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The Fusion of Erythrocytes by Fatty Acids, Esters, Retinol and α-Tocopherol

By Q. F. AHKONG, D. FISHER, W. TAMPION AND J. A. LUCY
Department of Biochemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter St., London WC1N 1BP, U.K.

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1. The ability of a number of carboxylic acids, their esters, retinol and α-tocopherol to induce fusion of hen erythrocytes *in vitro* was investigated. 2. Some 30 different fat-soluble substances (100μg/ml) were found to cause the formation of multinucleated erythrocytes with a suspension of 3×10^8 erythrocytes/ml. The most effective agents induced fusion within 5–10min at 37°C; some substances required about 1h. 3. Inclusion of Dextran 60C in the test medium minimized colloid osmotic lysis caused by exogenous lipids that induce cell fusion. 4. Cell swelling, followed by cell adhesion, was then seen to precede cell fusion. 5. Fusion occurred with C_{10}–C_{14} saturated carboxylic acids, with unsaturated, longer-chain carboxylic acids and their mono-esters; retinol, and to a lesser extent α-tocopherol, also caused cell fusion. 6. C_6–C_9, C_{15}, C_{16} and C_{18} saturated carboxylic acids did not induce fusion within 4h; glyceryl dioleate was only weakly active, and glyceryl trioleate was inactive in the test system. 7. Fusion was facilitated by a high ratio of chemical agents to cell number and by incubation between pH5 and 6. It was inhibited by EDTA and by serum albumin. 8. Glyceryl mono-oleate caused both a similar fusion of several species of mammalian erythrocyte and the interspecific fusion of human and chicken erythrocytes. 9. The term 'fusogenic' is proposed to describe chemical, viral and physical agents that cause membranes to fuse. 10. The biochemical mechanisms involved and the possible biological significance of membrane fusion by fusogenic lipids are discussed.

In previous work lysophosphatidylcholine was found to induce cell fusion without the aid of viruses (Howell & Lucy, 1969). Homokaryons were formed from hen erythrocytes in the presence of lysophosphatidylcholine whereas heterokaryons were produced with mixed cell populations (Poole *et al.*, 1970). In both instances, concomitant damage to membranes resulting from the treatment with lysophosphatidylcholine was substantially less when the lysophosphatidylcholine was present in microdroplets of fat rather than in aqueous solution (Ahkong *et al.*, 1972). The use of lysophosphatidylcholine in an aqueous glyceride–lecithin emulsion therefore allowed viable clones of hybrid mouse–hamster fibroblast cells to be obtained, although hybrid cells were produced with lysophosphatidylcholine only at twice the frequency of the hybrids formed spontaneously (Ahkong *et al.*, 1972). Croce *et al.* (1971) have confirmed that treatment of animal cells with lysophosphatidylcholine can induce fusion and, using lysophosphatidylcholine in the presence of protein, they observed that two different mutant cell lines fused to produce viable hybrid cells. DeBoer & Loyter (1971) have reported the formation of polynucleate avian erythrocytes after treatment with a mixture of polylysine and phospholipase C, and Toister & Loyter (1971) have found that fusion of avian erythrocytes can also be induced by Ca^{2+} at pH10.5.

Experiments have now been done to see if other lipids can reproducibly induce cell fusion. The present paper reports that the fusion of hen erythrocytes occurs with a variety of fat-soluble molecules under defined conditions. Conceivably, the ability of some of these substances to cause membranes to fuse may be important in relation to the induction and control of membrane fusion occurring *in vivo* at cellular and subcellular levels. Some of the physical and chemical factors facilitating and inhibiting the fusion of hen erythrocytes have also been studied. Preliminary communications on aspects of this work have been published (Lucy *et al.*, 1971; Fisher *et al.*, 1972).

Materials and Methods

Erythrocytes

Blood (approx. 2ml) was removed from the brachial vein of an adult hen and taken into a syringe containing citrate anticoagulant solution (1ml) (DeGowin *et al.*, 1949). It was then transferred to approx. 10ml of this solution, well mixed

and centrifuged gently. The pelleted cells were washed three times in a modified Eagle's basal salt solution buffered with sodium cacodylate (NaCl, 116mM; KCl, 5.36mM; $CaCl_2$, 1.80mM; $MgSO_4$, 0.81mM; Me_2AsO_2Na, 10.0mM; penicillin G, 200000units/l; streptomycin sulphate, 100000μg/l glucose, 1g/l). Dextran 60C was included in this buffer at a concentration of 80g/l, and its pH was adjusted with HCl (1M) as required before diluting to 1 litre. After each centrifugation, the extreme top layer of cells was removed by suction to remove the buffy coat, which contained most of the leucocytes. The packed cell volume was noted after the final centrifugation, and 1ml of packed cells was diluted to 8.5ml with buffered saline, containing Dextran 60C where appropriate (usually 80mg/ml). The final suspension of erythrocytes, which contained 7×10^8–8×10^8 cells/ml, was kept at about 4°C until used later on the same day.

