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# MEMBRANE MOBILITY AGENTS

## IV. THE MECHANISM OF PARTICLE-CELL AND CELL-CELL FUSION

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## Summary

Fusion processes are classified according to type and size of the fusing elements:I, particle-particle, vesicle-vesicle; II, vesicle-cell, particle-cell; III, cell-cell. Fusion may be direct (original elements merge) or indirect (transfer of components from one element to another).

Membrane mobility agents like $A_2C$ (structural formula below)

$$CH_3(CH_2)_7\underset{\diagdown}{\overset{\diagup}{CH}}\!\!-\!\!\underset{\diagup}{\overset{\diagdown}{CH}}(CH_2)_7COOCH_2CH_2OCH_2CH_2OCH_3$$
$$\qquad\qquad\quad CH_2 \qquad\qquad\quad A_2C$$

form small particles on dispersion in aqueous solutions, particles which can be made fluorescent by the addition of fluorescent probes, the Flomols (fluorescent probes of mobility in membranes). Fusions of these particles with cells (particle-cell fusion) and of cells with cells induced by membrane mobility agents (cell-cell fusion) fulfill the criteria for direct fusion.

The following stages in the overall process have been identified, using $A_2C$ and Flomol F20C in the fusion of hen red cells: 1, Approach (of particle to cell); 2, sticking (of particle to cell); 3, local entry (of particle contents into cell membrane); 4, membrane spread (of particle contents from original entry point throughout the rest of the cell membrane); 5, rounding (from ovals to cups to spheres). (These steps of particle-cell interaction are succeeded by those for cell-

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cell interaction.) 6, approach (of one rounded cell to another rounded cell); 7, sticking (cell to cell); 8, fusion (of two cells); 9, sticking (of binucleated cells to additional cells); 10, fusion (to multinucleated cells).

After contact, sticking is achieved through "hydrophobic bonding". It is proposed that the critical step which follows sticking and commits the elements irreversibly is the microfusion between the outer surface layers of the elements. Microfusion (for particle-cell and cell-cell) results from the permeation of water into the isolated surface contact region after sticking, followed by a rearrangement of the molecules in that contact region into inverted micelles. The latter arrangement constitutes a region of instability which is resolved by fusion of the outer surface elements (see diagrams). The entry of membrane mobility agent into the membrane and the spread of the reagent along with the motion of membrane components follows. The consequent alterations in membrane properties lead to a spherical cell, a morphological state with the minimum area.

Two phase temperature effects on the fusion process, as shown by experiments at different temperatures and by temperature jump, imply the presence of a barrier within the membrane more complex than phospholipids, possibly involving intramembranous particles and/or cytoskeletal elements which must be moved to form a "clear region" for lipid bilayer contact.

The applicability of the overall scheme to physiological processes like neurotransmitter release and mucocyst release, as well as parallels to "Sendai virus" (HVJ, or hemagglutinating virus of Japan)-induced fusion are discussed. The contribution of other factors (Dextran, pH, $Ca^{2+}$) to the progress of fusion is also examined.

## Introduction

In the course of explorations into the biological effects of membrane mobility agents [1], we discovered that such agents were efficient promoters of cell-cell fusion [2]. Experimental cell-cell fusion is useful for the investigation of the mechanisms of fusion. One of the most widely used means for achieving cell fusion is Sendai virus [3,4] and certain other viruses [5]. The desire to avoid some of the disadvantages of virus (irreproducibility, introduction of extracellular biological material) led to searches for suitable, defined chemical agents, called "fusogenic" by Lucy and coworkers [6]. The results of such work as well as on virus- and lipid vesicle-induced cell-cell fusion have pointed to membrane fluidity and membrane component rearrangement as playing roles in the fusion process [6—14]. In view of the importance of membrane fusion in a wide variety of biological processes, we have made an extensive study of the role of membrane mobility agents in the fusion of hen erythrocytes.

