl choline, egg, Sigma) was dissolved in absolute ethanol at 2.0 mg/ml. The solution was diluted in sterile GKN pH 7.4 to yield aqueous solutions of 400, 300, 200, and 100 µg/ml.

Lysolecithin solutions were also prepared in mineral oil (Squibb) at concentrations of 100 and 200 µg/ml.

Lysolecithin solutions were also tested in the presence of cholesterol at 5 µg/ml (Table 1).

Fusion Procedure. Freshly isolated cells were

TABLE 1. Lysolecithin test solutions

Lysolecithin (µg/ml)	Aqueous	Aqueous with 5 µg/ml cholesterol	Mineral oil	Mineral oil with 5 µg/ml cholesterol
0	*	*	x	x
100	*	*	x	x
200	*	*	x	x
300	*	*	ND	ND
400	*	*	ND	ND

* indicates solutions tested at 4, 25, 37, and 45 C

x indicates solutions tested at 25, 37, and 45 C

ND indicates solution was not tested

adjusted to a concentration of 1 X 10^7 cells per ml. One ml of this suspension was centrifuged at 800 rpm for five min and the supernatant discarded. The pellet was resuspended using a Pasteur pipette. Mineral oil samples were resuspended using the Vortex mixer. Solutions were neutralized after one min by the addition of one ml fetal calf serum followed by brief stirring on the Vortex mixer. The samples were immediately centrifuged at 800 rpm for five min and washed twice in Hank's BSS. All solutions were tested at 4, 25, 37, and 45 C.

Quantitation of Fusion Events. Immediately following treatment, the cells were stained with trypan blue to evaluate viability and cell concentration. To quantitate fusion events, permanent slides were made by first fixing the cells in methanol-acetic acid and dripping three to four drops onto a chilled wet slide. The slide was air dried and stained in hematoxylin and eosin. Cells were observed with the Wild M-20 and the number of single and multinucleated cell units recorded. At least ten randomly chosen fields were evaluated per slide at a magnification of 480 X.

Samples were prepared for electron microscopy by resuspending treated and washed samples in phosphate buffered 4% glutaraldehyde. Samples were placed in conical BEEM capsules and pelleted at 800 rpm for five min. Fresh glutaraldehyde was added prior to osmium tetroxide fixation and staining with uranyl acetate. Samples were embedded in

Epon 812 (6:4 hard). Sectioning was done using glass knives on an LKB Huxley ultramicrotome. Sections were placed on 200 mesh copper grids, stained with lead citrate, and observed on the Hitachi HS-8 transmission electron microscope. EM samples were photographed on Kodak 3.25 X 4.0 in glass photographic plates (high contrast).

Light microscope samples were photographed with the Wild-Heerbrugg photo-automat fixed to the Wild M-20 microscope. Black and white photographs were exposed on Kodak Plus-X Pan film and developed with Kodak Microdol-X. Color photographs were exposed on Kodak Ektachrome slide film and developed using the Kodak E-5 process. Color prints were printed from the Ektachrome slides by a commercial firm (Photomat). Plates were developed in Microdol-X. All black and white prints were on Kodabromide F-5 single weight paper.

Statistical analysis was performed using the Student T-test with the aid of an Atari 800 personal computer.

RESULTS

Aqueous systems with lysolecithin concentrations of 300 µg/ml yielded the highest percentage of binucleated and multinucleated cells observable by both light and electron microscopy (Fig. 5 and 6). At this concentration, up to 15% of the treated cells showed at least two nuclei. Fusion events decreased with both higher and lower lysolecithin concentrations (Fig. 7). Examination by electron microscopy indicated that the cell membranes were severely destabilized by higher lysolecithin concentrations. Cell membranes appeared highly deformed, broken, and rearranged (Fig. 8).

Cell loss due to lysis and cellular mortality was directly related to lysolecithin concentration (Fig. 9). The highest cell loss recorded was 83% in a preparation containing 400 µg/ml lysolecithin at a temperature of 45 C (Fig. 7). Temperature was not a strong determinant of cell fusion frequency (Fig. 10), though it did prove to be a strong determinant of cell viability and lysis in the high and low temperature assays (Fig. 11 and 12). The temperature extremes of 4 and 45 C generally increase cell loss, toxicity and decreased fusion rate in an equally proportionate manner. The ideal temperatures are at 25 and 37 C at a roughly equivalent efficiency rate.

The observed fusion rate was relatively unchanged by the presence of cholesterol in all preparations (Fig. 13). Less toxicity was observed in the aqueous cholesterol

Figure 5. Light photomicrograph of a binucleate cell after treatment with lysolecithin.

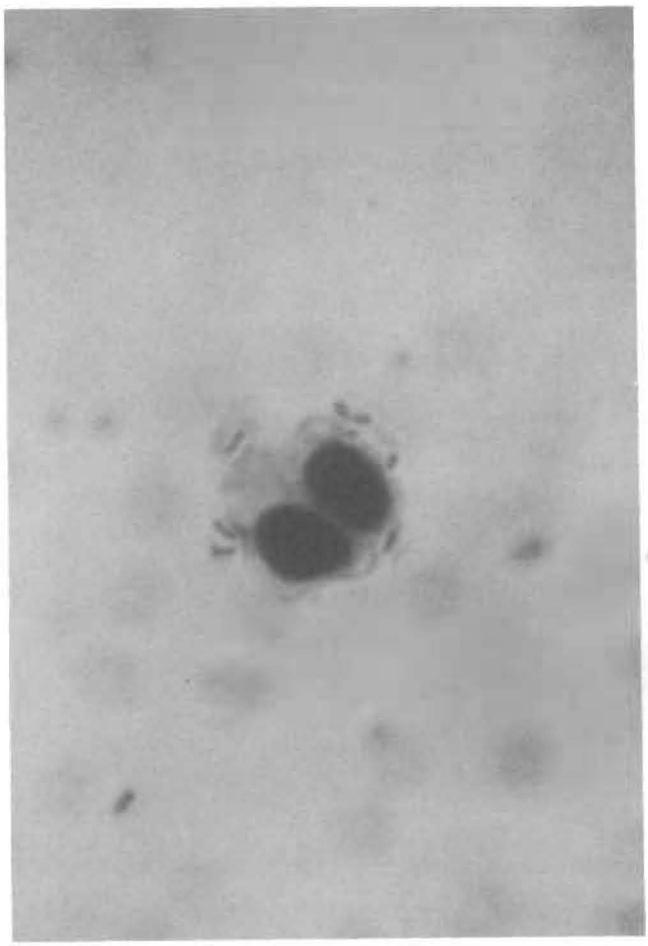


Figure 6. Electron micrograph of cells fused by treatment with lysolecithin.