Reagents

The lipids used were of the highest chemical purity readily available from commercial sources. Sigma (London) Chemical Co. (London S.W.6, U.K.) supplied oleyl alcohol and the following fatty acids and their methyl and glyceryl esters (99% purity): hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, dodecanoic acid, tridecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, octadecanoic acid, undec-10-enoic acid, tetradec-*cis*-9-enoic acid, hexadec-*cis*-9-enoic acid, octadec-*cis*-9-enoic acid, octadec-*trans*-9-enoic acid, octadeca-*cis,cis*-9,12-dienoic acid, octadeca-*cis,cis,cis*-9,12,15-trienoic acid and docosa-*cis*-13-enoic acid. The glyceryl and sucrose mono-esters of dodecanoic acid (lauric acid) were obtained from K & K Laboratories Inc. (Plainview, N.Y., U.S.A.). The ethylene glycol and propylene glycol esters of stearic acid and oleic acid were gifts from Leek Chemicals Ltd. (Bridge End Works, Leek, Staffs., U.K.); t.l.c. revealed the presence of mono- and di-esters in these four preparations. Glyceryl-1-octadecenyl ether (selachyl alcohol) was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. α-Tocopherol and synthetic crystalline retinol were from Roche Products Ltd. (Welwyn Garden City, Herts., U.K.). Fatty acid-free bovine serum albumin (fraction V) was from Pentex Biochemicals (Kankakee, Ill., U.S.A.), and Dextran 60C (average mol.wt. 60000–90000, clinical grade) was from Sigma (London) Chemical Co. Glutaraldehyde was from Edward Gurr Ltd. (London S.W.14, U.K.).

Lipid emulsions

Fatty acids, esters and selachyl alcohol. These lipids were stored at 4°C as solutions in hexane (10mg/ml). The required amount of lipid (0.1ml of stock solution) was transferred into a round-bottom flask and all traces of the solvent were removed, either by evaporation under reduced pressure in a rotary evaporator at 37°C or under a stream of N_2. The dried lipid was dispersed in the appropriate buffer (6ml) by sonication with a 150W ultrasonic disintegrator fitted with a 1.9cm diameter titanium probe (M.S.E. Ltd., Crawley, Sussex, U.K.), for 1min or until the lipid had dispersed. During this process the flask was cooled in ice and N_2 was blown over the surface of the buffer. The emulsion was used within 5min of preparation, as the ability of some lipids to cause cell fusion decreased if the emulsion was allowed to age.

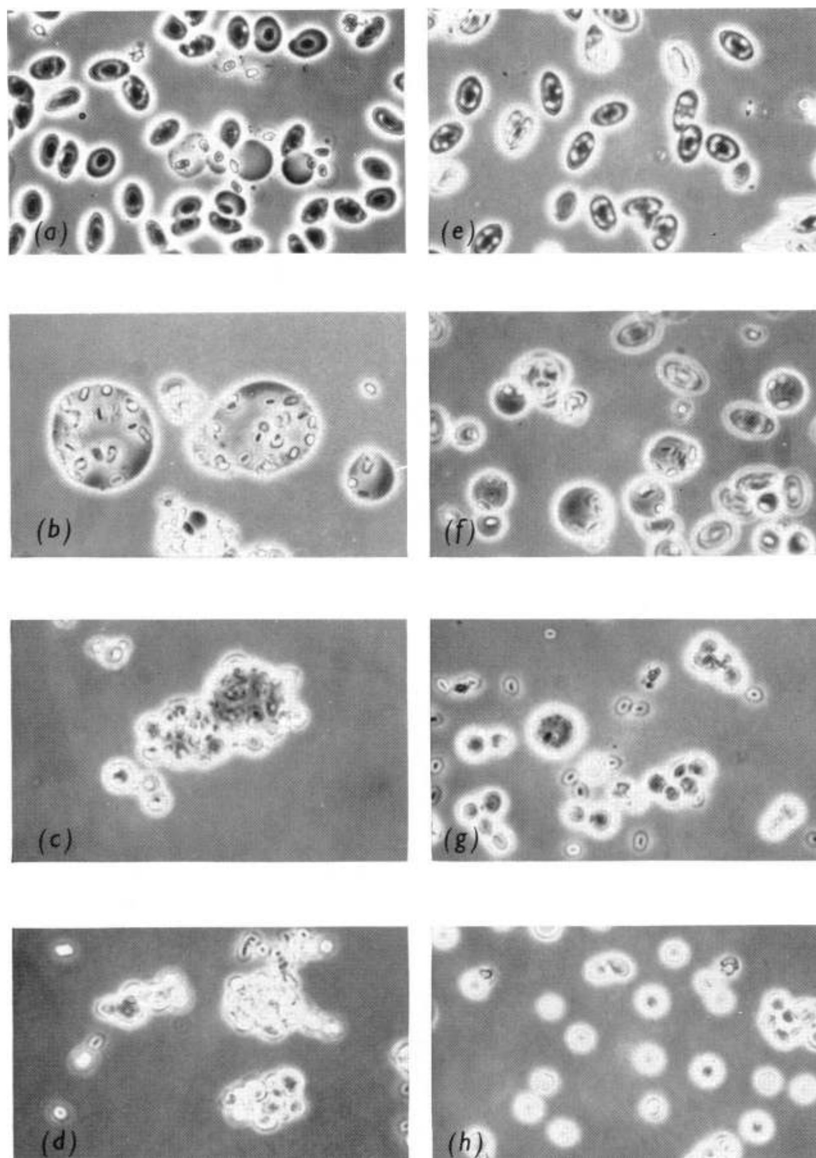
Retinol was stored and handled as described by Dingle & Lucy (1962). The stock solution in ethanol was diluted to give a working solution containing approx. 10mg of retinol/ml; the actual concentration of the stock solution was determined by using a molar extinction coefficient of 52400litre·mol^{-1}·cm^{-1} (Morton, 1962). A sample (25μl) of the working solution of retinol was injected, with a Hamilton syringe (Micromeasure N.V., The Hague, The Netherlands) into a glass vessel containing the appropriate buffer (1.5 ml) used for cell fusion and this suspension of retinol was used immediately. Suspensions of α-tocopherol were prepared similarly from a stock solution in ethanol. The final concentration of ethanol in experiments with retinol and α-tocopherol was 1%: this had no discernible effect on the erythrocytes.