This new class of reagents was designed to increase the mobility of components within cell membranes. The hydrophilic (or polar) portion of the molecules is of a length adequate to bridge the region between water and hydrocarbon region of the phospholipid bilayer present in cell membranes. The hydrophobic (nonpolar) portion of the membrane mobility agent molecule carries a cyclopropane ring for promoting disorder within the hydrocarbon region of the bilayer. Disorder of this type presumably increases the fluidity of the

domain within which the molecule is located. In the course of the work, we developed new fluorescent probes called Flomols (ref. 15 and Kosower, N.S., Kosower, E.M., Lustig, S. and Pluznik, D.N., unpublished) and utilized them to illuminate various stages of fusion processes. In our context, fusion represents the merging of two (or more than two) elements, a term which refers to particles (e.g., microdroplets), vesicles or cells. On the basis of the experiments we have carried out, the fusion process can be divided into four stages: (1) Approach, (2) sticking, (3) component mobility and (4) morphological change. Using our results and information from the literature, we try in this paper to present a molecular level interpretation of some of the stages in the fusion process.

## Materials and Methods

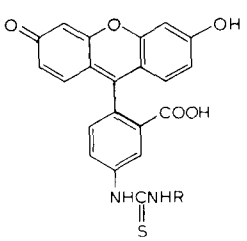
*Cells.* Lehorn hens were used as source of blood, which was obtained from a brachial vein and prevented from coagulating by heparin.

*Mobility agent.* The ester $A_2C$ (synthetic) is an oil which can readily be dispersed in water and the usual buffers. (The substance is now available from Makor Ltd., P.O.B. 6570, Jerusalem, Israel)

$$\underset{A_2C}{CH_3(CH_2)_7\overset{CH_2}{\diagup\!\diagdown}CH(CH_2)_7COOCH_2CH_2OCH_2CH_2OCH_3}$$

$A_2C$ is measured into a tube with a Hamilton 10 $\mu$l syringe, the appropriate amount of 0.15 M NaCl solution added to give a concentration of 0.1—0.8 $\mu$l $A_2C$/ml and the mixture sonicated for 15—30 s. The dispersions consist of particles between 0.4 $\mu$m and 1.6 $\mu$m in size.

*Flomol-mobility agent combination.* During the investigation of the interaction of the membrane mobility agent with the red blood cell, it became apparent that it would be useful to have a fluorescent label for the particles of the agent in the dispersions. We designed and synthesized probes called Flomols (fluorescent probes of mobility in membranes). Details of the preparation will be given elsewhere (Kosower et al., in preparation for publication). In the present work, we have used the Flomol F20C:




$$R = CH_3(CH_2)_7\overset{CH_2}{\diagup\!\diagdown}CH(CH_2)_9-$$

Flomol F20C ($2 \cdot 10^{-3}$ $\mu$mol/$\mu$mol of $A_2C$) in methanol was mixed with $A_2C$ measured as stated above. The methanol was removed with a stream of $N_2$, NaCl (0.15 M) added and the mixture sonicated to yield fluorescent $A_2C$ particles.

*Buffers.* The usual buffer (Buffer A) contained NaCl (70 mM), sodium acetate (75 mM), $CaCl_2$ (between 0.2 and 2.0 mM) and Dextran (T-70, Pharmacia)

(100 mg/ml), pH 5.7. Buffer A with Dextran omitted; buffer A with $CaCl_2$ omitted; buffer A with EDTA added. Other buffers (higher pH, see Table I); sodium cacodylate, $(CH_3)_2As(O)ONa$; Tris $[(HOCH_2)_3CNH_2]$; glycylglycine. Since, in all cases, equal volumes of buffer containing red blood cells and mobility agent suspension were mixed to initiate an experiment, the final buffer concentrations in the incubation mixtures were half of those cited here.

*Other reagents* will be noted when necessary in the text.

*Preparation and utilization of cell suspension*

*Incubation.* Hen blood drawn as noted above was centrifuged, the buffy coat removed, the red cells washed twice with NaCl (0.15 M), and then once with buffer A. Cells were resuspended in buffer A to a hematocrit of 6—8%, and the incubation mixture prepared by mixing of the cell suspension with an equal volume of $A_2C$ suspension or $A_2C$/Flomol F20C suspension. The mixture was held at the desired temperature for the duration of the experiment, unless otherwise noted.