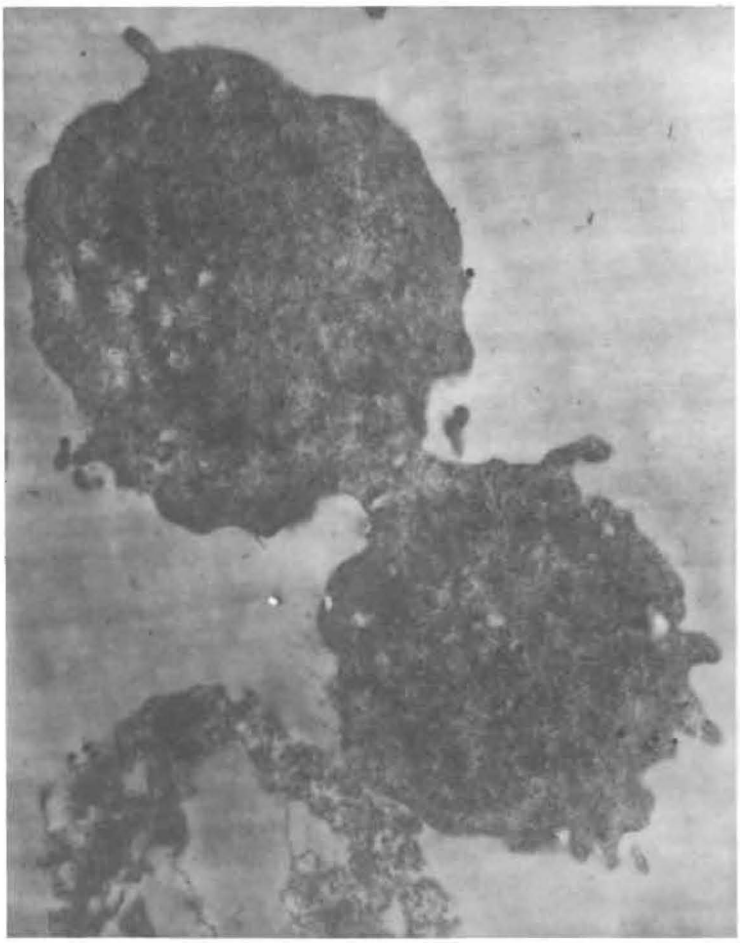


Figure 7. Effect of aqueous lysolecithin with 5 µg/ml cholesterol upon cell count and viability. A, B, C, and D refer to the temperatures 4, 25, 37, and 45 C respectively. 0, 100, 200, 300, and 400 refer to the µg/ml concentration of lysolecithin. ■ represents fused cells, ▨ represents cell count after treatment, □ represents viable cell count

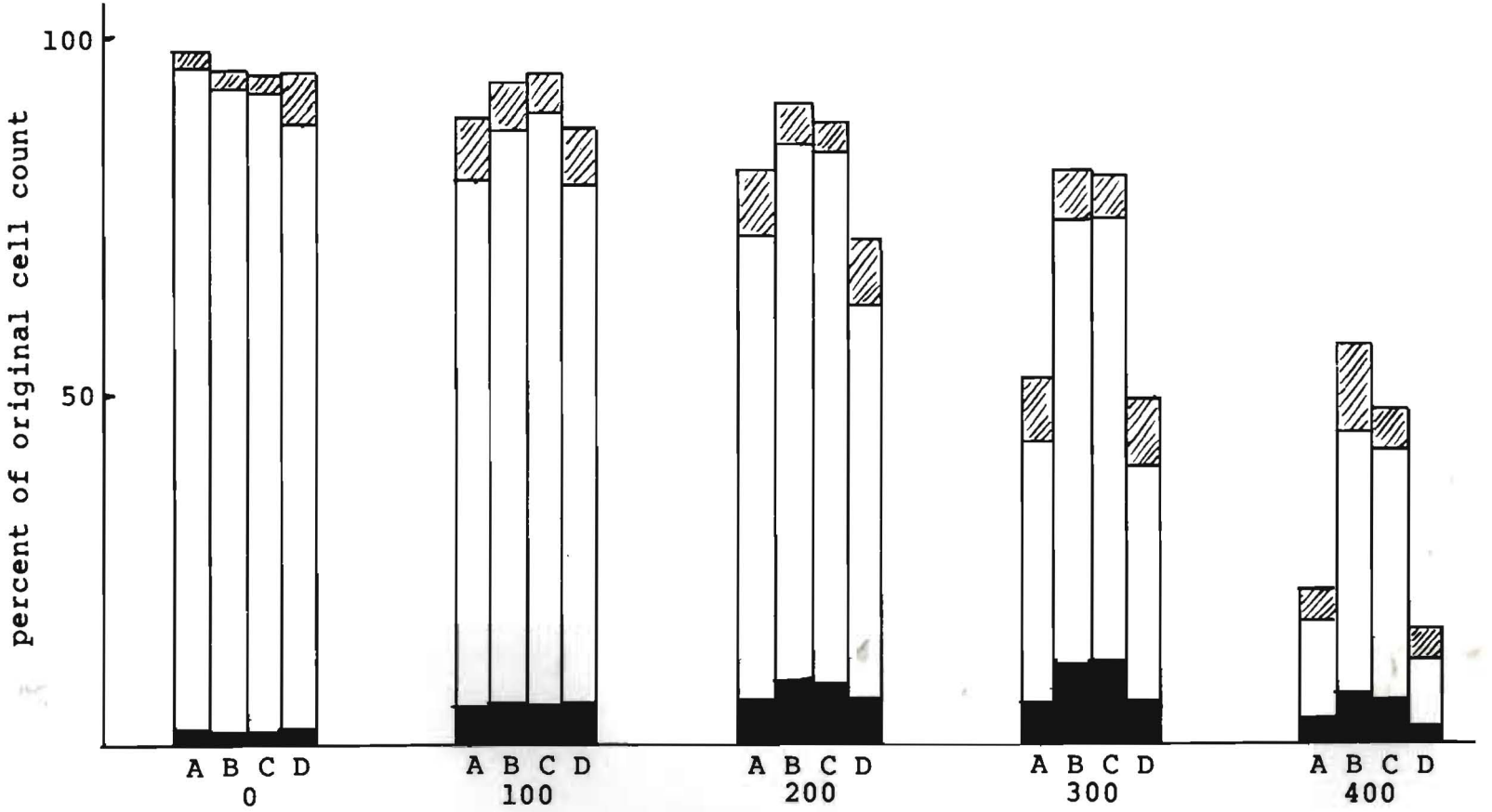

percent of original cell count
100
50
A B C D
0
A B C D
100
A B C D
200
A B C D
300
A B C D
400

Figure 8. Disorganized cell membranes resulting from treatment with lysolecithin at 400 µg/ml.