Cell fusion

Unless otherwise stated, the freshly prepared emulsion of lipid (0.8ml) was mixed with a suspension of washed hen erythrocytes (0.5ml) that had been preincubated at 37°C for about 5min. The treated cells (3×10^8 hen erythrocytes/ml and 100μg of lipid/ml) were maintained at 37°C for up to 4h, and small samples were removed at frequent intervals for examination with a phase-contrast microscope (Standard WL Research Microscope; Carl Zeiss, Degenhardt and Co. Ltd., London, U.K.). The incubation medium used was either Eagle's tissue-culture M.E.M. medium (Wellcome Reagents Ltd., Beckenham, Kent, U.K.), or more often the modified Eagle's basal salt solution buffered with sodium cacodylate (10mM) (see above). When other buffers were used instead of cacodylate, their concentrations were also 10mM.

Haemolysis

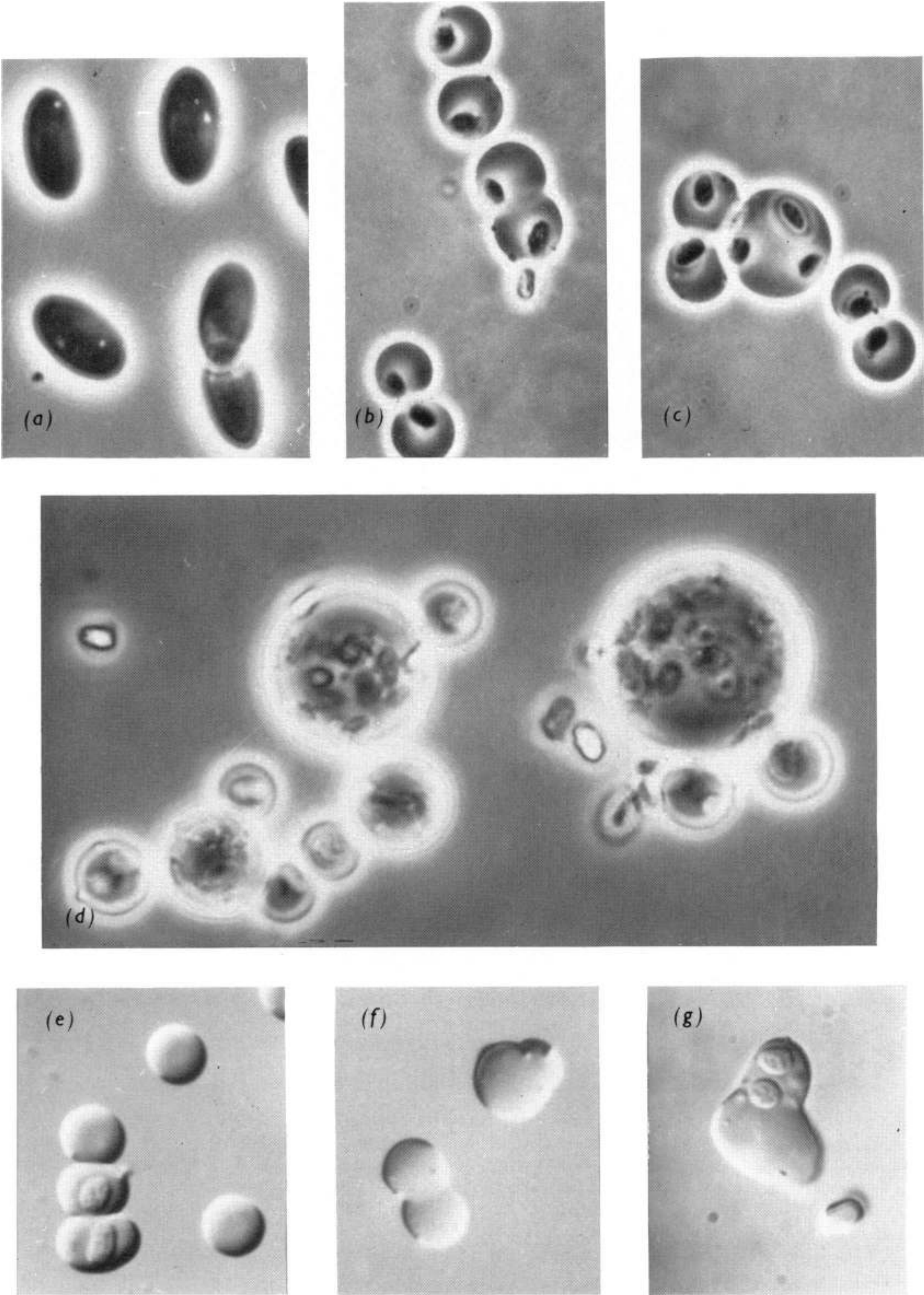
Percentage haemolysis of erythrocytes was determined by measuring the extinction of haemoglobin as cyanomethaemoglobin at 540nm in the supernatant obtained by centrifuging the incubation



EXPLANATION OF PLATE 1

Effects of Dextran 60C on the fusogenic activity of glyceryl mono-oleate

A suspension of hen erythrocytes (3×10^8 cells/ml) with ultrasonically dispersed glyceryl mono-oleate (100μg/ml) was incubated at 37°C in Eagle's basal salt solution buffered with sodium cacodylate, pH5.6 (*a–d*) or pH7.4 (*e–h*), containing differing concentrations of Dextran 60C; 0 (*a* and *e*), 40 (*b* and *f*), 80 (*c* and *g*) and 120mg/ml (*d* and *h*). Cell fusion occurred most extensively in the presence of 40 and 80mg of Dextran 60C/ml, with fusion at pH5.6 (*b* and *c*) being greater than at pH7.4 (*f* and *g*). With 120mg of dextran/ml, the cells were extensively aggregated at pH5.6 (*d*) and shrunken at pH7.4 (*h*). Glyceryl mono-oleate caused little cell-fusion in the absence of dextran (*a* and *e*) and the cells remained oval in shape, with evidence of crenation at pH7.4. Phase-contrast microscopy: ×300.