*Observation, photography and counting.* Aliquots were removed from the incubation mixture at intervals for viewing and photography, using a Leitz Orthoplan microscope equipped with an incident light fluorescence unit, Ploempak 2.1, a constant temperature stage and an Orthomat-W camera. 3—6 fields were photographed for each aliquot examined. Changes in cell shape and in the degree of fusion were then determined from the pictures. The former was quantitated by counting the number of cells in the process of rounding; quantitation of the latter was accomplished by counting the number of nuclei in fused cells and dividing by the number of nuclei in the field photographed. It was also necessary to note the stage of fusion, e.g., whether or not the majority of fused cells had either two or three nuclei, or many nuclei (multinucleated cells).

## Results

*Overall characteristics of fusion*

On mixing a hen red cell suspension with a suspension of $A_2C$ particles, no immediate change in the oval shaped hen cells is noted at any temperature between 19 and 42°C. At the higher temperatures in this range, particles become attached to cells fairly soon (2—5 min), a process that is easily seen with Flomol-loaded $A_2C$ in a stage we call "sticking". At this point, no fluorescence can be seen in the cell membrane. Later (10 min or more, depending on the temperature) fluorescence is seen in the cell membrane (Fig. 1).

The entry and spread of the $A_2C$ particle content into the membrane is associated with pronounced morphological changes. The earliest stage, cup formation, has been observed as the result of $A_2C$-human red cells interaction in buffer A (Fig. 2). An apparently similar, but less well defined, shape change is observed for hen red cells. The hen red cells then become rounded, but only in the case of those cells to which $A_2C$ particles have become attached (Fig. 1). In the usual experiment carried out at 37°C, some cells have changed their shape within 20—30 min and most of the cells have become spherical within 45—60