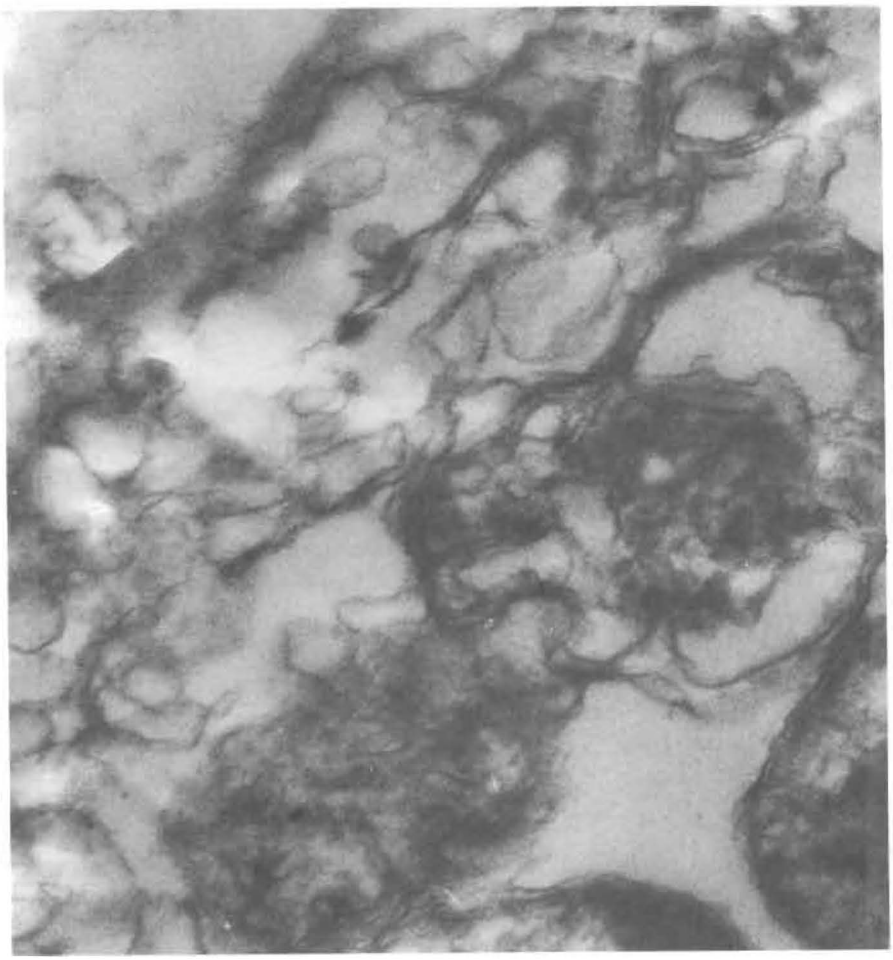


Figure 9. Effect of lysolecithin upon cell viability at 25 C. Aqueous lysolecithin, ● ; aqueous lysolecithin + cholesterol, Δ ; lysolecithin in mineral oil, o ; lysolecithin in mineral oil + cholesterol, ▲ .