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mixture. Measurements were made at least 10min after the addition of 0.2ml of this supernatant, or the uncentrifuged mixture, to 4.8ml of a solution containing $NaHCO_3$ (1g/l), $K_3Fe(CN)_6$ (0.2g/l) and KCN (0.2g/l) (Baker *et al.*, 1966).

Results

Test system for studying cell fusion: use of dextran

Chemical agents that are capable of causing a sufficient change in the organization of erythrocyte plasma membranes to allow the cells to fuse also increase membrane permeability. This was seen previously in extreme form on treatment of hen erythrocytes at 37°C with lysophosphatidylcholine in aqueous solution, when the cells not only fused to form syncytia within 30s but the syncytia produced also disintegrated within 1min (Ahkong *et al.*, 1972). In an attempt to minimize osmotic effects, sucrose was initially included in the incubation medium in the present work, but without success. Improved results were obtained when dextran was investigated because of the report by Sears *et al.* (1964) that dextrans protect human erythrocytes against colloid osmotic lysis after treatment with complement. Dextran 60C was therefore added (80mg/ml) to either NaCl solution buffered with sodium cacodylate, or to Eagle's medium, during treatment of erythrocytes at 37°C for up to 4h with a chemical capable of inducing cell fusion, e.g. glyceryl mono-oleate. That the added dextran could inhibit colloid osmotic lysis was shown by the fact that lysis immediately occurred when the resulting multinucleated erythrocytes were resuspended in a medium not containing dextran.

The behaviour of dextran in the test system is, however, not yet fully understood. High concentrations (10%, w/v) of dextrans having molecular weights greater than about 40000 are known to aggregate human erythrocytes (Brooks & Seaman, 1972). In the absence of glyceryl mono-oleate, 80mg of Dextran 60C/ml (mol. wt. approx. 77000) caused neither agglutination nor fusion of hen erythrocytes in our experiments. Contrary to expectation, however, it has been found that the dextran positively facilitates cell fusion rather than delaying cell lysis during treatment with chemical agents. Fig. 1 shows that lysis occurred more, not less, rapidly in the presence of dextran when hen erythrocytes at 37°C were treated with oleic acid at pH5.6 for up to 40min. Despite this, extensive cell fusion was observed after only 5min in the presence of dextran. Without dextran the cells were almost completely lysed after 40min and there was negligible cell fusion. Plate 1 shows the appearance of hen erythrocytes treated with glyceryl mono-oleate (which is less haemolytic than oleic acid) in the presence of different concentrations of dextran

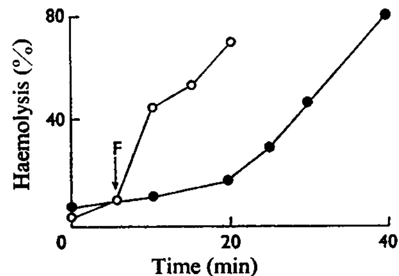

Fig. 1. *Effects of Dextran 60C on lysis and on cell fusion*

Hen erythrocytes (3×10^8 cells/ml) were incubated at pH5.6 with oleic acid (370nmol/ml) in Eagle's basal salt solution buffered with sodium cacodylate in the presence (○) and absence (●) of Dextran 60C (80mg/ml). Fusion was observed as indicated (F) in the presence of dextran after 4–6min but not, within 40min, in its absence.

EXPLANATION OF PLATE 2

Morphological changes during cell fusion

(*a*) Oval-shaped hen erythrocytes (3×10^8 cells/ml) after incubation at pH7.4 for 15min at 37°C in Eagle's basal salt solution buffered with sodium cacodylate, containing Dextran 60C (80mg/ml). Phase-contrast miscoscopy: ×1000. (*b–d*) The erythrocytes (3×10^8 cells/ml) were treated at pH7.4 with ultrasonically dispersed glyceryl mono-oleate (100μg/ml) in the buffered salt solution containing Dextran 60C (80mg/ml) at 37°C. With (*b*) and (*c*), a buffered solution (pH7.4) of bovine serum albumin (6mg/ml) was added after 15min, and the cells were then incubated for a further 60min. With (*d*), the cells were treated with the dispersed glyceryl mono-oleate for 30min. Phase-contrast microscopy: ×1000. (*e–g*) A suspension of hen and human erythrocytes (approx. 1.5×10^8 cells of each species/ml) was treated at pH5.6 with ultrasonically dispersed glyceryl mono-oleate (100μg/ml) in Eagle's basal salt solution buffered with sodium cacodylate, containing Dextran 60C (80mg/ml) at 37°C. (*e*) Shows a human erythrocyte and two hen erythrocytes (all swollen) in close contact after 5min. (*f*) Shows stages in the fusion of human erythrocytes with each other after 15min. Interspecific fusion between human and hen erythrocytes is seen in (*g*). Nomarski differential-interference-contrast microscopy: ×1000.

at pH 5.6 and 7.4; the optimum concentration of Dextran 60C is in the range 40–80 mg/ml at pH 5.6. The most effective concentration within this range is dependent on the concentration of lipid, and the higher concentration of 80 mg of Dextran 60C/ml has been used in most of our experiments.