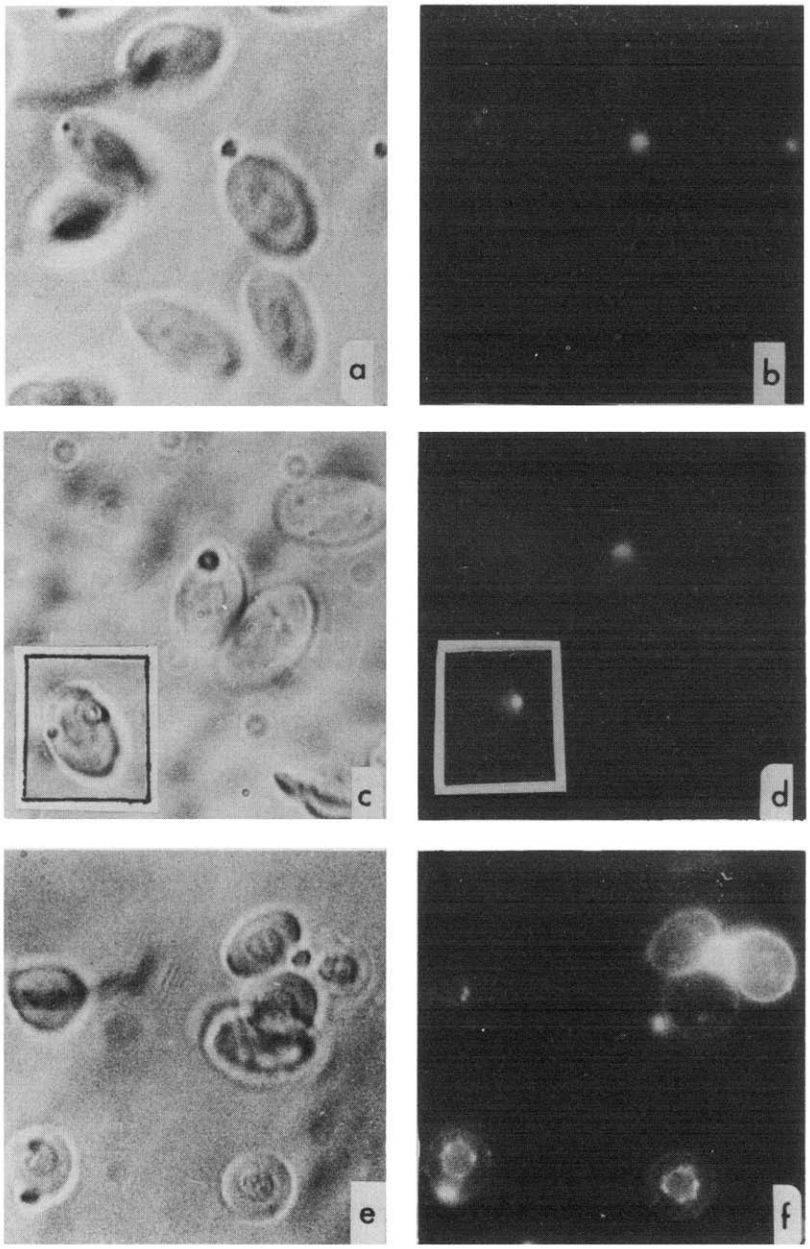



Fig. 1. Interaction of $A_2C$ particles with hen red cells. Hen red cell suspension was mixed with an $A_2C$/F20C suspension (final concentrations: red cells, $5 \cdot 10^8$/ml; $A_2C$, $5.8 \cdot 10^{-4}$ M; F20C, $1.2 \cdot 10^{-6}$ M) and aliquots were incubated at various temperatures. Samples were removed at timed intervals for viewing and photography. Fading during photography of samples made partially fluorescent cells (see d) technically difficult to record. Photographs illustrating the results are given as follows, using the same field photographed first with the exciting light for fluorescence and then with ordinary light. a and b: A group of cells incubated briefly at 37°C (5 min) after preparation of the final suspension. In a, the particles of $A_2C$-Flomol may be seen resting on the cells. In b, only the fluorescent particles are seen. c and d: Two examples of particle-bearing cells in which partial spread of fluorescence is seen. Incubation, 120 min at 19°C. e and f: Cells carrying particles in which the fluorescence has spread through the whole membrane. The cells are rounded and some are sticking to one another. The group at the upper right is a particularly vivid example. An unusual phenomenon which shall be taken up further elsewhere is the presence of fluorescent regions on what appears to be the nuclear membrane. Incubation, 25 min at 37°C.

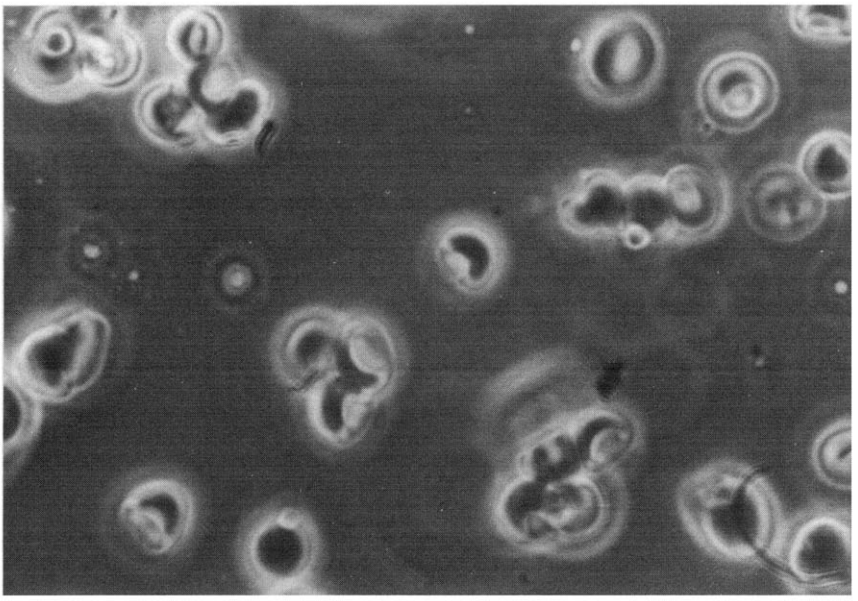


Fig. 2. Cup formation in $A_2C$-treated human red cells. Human red cells were washed and suspended in buffer A, then treated with $A_2C$-dispersion in the same way as the hen red cells. (See Fig. 1 and text). Samples were taken at various times and examined under the microscope. Obvious cups are illustrated in the photograph shown. These formed between 20 and 40 min after the beginning of incubation. Rounding and fusion followed between 60 and 120 min after incubation at 37°C.

min. Rounded cells (2 or occasionally more) then approach and stick to one another. With Flomol F20C present in both cell membranes, a thick fluorescent line can be seen dividing the cells which are stuck to one another (Fig. 3). After some time (approx. 5 min), the membrane which separates the cytoplasms of the participating cells disappears, and a single binucleated cell is produced. The fusion process can then occur between multiple nucleated cells to yield cells with large numbers of nuclei. The stage of huge cells with many nuclei is succeeded by lysis, about 30 min later.