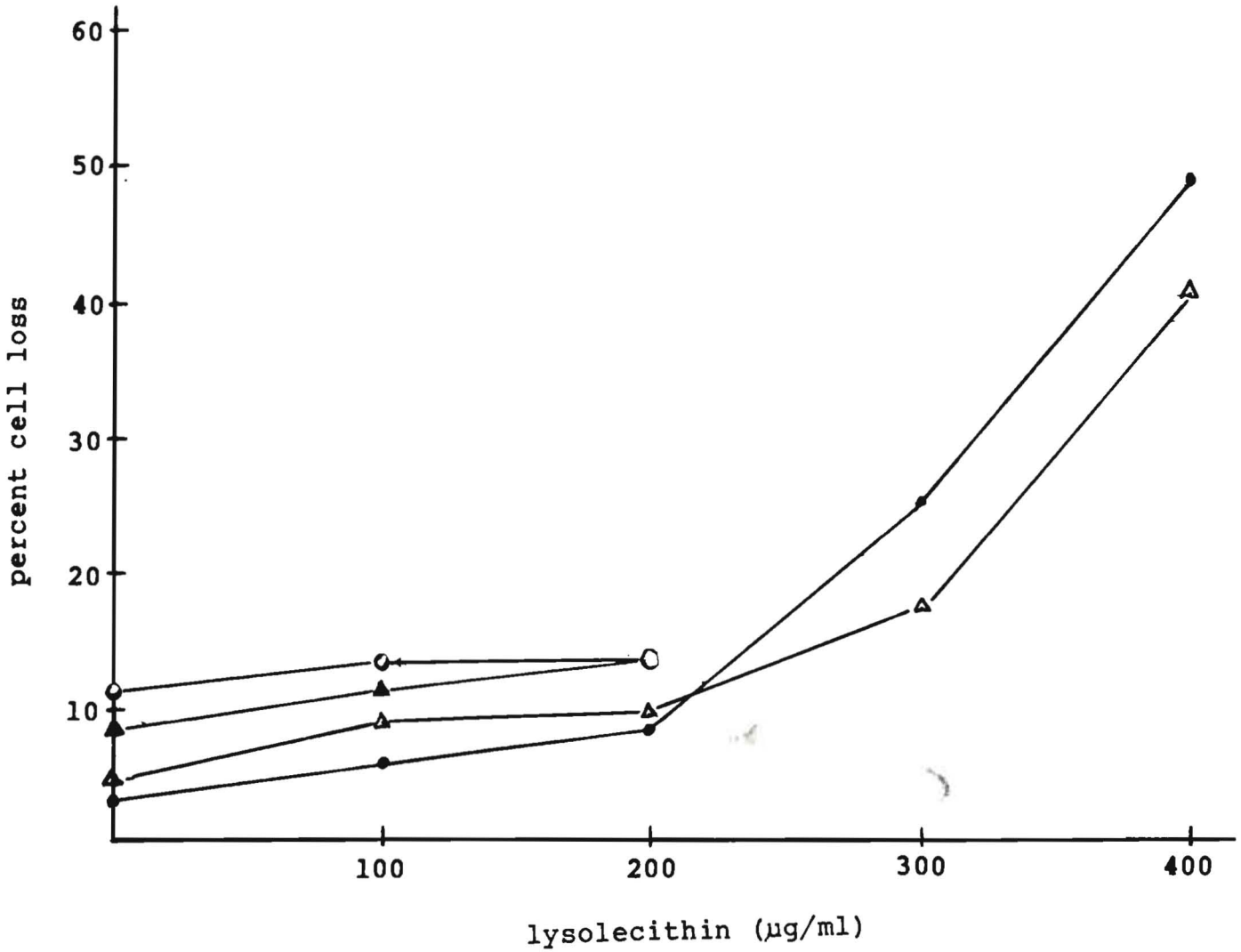

60
50
40
30
20
10
percent cell loss
100
200
300
400
lysolecithin (µg/ml)

Figure 10. Effect of temperature upon cell fusion at a lysolecithin concentration of 200 µg/ml. Aqueous lysolecithin, ● ; aqueous lysolecithin + mineral oil, o ; lysolecithin in mineral oil, △ ; lysolecithin in mineral oil + cholesterol, ▲ .

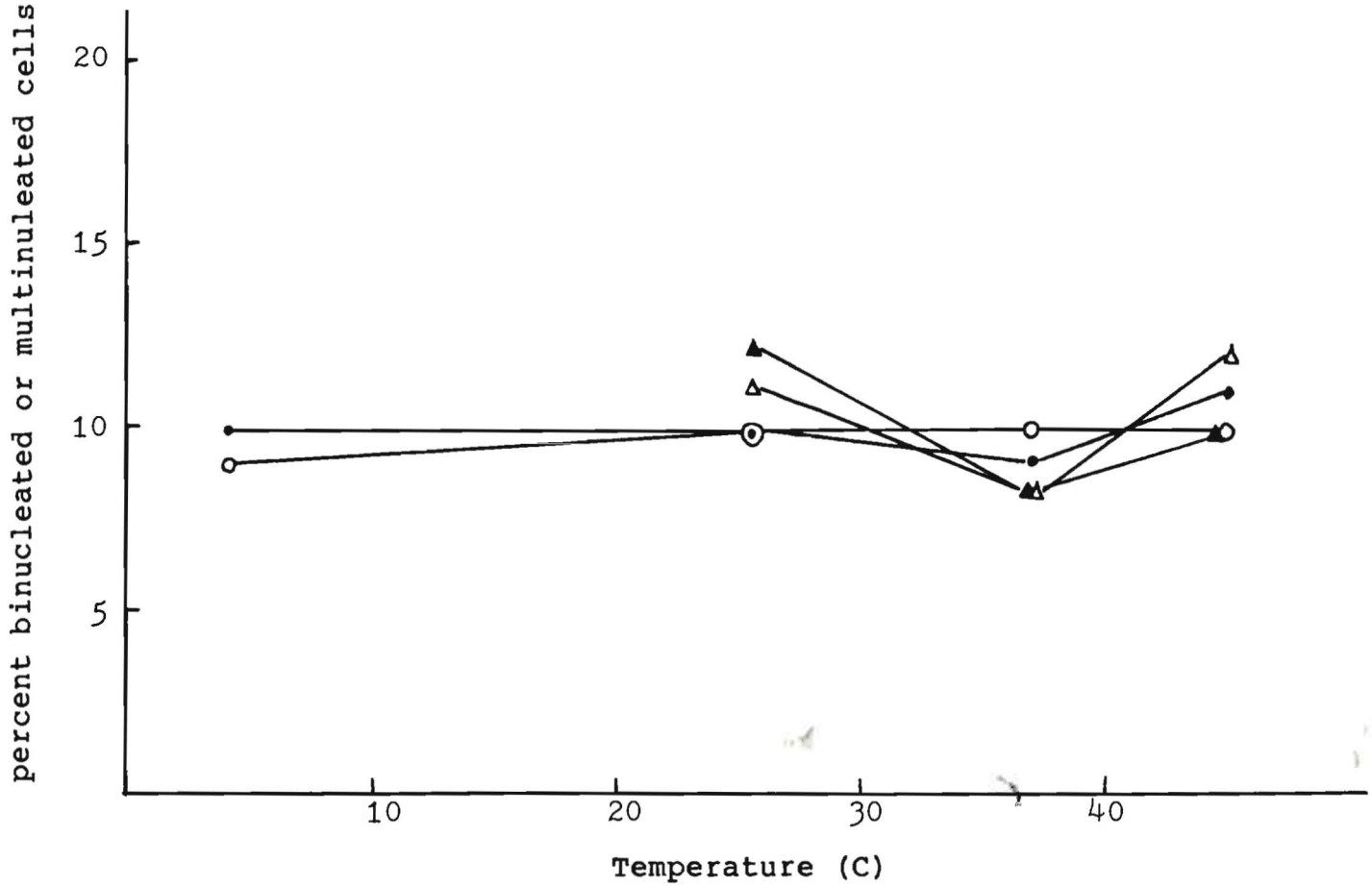

percent binucleated or multinuleated cells
20
15
10
5
10
20
30
40
Temperature (C)

Figure 11. Effect of temperature upon cell viability at a lysolecithin concentration of 200 µg/ml. Aqueous lysolecithin, ● ; aqueous lysolecithin + cholesterol, o ; lysolecithin in mineral oil, Δ ; lysolecithin in mineral oil + cholesterol, ▲ .