Cell fusion induced by fatty acids, esters, retinol and α-tocopherol

In previous work, lysophosphatidylcholine was incorporated into micro-droplets of glycerides to localize its action and to minimize generalized membrane damage. Small numbers of fused erythrocytes were then occasionally seen when cells were incubated for 15 min at 37°C with emulsions of commercial preparations of glyceryl trioleate or dioleate (Ahkong *et al.*, 1972). This low background of cell fusion occurring in the absence of lysophosphatidylcholine indicated that the glyceride preparations contained one or more agents capable of causing fusion. T.l.c. has shown that glyceryl mono-oleate and traces of oleic acid are present in the commercial samples of glyceryl tri- and di-oleate used. Fractionations with ion-exchange and adsorption column chromatography were undertaken, followed by tests of the action of purified fractions, on hen erythrocytes (Q. F. Ahkong & W. Tampion, unpublished work). In preliminary experiments in which dextran was not used, it was found that glyceryl trioleate was unable to cause cell fusion; glyceryl dioleate caused cell agglutination but apparently not fusion; glyceryl mono-oleate induced extensive cell fusion.

Subsequently, a series of fatty acids, esters and other fat-soluble compounds were tested for ability to fuse hen erythrocytes during incubation at 37°C for up to 4 h in test medium containing Dextran 60C. The results of these experiments are shown in Table 1. It may be significant that, of the fatty acids tested, fusion was most readily demonstrated with unsaturated acids, and with medium chain-length saturated compounds. Elaidic acid was markedly less effective than oleic acid. Both short-chain and long-chain saturated acids were ineffective. Most of the active compounds caused cell fusion within 15 min but, as indicated in Table 1, some agents induced fusion only after a lag period of about 45 min. However, those lipids capable of causing cells to fuse, unlike those that were inactive, gave rise to increasing numbers of multi-nucleated cells with increasing time of incubation at 37°C. In confirmation of an earlier suggestion concerned with the haemolytic properties of retinol and its actions on the lysosomal vacuolar system (Lucy, 1969), retinol has been found to cause extensive cell fusion. α-Tocopherol was active, but only when tested at a concentration of 200 μg/ml.

Morphological changes accompanying cell fusion

Light-microscopy revealed that the oval-shaped hen erythrocytes treated with glyceryl mono-oleate, for example, in buffered saline at pH 5.6 first become swollen and then spherical (Plate 2*b*). This occurs both in the presence and in the absence of dextran, but without dextran most cells proceed to lyse rather than fuse. In the presence of dextran, regions of contact between adjacent, adhering cells enlarge, a neck forms between the cells, and the membranous barriers between individual cells become indistinct as fusion occurs (Plates 2*b* and 2*c*). After fusion, the nuclei of the fused cells are often to be seen moving randomly through the enlarged, 'common' cytoplasm of the multinucleated cells (see Plate 2*d*). Fusion between oval-shaped unswollen cells has never been observed in our experiments. As mentioned below, these observations parallel aspects of the behaviour of plant protoplasts during chemically induced fusion.

The morphological changes occurring during fusion allow two chronological stages to be distinguished. In the first stage, while cell swelling and cell–cell interactions occur, no fusion is observed. During the second stage, a high proportion of the erythrocytes fuse at about the same time. The time before onset of extensive cell fusion is thus readily measured. We have defined the 'time to fusion', t_f, as the number of minutes of incubation that elapse before fused cells are observed in high incidence, with the light microscope, in samples taken at frequent intervals from a preparation of treated cells. The time to fusion can normally be determined to within $\pm 1\frac{1}{2}$ min.

Fusion by glyceryl mono-oleate and retinol: dependence on concentration

The time to fusion, t_f, may be used both to study the dependence of cell fusion on the concentration of the added chemical agent, and also to compare the effectiveness of different agents. Fig. 2 shows the dependence of t_f on the concentration of glyceryl mono-oleate and retinol. As the concentration of agent is increased, fusion occurs at earlier and earlier times. Fig. 3 shows that, if the concentration of glyceryl mono-oleate is kept constant, fusion occurs more quickly as the number of erythrocytes is decreased. A high ratio of chemical agent to cell number therefore facilitates rapid cell fusion.

Effects of pH

Experiments in this laboratory on cell fusion induced by lysophosphatidylcholine were undertaken at pH 5.6 to minimize the electrostatic repulsion between cells as much as possible without damaging the cells by using too acid conditions

Table 1. *Fusion of hen erythrocytes by fat-soluble compounds*

All lipids were used at a concentration of 100 μg/ml unless otherwise stated, assuming complete dispersion (see the Materials and Methods section). Each lipid was tested at pH 5.6 with erythrocytes at 37°C in Eagle's basal salt solution buffered with sodium cacodylate containing Dextran 60C (80 mg/ml). The cells and dispersed lipids were prepared as described in the Materials and Methods section.

Chain length of parent compound	Agent	Ability to induce fusion within 4 hours
C_6	Hexanoic acid (caproic acid)	Negative
C_7	Heptanoic acid (enanthic acid)	Negative
C_8	Octanoic acid (caprylic acid)	Negative
C_9	Nonanoic acid (pelargonic acid)	Negative
C_{10}	Decanoic acid (capric acid)	Positive†
C_{11}	Undecanoic acid (hendecanoic acid)	Positive
	Methyl undecanoate	Positive*
	Undec-10-enoic acid (undecylenic acid)	Positive†
C_{12}	Dodecanoic acid (lauric acid)	Positive
	Methyl docecanoate	Positive*
	Glyceryl monolaurate	Positive
	Sucrose monolaurate	Positive
C_{13}	Tridecanoic acid (tridecoic acid)	Positive
	Methyl tridecanoate	Positive*
C_{14}	Tetradecanoic acid (myristic acid)	Positive†
	Methyl tetradecanoate	Positive*
	Tetradec-*cis*-9-enoic acid (myristoleic acid)	Positive
C_{15}	Pentadecanoic acid	Negative
C_{16}	Hexadecanoic acid (palmitic acid)	Negative
	Glyceryl monopalmitate	Negative
	Methyl hexadecanoate (methyl palmitate)	Positive*
	Hexadec-*cis*-9-enoic acid (palmitoleic acid)	Positive
	Methyl palmitoleate	Positive
C_{18}	Octadecanoic acid (stearic acid)	Negative
	Glyceryl monostearate	Negative
	Ethylene glycol monostearate	Negative
	Propylene glycol monostearate	Negative
	Methyl octadecanoate (methyl stearate)	Positive*
	Octadec-*cis*-9-enoic acid (oleic acid)	Positive
	Octadec-*trans*-9-enoic (elaidic acid)	Positive
	Methyl oleate	Positive
	Glyceryl mono-oleate	Positive
	Glyceryl dioleate	Positive (200 μg/ml)
	Glyceryl trioleate	Negative
	Ethylene glycol mono-oleate	Positive
	Propylene glycol mono-oleate	Positive
	Glyceryl octadec-1-enyl ether (selachyl alcohol)	Positive
	Oleyl alcohol	Positive
	Octadeca-*cis*,*cis*-9,12-dienoic acid (linoleic acid)	Positive
	Octadeca-*cis*,*cis*,*cis*-9,12,15-trienoic acid (linoleic acid)	Positive
C_{22}	Docosa-*cis*-13-enoic acid (erucic acid)	Positive
C_{20}	Retinol (vitamin A_1 alcohol)	Positive
C_{29}	α-Tocopherol (vitamin E alcohol)	Positive (200 μg/ml)
	α-Tocopheryl acetate (vitamin E acetate)	Negative