*Factors affecting the fusion process*

Certain conditions have been found to favor the overall process of cell-cell fusion. We have investigated cell and $A_2C$ concentration, buffer composition, calcium and Dextran content, pH and temperature variation with respect to both the rate and extent of cell-cell fusion.

*A. Cell and $A_2C$ concentration.* Efficient and observable fusion can be carried out with $3–5 \cdot 10^5$ cells/ml. If the cell concentration is much lower than $3 \cdot 10^8$ cells/ml, the cells round and lyse without fusion.

The membrane mobility agent, $A_2C$, was normally used at a final concentration of 0.25 μl/ml. Concentrations of $A_2C$ as low as 0.05 μl/ml could induce fusion, but the rate was low and the extent of fusion limited (10% in 2–3 h). Final concentrations of $A_2C$ as high as 0.4 μl/ml could be used, with no advantage apparent over the use of 0.25 μl/ml, (70–90% fusion within 45–60 min).

Dispersions of $A_2C$ vary in stability with the concentration of $A_2C$, with the

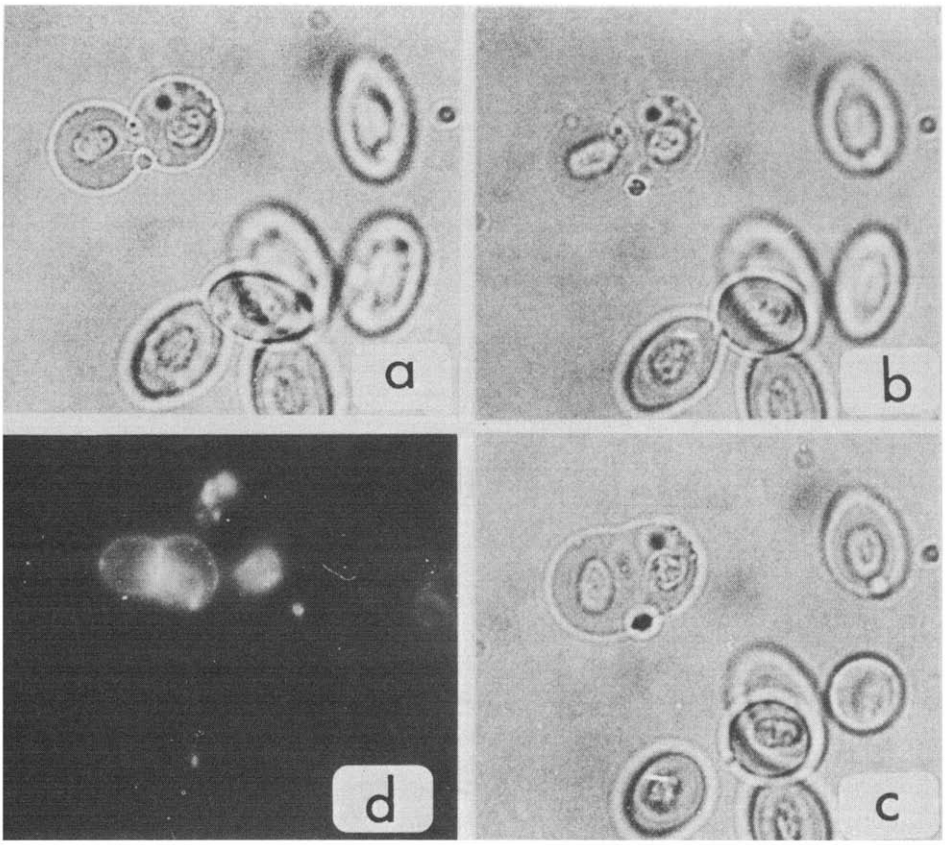



Fig. 3. Cell-cell interaction of $A_2C$-treated cells. Conditions are described in the caption to Fig. 1 and in the text. An aliquot of an incubation mixture, incubated at 27°C, was photographed sequentially over a period of some minutes. a, Two rounded cells are seen in the upper left portion of the photograph immediately after sticking; b, the two rounded cells are much more in contact. The membranes are barely visible, and it is not possible to decide whether they are full or partial; c, the two rounded cells now have fused to one cell, but have not fully rounded to the final shape of a binucleated cell; d, two rounded cells in intimate contact, before the fusion membrane has disappeared. The cells are labeled with F20C and the fluorescence makes the fusion membrane easily visible. These cells, from a separate sample, were chosen to illustrate a stage near that shown in b. The brightness of the fusion line suggests that both of the original membranes are present in the "fusion membrane". In a—c, several examples of cells progressing from oval shape to rounded shape under the influence of the membrane mobility agent, $A_2C$ may be seen. Magnification, a—c, ×1000; d, ×630.

viscosity of the solution, with the nature of the dispersing method, with the temperature, and according to whether or not a stabilizing agent (like phospholipid) is present.