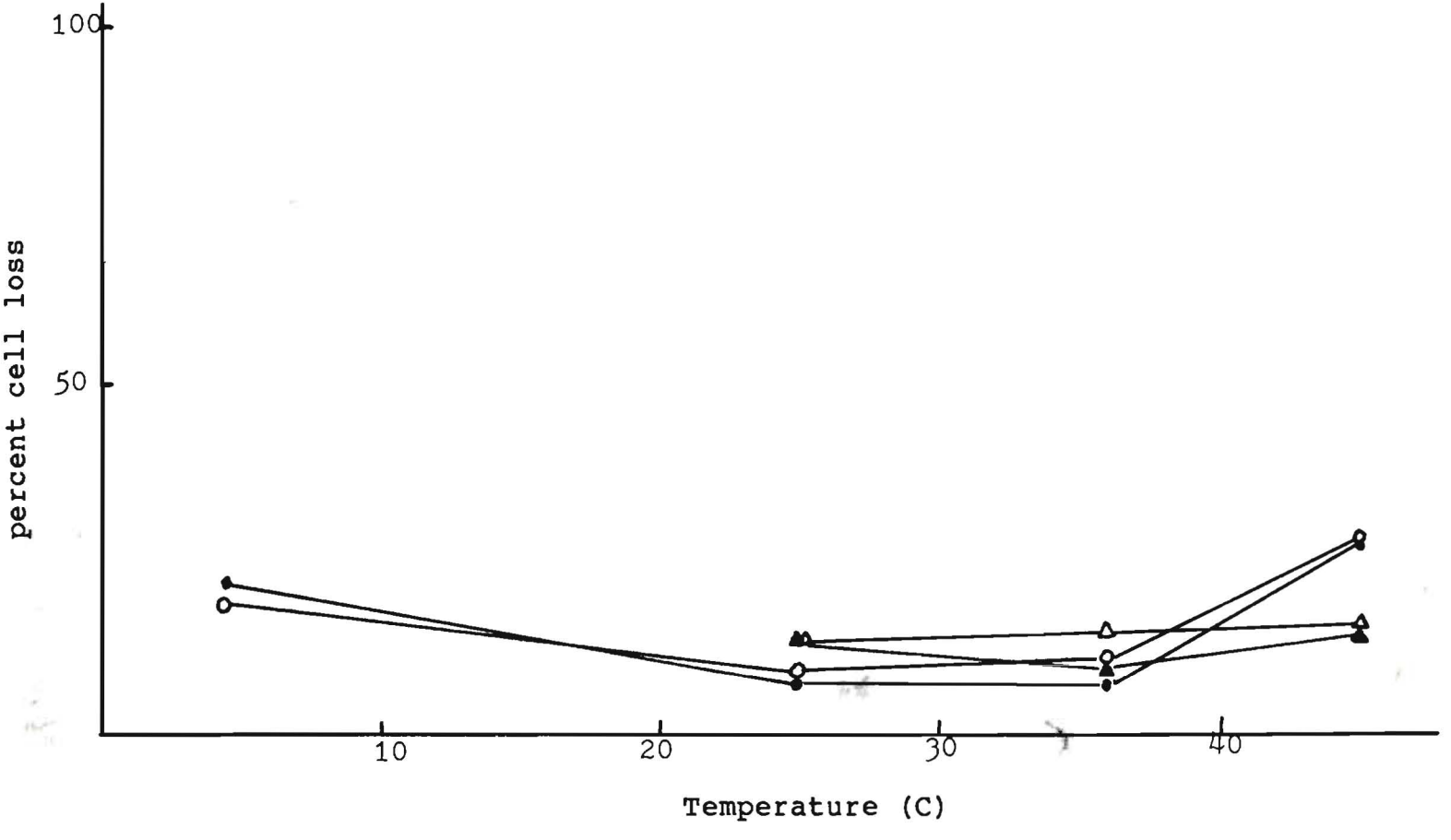

100
50
percent cell loss
10
20
30
40
Temperature (C)

Figure 12. Effect of aqueous lysolecithin treatment and temperature upon cell viability, cell fusion and cell count. A, B, C, and D refer to the temperatures 4, 25, 37, and 45 C respectively. 0, 100, 200, 300, and 400 refer to the µg/ml concentration of lysolecithin. ■ represents fused cells, ▨ represents cell count after treatment, □ represents viable cell count.