*Fusion observed after a delay of about 45 min.
†Very low incidence of fusion.

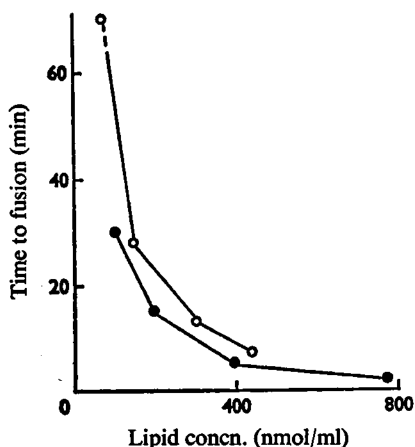

Fig. 2. *Effect of concentration of fusogenic lipid on cell fusion*

Hen erythrocytes (3×10^8 cells/ml) in Eagle's basal salt solution buffered with sodium cacodylate (pH 5.6) containing Dextran 60C (80 mg/ml) were incubated with retinol (●) or glyceryl mono-oleate (○). The time to fusion is defined in the text.

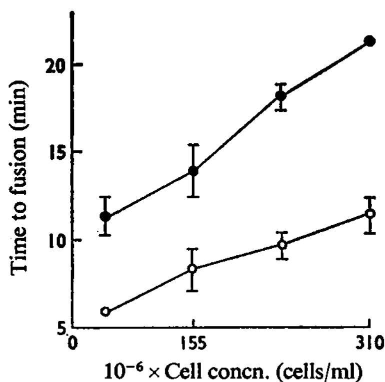

Fig. 3. *Effect of cell concentration on cell fusion*
Hen erythrocytes at different concentrations were incubated at pH 5.6, in Eagle's basal salt solution buffered with sodium cacodylate containing Dextran 60C (80 mg/ml), with glyceryl mono-oleate at 147 nmol/ml (●) or at 294 nmol/ml (○). The time to fusion is defined in the text. Each point is the mean of results from two experiments.

(Howell & Lucy, 1969; Poole *et al.*, 1970; Ahkong *et al.*, 1972). Croce *et al.* (1971, 1972) have reported that cell fusion and the formation of hybrid cells in experiments with lysophosphatidylcholine and Sen-

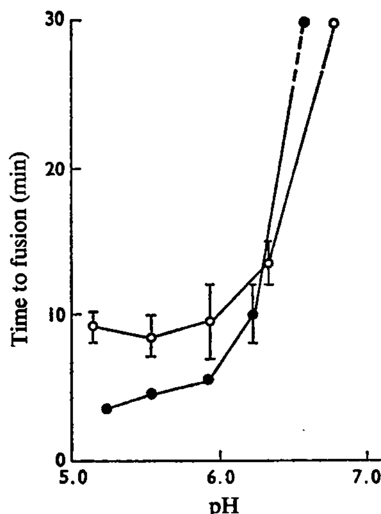

Fig. 4. *Effect of pH on cell fusion*

Hen erythrocytes (approx. 3×10^8 cells/ml) in Eagle's basal salt solution buffered with sodium cacodylate at various pH values containing Dextran 60C (80 mg/ml), were incubated with oleic acid (185 nmol/ml) (●) or with retinol (270 nmol/ml) (○). The time to fusion is defined in the text. Points given are the means from two experiments where indicated.

dai virus is greater at pH 7.2 than at pH 5.5, with a maximum yield of hybrid cells between pH 7.6 and 8.0. In the present experiments, changes in t_f with pH have been determined with hen erythrocytes for several chemical agents, excluding lysophosphatidylcholine because of its drastic effects on membrane permeability and stability.