*B. Dextran.* In the absence of Dextran, $A_2C$ did not lead to cell fusion. Examination of an incubation mixture containing red cells, and the $A_2C$/F20C combination in buffer A without Dextran, revealed that many of the $A_2C$ particles were to be seen in a plane above the plane within which the red cells were located. The few cells which respond in the absence of Dextran, exhibit particle sticking, rounding and then lysis.

*C. Effect of pH and buffer variants.* pH 5.7 was found to promote both

TABLE I

EFFECT OF pH AND OF BUFFER ON HEN RED CELL FUSION BY $A_2C$ *

| pH | Buffer | Rounding ** | Fusion ** |
|---|---|---|---|
| 5.7 | Sodium acetate | +++ | +++ |
| 6.7 | Sodium cacodylate | + | + |
| 7.5 | Glycylglycine | + | ± |
| 7.5 | Tris | + | ± |
| 8.1 | Sodium cacodylate | ± | 0 |
| 8.5 | Glycylglycine | ± | 0 |
| 8.5 | Tris | ± | 0 |

* Cell/$A_2C$ mixtures incubated at 37°C, and followed for 120 min.

** Rounding and fusion evaluated on a scale of +++ to 0; +++ means >50% of cells involved in the process of rounding and subsequent fusion to multinucleated cells; + means 10—25% of cells involved; ± means 5% or less.

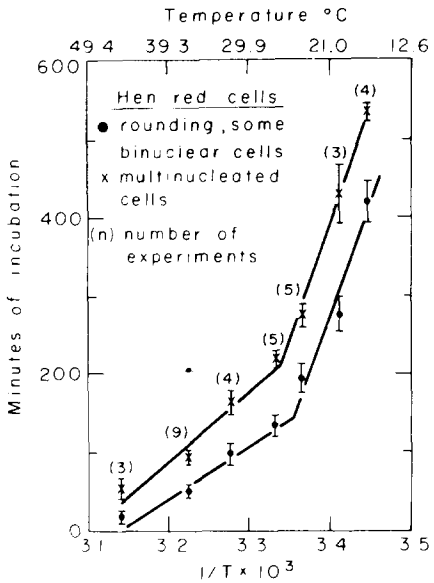
rounding and fusion. Raising the pH to 6.7 markedly diminished the amount of rounding and fusion within a standard observation time. At pH 7.5, some rounding could be seen but little or no fusion, and above pH 8.0, there was little rounding to be seen in an experimental sample and no fusion in any of the buffers used. The results are summarised in Table I.

*D. Effect of calcium.* No significant difference in the rate or extent of fusion is observed if calcium concentration is changed from 1.0 mM to 0.4 mM. Lower calcium concentrations (e.g., 0.12 mM) lead to diminished fusion rates, but the decrease is not as great as would be expected for the decrease in concentration. If EDTA (1 mM) is added to a calcium-poor medium (0.05 mM $Ca^{2+}$, buffer A) little or no fusion is observed.

*E. Effects of albumin and serum.* Albumin (utilized as a solution of 50 mg/ml in 0.15 M NaCl and added to give a final concentration of 5 mg/ml) markedly inhibits all stages of cell fusion. If added to the incubation mixture at the time incubation is begun, neither rounding nor fusion is seen. If added at the time at which many cells are rounded, the cells are arrested at this point and few continue on to give fusion. If albumin is added after the time at which bi- and trinucleated cells can be observed, a significantly lower rate of further fusion is seen.