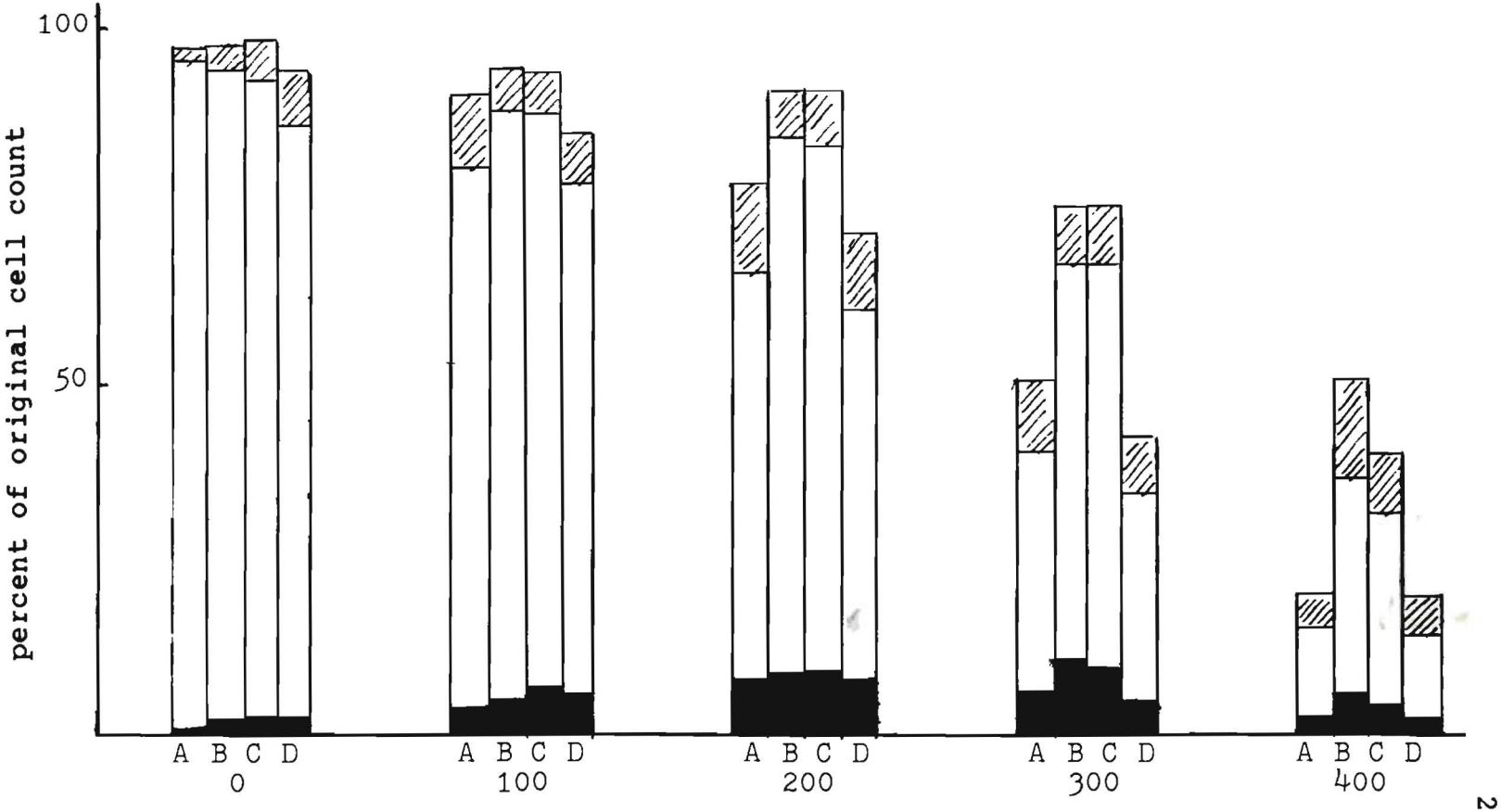
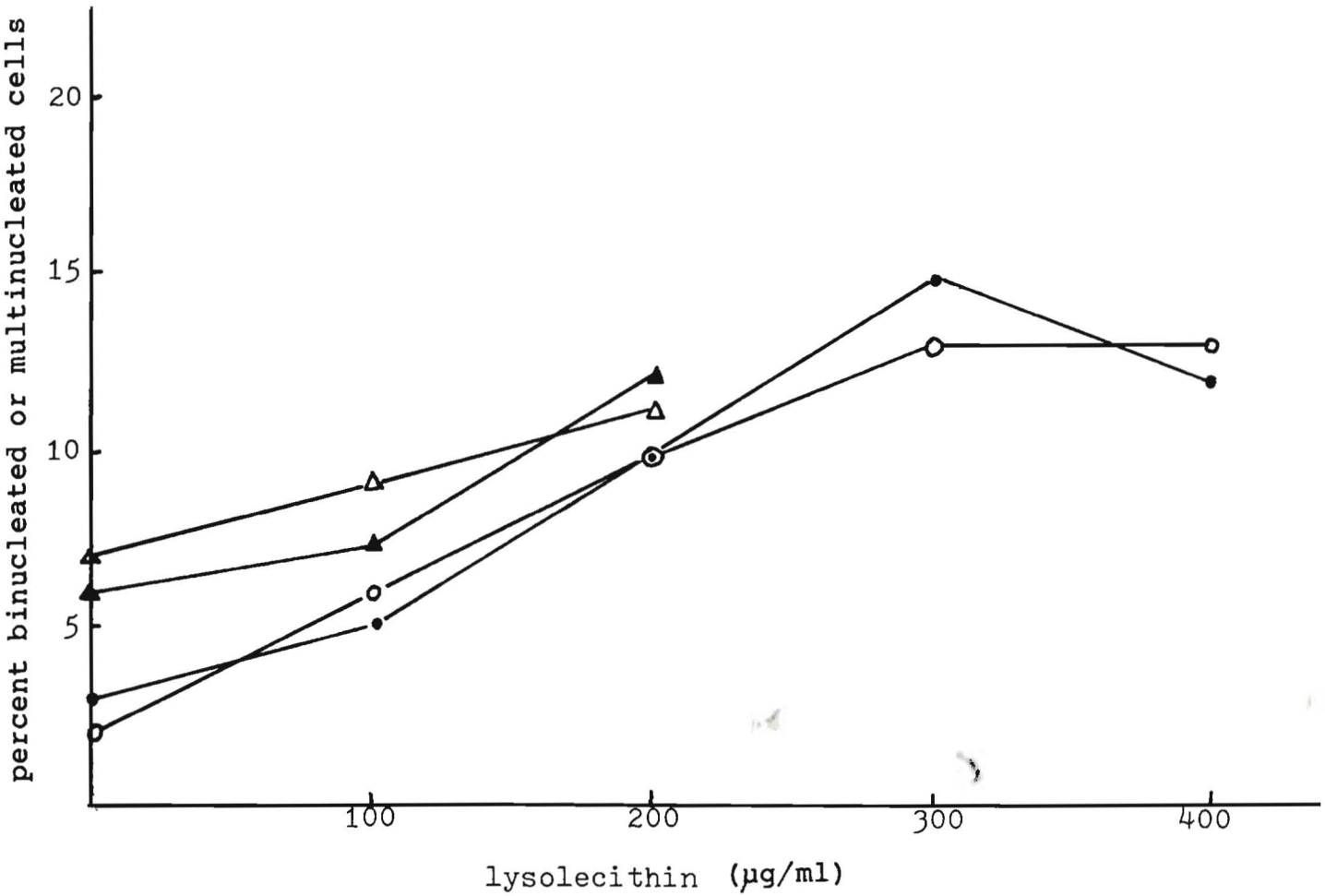

100
50
percent of original cell count
A B C D
0
A B C D
100
A B C D
200
A B C D
300
A B C D
400