Fig. 4 shows that the onset of cell fusion with cells treated with retinol and oleic acid at 37°C occurs most rapidly between pH 5 and 6. At pH 6.5 and above, t_f is greater than 30 min. Similar results have been obtained with glyceryl mono-oleate, although with this substance t_f increases less rapidly between pH 6 and 7. The values of t_f observed between pH 5 and 6 with glyceryl mono-oleate and retinol were unchanged when cacodylate buffer was replaced by phosphate or acetate buffers. It is unlikely that the pH-dependence of cell fusion induced by oleic acid is related to the dissociation of the acid, since the values of pK for emulsified fatty acids are as high as 8–9 (Heikkila *et al.*, 1970). In addition, since oleic acid, glyceryl mono-oleate and retinol are all most effective between pH 5 and 6, it would seem that the observed dependence of cell fusion on pH is a feature of the state of the erythrocytes, rather than of the

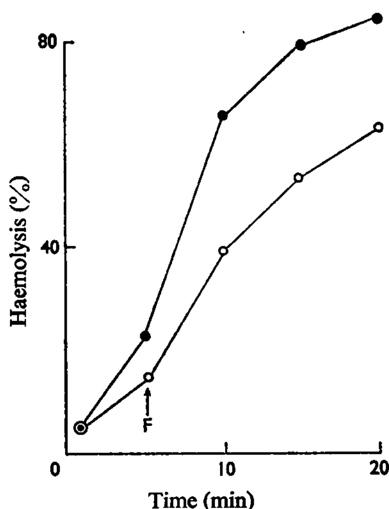

Fig. 5. *Effects of EDTA on cell fusion and lysis*

Hen erythrocytes (approx. 3×10^8 cells/ml) that were incubated at pH5.6 in Eagle's basal salt solution buffered with sodium cacodylate containing Dextran 60C (80mg/ml) and oleic acid (185nmol/ml) (○) began to fuse (F) at 5min. In the presence of EDTA (3.5mM) (●) no fusion was seen within 20min but cell lysis increased.

fusogenic agents. This interpretation is consistent with the actions of glyceryl mono-oleate, in the absence of dextran, at pH5.6 and 7.4. At the higher pH value, glyceryl mono-oleate induces the changes in the shape of hen erythrocytes that are shown in Plate 1.

Effects of Ca^{2+}, EDTA and serum albumin

In the standard incubation medium used for cell fusion the concentration of Ca^{2+} is 1.8mM. The addition of EDTA (3.5mM) not only inhibits fusion by glyceryl mono-oleate, oleic acid and retinol but also increases cell lysis (Fig. 5). Interestingly, the cells swell in the presence of EDTA, but the swollen cells then lyse more readily than they fuse. Ca^{2+}, in a sufficient concentration, overcomes the inhibitory effect of EDTA on fusion. This suggests either that Ca^{2+} is needed for the fusion process itself, and/or that it is required to stabilize the membranes of the resulting multinucleated cells. Preliminary experiments with fusion induced by glyceryl mono-oleate indicate that Mg^{2+} and Ba^{2+} can be substituted for Ca^{2+}. Comparable effects of EDTA have been noted in virus-induced cell fusion (Okada & Murayama, 1966). In addition, Toister & Loyter (1970) have reported that the haemolysis associated with virus-induced fusion of hen erythrocytes is markedly decreased by the addition of Ca^{2+}.

The inhibitory effect of serum albumin on changes in membrane systems induced by hydrophobic substances is well documented. For example, the swelling of mitochondria caused by fatty acids (Wojtczak & Lehninger, 1961) and by retinol (Lucy *et al.*, 1963) and the lysis of erythrocytes by retinol (Dingle & Lucy, 1962) are inhibited by the presence of serum albumin, which traps the lipids and effectively prevents them from reaching the membrane concerned. Since unsaturated fatty acids and retinol have now been shown to cause cell fusion, the ability of serum albumin (6mg/ml) to prevent fusion was investigated. As expected, the protein inhibited cell fusion induced by glyceryl mono-oleate, oleic acid and retinol.

The behaviour of serum albumin is consistent with it acting as a trap for the fusing agents concerned. For example, with glyceryl mono-oleate, hen erythrocytes maintained their oval shape and no fusion occurred if bovine serum albumin was added to the erythrocytes before the addition of the fusing agent. When the cells were incubated with the monoglyceride for 30s at 37°C, and the cells then added to a solution of the protein and incubated for a further 5h at 37°C, most of the cells were oval-shaped. Some spherical cells were observed, but cell fusion was absent. Thus glyceryl mono-oleate was effectively removed from the system by the presence of serum albumin. When the protein was added after the cells had been incubated with glyceryl mono-oleate for 30min, those cells that had begun to fuse proceeded to complete the process during the next several hours. If these multi-nucleated cells were subsequently washed four times with a solution of the protein all traces of glyceryl mono-oleate were apparently removed, since many of the cells then survived for a further 72h at 37°C, without lysis, in Eagle's medium containing added dextran.

Mammalian erythrocytes

Hen erythrocytes were used in early experiments on cell fusion with lysophosphatidylcholine (Howell & Lucy, 1969), because the presence of a nucleus in these cells enables the occurrence of fusion to be followed relatively easily. After we had become familiar with the sequence of morphological changes that characterize the fusion process with hen erythrocytes (see above), it was possible to study fusion with non-nucleated cells by looking for these characteristic changes as well as for the formation of giant cells. The effects of glyceryl mono-oleate on mammalian erythrocytes have been investigated. This lipid fused mammalian cells, and giant-cell formation has been observed with all the species of mammalian erythrocytes so far tested. These include man, cat,

rat, sheep and rabbit. Again fusion occurred more quickly at pH5.6 than at pH7.4. Plate 2 shows fusion between human erythrocytes, and also the fusion of human erythrocytes with hen erythrocytes.

Discussion

To enable comparisons to be made readily between the ability of different lipid molecules to fuse hen erythrocytes into multinucleated cells under defined conditions a test system has been developed that, unlike previous experiments reported from this laboratory (Howell & Lucy, 1969; Poole *et al.*, 1970; Ahkong *et al.*, 1972), does not rely on electron microscopy. The inclusion of Dextran 60C in the test medium used in the present work significantly decreased the colloid osmotic lysis that accompanies the fusion of hen erythrocytes induced by exogenous lipid agents. As a result, light-microscopy has been employed successfully, since the fusing cells were sufficiently stable to allow their progress to be followed continuously with phase-contrast optics over a period of several hours.