Total plasma protein (serum) inhibits fusion when added to the $A_2C$-red cell suspension at the beginning of incubation. Unwashed cells, i.e., whole blood, did not give rise to fused cells when treated with $A_2C$ in the usual way.

*F. Effect of temperature.* Raising the temperature increased the overall rate of fusion and especially enhances the first part, up to rounding. In addition, the rate of rounding and fusion increases rapidly between 17 and 25°C; above 25°C, the rate of fusion increases with temperature less rapidly. For example, to reach the criterion of rounding (40—60%) and some fusion (<10%) to binucleated cells at 17°C requires about 7 h. At 23°C, the time to arrive at a similar degree of rounding and fusion is reduced to a little more than 3 h. At 31°C, 1.66 h is needed. The rates for reaching the stage of multinucleated cells are parallel to those cited for rounding and some binuclear cell formation. The dramatic effect of temperature on the rate of fusion and the break in the temperature coefficient for the process are illustrated in Fig. 4.



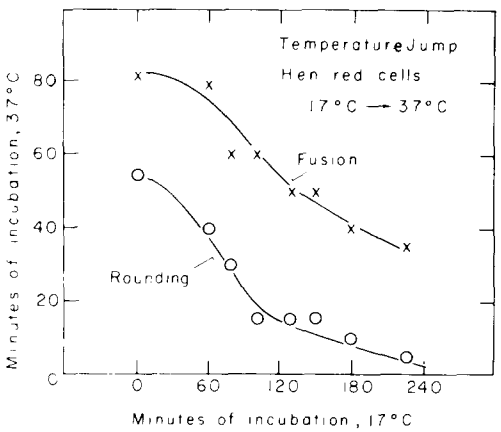




Fig. 4. The effect of temperature on the rounding and fusion of hen red cells by $A_2C$. The red cell suspension, washed and resuspended in buffer A, was brought to the desired temperature, mixed with $A_2C$ dispersion of the same temperature and incubation continued. (Final concentration, $3—5 \cdot 10^8$ cells/ml; $A_2C$, 0.25 µl/ml). The vertical bars represent the magnitude of the standard error of the mean, and the figures in parentheses show the number of different experiments which were carried out for each temperature. ●, Rounding: 40—60% of cells; ×, fusion of 60—80% of cells to multinucleated cells. Two temperature ranges are clearly evident. In the low temperature range, the rate of fusion increases rapidly with temperature. In the high temperature range, the rate of fusion increases but less rapidly than in the low temperature range. "Activation energies" can be derived from these numbers, and are noted in the text.

Fig. 5. Temperature jump for $A_2C$-treated hen red cells. A red cell-$A_2C$ suspension was incubated at 17°C in numerous separate tubes, which were individually transferred to 37°C at different times for further incubation. Samples were taken for observation and the times at which rounding (40—60% of cells) or fusion (60—80% of cells) could be seen were recorded. In the experiment illustrated in the figure, very little rounding was noted after 225 min at 17°C, although jumping to 37°C at that time led to rounding within 5 min. Samples at 17°C showed rounding at 400 min and fusion at 550 min.

By taking the time needed to reach the experimental criterion as a measure of rate, an "activation energy" can be estimated for each temperature range. For the low temperature range, the "activation energy" is between 19 and 26 kcal/mol; for the high temperature range, the "activation energy" is about 10 kcal/mol.

No fusion could be observed at 2°C over 8 h. In fact, various stages of the fusion process can be arrested by cooling to 2°C, and such cooling is a convenient way of retaining samples in a desired stage for further examination.

*G. Effect of temperature jump.* Incubations of $A_2C$-red cell suspensions below 20°C produce little change in the appearance of the cells for 3—4 h. In comparison, comparable samples incubated at 37°C show rounding and fusion in about 1 h. If a sample which had been maintained at 17°C for 3—4 h is transferred to 37°C, rounding begins within 5—10 min; fusion follows at a time somewhat less than usual, within about 40 min. A series of experiments, in which the incubation at 17°C is maintained for various lengths of time before "temperature jump", is illustrated in Fig. 5.