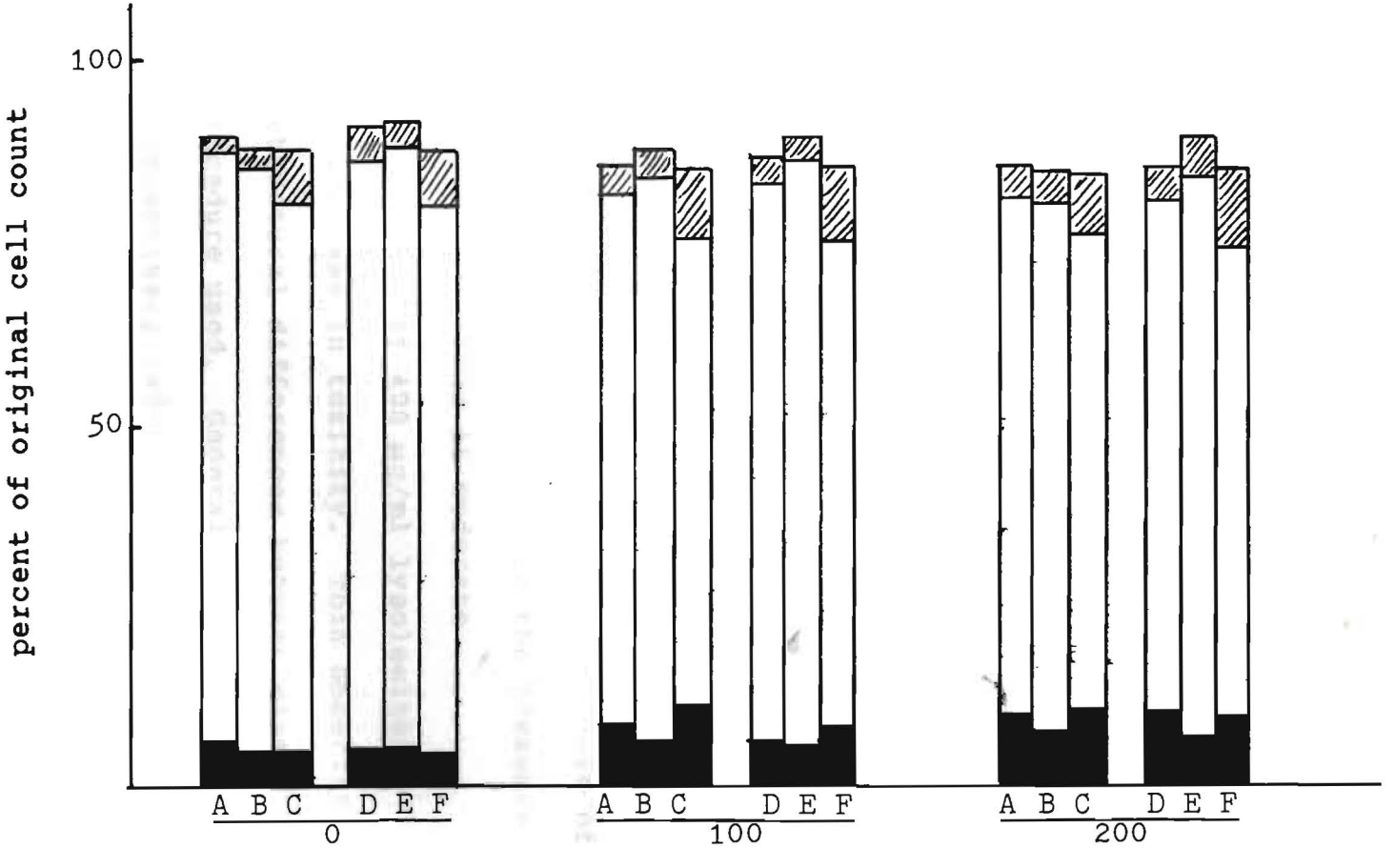
Figure 13. Effect of lysolecithin upon cell fusion at 25 C. Aqueous lysolecithin, ● ; aqueous lysolecithin + cholesterol, o ; lysolecithin in mineral oil, Δ ; lysolecithin in mineral oil + cholesterol, ▲ ;


percent binucleated or multinucleated cells
20
15
10
5
100
200
300
400
lysolecithin (µg/ml)

preparations when the lysolecithin concentration was 400 µg/ml at 25 and 37 C. At 45 C for the same lysolecithin concentration, toxicity was higher. The mineral oil preparation, 100 µg/ml lysolecithin at 25 C, showed a lower fusion rate in the presence of cholesterol.

Mineral oil preparations generally displayed a higher rate of cell loss except in the higher temperature assays where cell loss was considerably lower than aqueous preparations. Fusion rates in mineral oil controls were higher than aqueous controls by about three to four percent. This elevated fusion rate was also observed with lysolecithin at 100 and 200 µg/ml in mineral oil (Fig. 14).

Figure 14. Effect of lysolecithin in mineral oil upon cell viability, cell fusion, and cell count. A, B, and C refer to lysolecithin solutions without cholesterol at 25, 37, and 45 C respectively. D, E, and F represent lysolecithin solutions with 5 ug/ml cholesterol at 25, 37, and 45 C respectively. 0, 100, and 200 refer to the µg/ml concentration of lysolecithin. ■ represents fused cells, ▨ represents cell count after treatment, ▭ represents viable cell count.


percent of original cell count
100
50
A B C D E F
0
A B C D E F
100
A B C D E F
200

DISCUSSION

Lysolecithin concentration was the primary determinant of both cell fusion events and cell toxicity. The optimal concentration of 300 µg/ml showed significantly higher cell fusion than the lower concentrations with significantly less cell toxicity than the higher concentrations. The effects of lysolecithin concentration were consistent with the results reported by Croce et al. (1971). Electron microscopy observations support the theory of Lucy (1969) that the appearence of severely disrupted membranes occurred in the presence of high lysolecithin concentrations.

The cholesterol effects described by Papahadjopoulos et al. (1973) and Kwak et al. (1975) were not as readily observable in this investigation. Although the presense of cholesterol significantly reduced toxicity in the presence of high lysolecithin concentrations at moderate temperatures, the 45 C sample at 400 µg/ml lysolecithin showed a significant increase in toxicity. This observation is probably due to structural differences between plasma membranes and the procedure used. Generally the presence of cholesterol did not selectively reduce toxicity while allowing a higher fusion rate. Cholesterol probably reduces the fusion event proportionately with the reduction of cell toxicity making its use impractical in conjunction with lysolecithin for the induction of cell fusion.

The effects of temperature on toxicity and fusion were significant for the extremes of 4 and 45 C when compared to the 25 and 37 C assays. The assays at 25 and 37 were not significantly different from one another. At the high temperature end, cell stability was greatly reduced resulting in significant cell loss which was enhanced by increasing lysolecithin concentration. Membrane fluidity was most likely increased to the point that further destabilization due to lysolecithin resulted in complete lysis. The low temperature end (4 C) showed a significant increase in cell loss and toxicity. This is opposite to the observations of Breisblatt and Ohki (1975). Why these results are in opposition is not totally clear. Breisblatt and Ohki (1975) used phospholipid spherical membranes rather than biological membranes in their investigation. They noted a significant increase in stability at lower tempertures. They used 25 C as their lowest temperature. When biological membranes were used, as in this investigation, this stability was not significantly greater at 25 C although the instability at higher temperatures was supportive of his results. The strong destabilization observable at temperatures lower than 25 C supports a theory of membrane "brittling" at very low temperature. As the temperature deviates below physiological optimum the cell membrane probably becomes brittle and less resilient to mechanical pressure exerted by laboratory handling. The use

of the vortex mixer under brittle or non-resilient membrane conditions could result in a great deal of mechanically induced cell lysis. Generally, a higher temperature will enhance membrane fluidity and optimally should be in the physiological range for maximum fusion efficiency and low cytotoxicity.