At present, the role of cell swelling in the process of cell fusion is not clear. Conceivably, it may simply reflect an increased membrane permeability that is necessarily associated with the changes in membrane structure that allow membrane fusion to occur. It is possible, however, that physical factors are involved that are similar to those controlling the fusion of plant protoplasts (Power *et al.*, 1970). With protoplasts, it has been found that there must be a sufficient 'depth' of cytoplasm at the point of contact in each of the fusing cells before fusion can take place (Power & Cocking, 1971). Alternatively, it also seems possible that movements of the proteins, glycoproteins and lipids of the erythrocyte membrane occur during cell swelling, and that these movements may play an integral part in membrane fusion in the system under study (Ahkong *et al.*, 1973).

With regard to the molecular specificity for successful cell fusion that we have observed in our experiments (Table 1), it is noteworthy that, in general, fusion of hen erythrocytes by saturated fatty acids occurs only when they contain between 10 and 14 carbon atoms. Saturated acids with fewer, or more, carbons atoms are inactive. It is probably significant that both medium-chain-length saturated acids, and unsaturated C_{16} and C_{18} acids are active, since Van Deenen (1969) and his colleagues have shown that these two classes of carboxylic acids behave similarly at the air–water interface and that they also have similar effects on the permeability properties of liposomes. As has been suggested elsewhere (Howell *et al.*, 1972; Ahkong *et al.*, 1973), fusion induced by these carboxylic acids may involve, at least initially, an increase in membrane fluidity. Additionally, however, it is possible that only medium-chain-length saturated acids, and unsaturated acids, and their derivatives, are sufficiently dispersable in an aqueous medium to allow them to penetrate into the erythrocyte membrane and hence induce membrane fusion.

In relation to cell fusion caused by the esters of unsaturated fatty acids, e.g. glyceryl mono-oleate, it is not yet apparent whether the ester itself is solely responsible for fusion or whether fatty acids are formed by hydrolysis. That fusion is induced by selachyl alcohol, which closely resembles glyceryl mono-oleate but contains an ether linkage, would seem nevertheless to indicate that the liberation of unsaturated fatty acids may not necessarily be involved in fusion observed with fatty acid esters. The abilities of the methyl esters of the saturated palmitic acid and stearic acid to cause cell fusion in our experimental system after a delay of about 45min provide exceptions to the general behaviour of the compounds studied, for which no satisfactory explanation can be offered at present.

It has recently been proposed that α-tocopherol may participate in specific complexes in biological membranes with some molecules of polyunsaturated phospholipids. Without the vitamin, membranes may have an abnormally high permeability and they may be subject to degradation by endogenous phospholipases *in vivo*, as well as being abnormally susceptible to damage *in vitro* by 5-hydroxybarbituric acid and hydrogen peroxide (Diplock & Lucy, 1973). However, as the concentration of α-tocopherol used to cause cell fusion greatly exceeds that normally present in the erythrocyte membrane, there is no conflict between a role for the stabilization of membranes by endogenous α-tocopherol and the present observations.

There appear to be no scientific terms available at present that may conveniently be used to describe the behaviour of agents or conditions that give rise to membrane fusion in general, including cell fusion. Without prejudice to the differing mechanisms by which membrane fusion may occur, it is suggested that the adjective 'fusogenic' and the noun 'fusogen' may be appropriately used for this purpose. Glyceryl mono-oleate and Sendai virus would thus both be referred to as fusogens. The term fusogenic may be applied not only to the behaviour of certain chemicals and viruses, but also to the conditions necessary for the induction of cell fusion by thermal (Ahkong *et al.*, 1973) or mechanical means (Diacumakos & Tatum, 1972).

An important feature of the fusion of hen erythrocytes by fusogenic lipids in the present work is the fact that the pH optimum for fusion lies between pH5 and 6. This pH-dependence contrasts markedly with the fusion of cells by Sendai virus

which occurs best between pH 7.6 and 8.0 (Croce *et al.*, 1972). Recent experiments in our laboratory indicate, however, that fusion at pH 7 by, for example glyceryl mono-oleate, is facilitated by pre-treating the erythrocytes with neuraminidase (F. C. Cramp & J. A. Lucy, unpublished work).

Whether or not one or more of the lipids listed in Table 1, which have been observed to induce erythrocyte fusion, is actually involved either directly or indirectly in membrane fusion occurring *in vivo* under normal biological or pathological circumstances remains a matter for future investigations. The ability of both lysophosphatidylcholine and unsaturated fatty acids to fuse hen erythrocytes is paralleled by small quantities of these substances being able to activate the calcium-dependent ATPase (adenosine triphosphatase) of fragmented sarcoplasmic reticulum (Fiehn & Hasselbach, 1970). This relationship is consistent with the suggestion put forward by Poste & Allison (1971), that fusion in general depends on the activity of Ca^{2+}-dependent membrane-bound enzymes with ATPase activity. Alternatively, however, it is also possible that lysophosphatidylcholine induces the phospholipids of biological membranes to form micelles (Lucy, 1970), and that the addition of exogenous unsaturated fatty acids increases membrane fluidity (Ahkong *et al.*, 1973; Seelig & Hasselbach, 1971). These changes may both alter the activity of membrane-bound enzymes and facilitate the process of membrane fusion.

Retinol is well-known to labilize lysosomes and to be active in facilitating the extracellular secretion of lysosomal enzymes (Dingle, 1969), and it was for these reasons that it was previously suggested that retinol might be expected to cause cell fusion (Lucy, 1970). It is relevant that Muto *et al.* (1972) have reported experiments which indicate that vitamin A deficiency primarily interferes with the secretion, rather than the synthesis, of retinol-binding protein by the liver, and that the deficient liver contains a pool of previously formed apo-(retinol-binding protein), which can be released rapidly into the serum as the holo-protein, when vitamin A becomes available.

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