The mineral oil assays were more troublesome in fusion procedures than aqueous systems. Cell recovery was decreased and difficulty of handling was increased. It was theorized that the hydrophobic nature of mineral oil would allow greater destabilization of the cell membrane without subsequent loss of cell contents. This, however was not apparent in the observations. Cell toxicity, or loss, was not significantly different than in the aqueous assays. One question unanswered is how much cell loss was due to incomplete recovery from the mineral oil medium rather than lysis induced by destabilization?

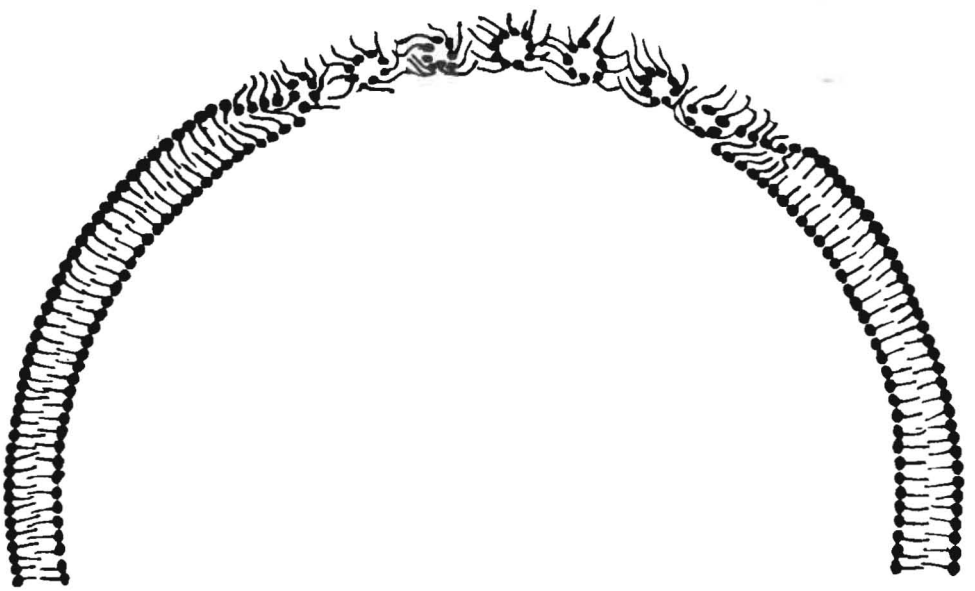
A significant increase in cell fusion was observed in the mineral oil controls when compared to the aqueous controls. This conceivably could be cell fusion induced by the presence of mineral oil, a mechanism not previously described. This investigator proposes a "reverse micelle" theory as a possible mechanism for mineral oil induced cell membrane fusion.

In keeping with the generally accepted theory of the lipid bilayer structure of cell membranes (Singer and

Nicholson, 1972), the mineral oil may cause a reversal in the orientation of lipids in the cell membrane. In the normal aqueous cell environment, the hydrophilic ends of the membrane lipid molecules are oriented to the external and cytoplasmic interfaces of the membrane. When placed in a mineral oil hydrophobic solution, the hydrophilic surface of the cell membrane would be repelled by the oil. The fluidity of the membrane allows reorientation of the external bilayer lipids so that the hydrophobic ends are brought to the surface where they are miscible with the mineral oil. At this point one of two things may occur. Either a point defect interaction occurs as described by Hui et al. (1981) or, destabilization occurs in the area forming "reverse" micelles with a hydrophilic core and a hydrophobic shell (Fig. 15). Hui et al. (1981) described the presence of similar structures which he termed the hexagonal phase during freeze-thaw induced fusion. Intermingling of these micelles and restabilization, as previously shown (Fig. 4), results in the union of two or more cells. The exposure of the comparatively longer hydrophobic ends probably results in a "stickier" surface to enhance micellar intermingling. Restabilization occurs when the cells are returned to an aqueous environment.

Mineral oil induced fusion occurs with a very low frequency. The low effectiveness of its activity and the difficulties encountered when working with mineral oil make

Figure 15. Reverse micelle formation in a membrane treated with mineral oil. The black circles represent hydrophilic components. The black tails represent the hydophobic components.



it impractical for use as a fusion inducing agent. Lysolecithin appeared to be an effective agent in producing somatic cell homokaryons although its toxicity is a major drawback. It consistently induced cell fusion at moderate concentrations with relatively little difficulty in procedure and workability. The results, from the viewpoint of cell loss, tend to reemphasize the need for a less toxic, highly reproducible chemical method of cell fusion.

SUMMARY

The phospholipid lysolecithin was tested in various concentrations, temperatures and environments for its effects on cell fusion, disintegration and mortality. Cholesterol and mineral oil were both assayed for their actions in reducing cell loss. Temperature extremes both high and low proved to be cytotoxic and cytolytic although higher temperatures increased cell fusion events. Cholesterol was found to be of little significance in decreasing lysolecithin's cytotoxic effects. Mineral oil solutions of lysolecithin did not significantly improve cell survival, but these mineral oil solutions proved to be significant in the fusion of cells without the presence of lysolecithin. A mechanism is proposed for this mineral oil induced cell fusion.

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APPENDIX

SOLUTION 1

Hank's Balanced Salt Solution

Unit 1: $NaHCO_3$ 3.5g dissolved in 250 ml deionized water

Unit 2:

NaCl	80.0g
KCl	4.0
$MgSO_4 \cdot 2H_2O$	2.0
$Na_2HPO_4 \cdot 2H_2O$	0.6
Glucose	10.0
KH_2PO_4	0.6

dissolve in 800 ml deionized water

Unit 3: $CaCl_2$ 1.4g dissolved in deionized water

Unit 4: Phenol red 0.4g mix phenol red in a small amount of water until a paste, dilute to 150 ml with deionized water.

titrate to pH 7.0 with 0.05 N NaOH (2.0g per liter) make up to a final volume of 200 ml.

Add 100 ml of unit #4 to unit #2 and add enough unit #3 to make 1000 ml. Add 3-4 ml of chloroform as a preservative.

The working BSS is prepared by diluting 10X stock solution 1:10 with deionized water. Autoclave and aseptically add sufficient unit #1 (1.3 ml) to turn 1X BSS to a cherry red color.

SOLUTION 2

GKN

NaCl	80.0 g
KCl	4.0 g
Glucose	10.0 g

Add deionized water to make one liter