## Discussion

### *Classification of fusion processes*

A variety of fusion processes have been described. In order to provide a basis for understanding this variety, we adopt a criterion for classification based on the type and size of the fusing element. "Small" elements are those elements of approx. 1 $\mu$m or less in diameter, thus excluding cells from this category. "Large" elements are those elements with diameters greater than 1 $\mu$m with cells as the primary example in this category. Three primary groups of fusion processes can thus be recognized: I. Small element-small element; II, small element-large element; III, large element-large element. "Small" elements include vesicles (bilayer structures containing an isolated segment of the solvent in which they were prepared) and particles (according to the dictionary definition, "a body whose spatial extent and internal motion and structure, if any,

TABLE II

CLASSES OF FUSION

| Class | Fusing elements | Examples |
|---|---|---|
| I | Particle-particle | Coalescence of water dispersion of oil [46] |
| | Vesicle-vesicle | Phospholipid vesicle fusion [a]<br>Secretory vesicle fusion [47] |
| IIA | Vesicle-cell [48] | Neurotransmitter release [49] |
| | | Mucocyst and trichocyst release [37] |
| (Exocytosis) | | Neurohypophysis secretion [45] |
| | | Pancreatic zymogen granule release [50] |
| | | Insulin release [51,52] |
| | | Acrosomal granule release [53] |
| IIB | Vesicle-cell | Vesicle-cell fusion [b] |
| | Particle-cell | Membrane mobility agent-cell [c] |
| | | Chylomicron remnant-hepatocyte [54] |
| III | Cell-cell | A. Natural |
| | | 1. Physiological |
| | | Myotube formation [55] |
| | | (myoblast-muscle cell) |
| | | 2. Pathological |
| | | Multinucleated giant cells [56,57] |
| | | Tumor cell fusion [4,58] |
| | | B. Promoted (by) |
| | | Sendai (HVJ) virus [8] |
| | | Membrane mobility agents [2] |
| | | Fusogenic lipids [34] |
| | | Polyethylene glycol [59–61] |
| | | Glycerol [10] |
| | | Dimethyl sulfoxide [10] |
| | | Pederine [62,63] |
| | | Heat [34] |

[a] See text.
[b] For criteria, see text.
[c] Present work.

are irrelevant in a specific problem"). The classes of fusion processes along with examples are listed in Table II.

At least two general fusion mechanisms can be recognized, direct and indirect. The direct mechanism includes all cases in which the two original elements merge to yield the fused product after direct contact and in which an appropriate internal label is not lost during the merging. The indirect mechanism includes the cases in which one element unfolds or from which components dissociate before combining with a second element, so that some or all of an internal label is lost during the fusion process.

The fusion of membrane mobility agent particles with the hen red cell (particle-cell) fulfills the criteria for direct fusion, as attested by the merging of the $A_2C$ particle, together with its fluorescent label, into the cell membrane after direct contact. The subsequent cell-cell fusion is also direct, occurring after contact between the cells, and with the conserved nuclei serving as internal markers. Vesicle-cell fusion also appears to be direct, with appropriate internal labels (large molecules) being retained [16—18]. Membrane leakiness (revealed by small molecule internal labels) is apparently a concomitant of vesicle-cell fusion [16—19] and cell-cell fusion promoted by Sendai virus [8,20,21].

On the other hand, for vesicle-vesicle fusion, indirect fusion appears to be common as shown by the transfer of spin-labeled phospholipids [22] and monomeric units [23] from one element to a second, thereby causing the growth of the latter at the expense of the former. The facilitation of vesicle-vesicle fusion near the transition temperature by impurities present in the phospholipids [24] may arise from the fact that monomeric units may "evaporate" easily from defect sites. (Increased permeability of vesicles at the transition temperature has been attributed to a similar cause [25].) That vesicles resist direct fusion is underlined by the reported failure of the attempt to observe reaction of potential reactants incorporated into separate liposomes [26].

*Stages in fusion*

Fig. 6 presents a summary of the stages of fusion along with an indication of the most important factors and features of the overall process. The scheme applies to both particle-cell and cell-cell fusion, which, as we shall see, go through rather similar stages. The scheme may well be valid for vesicle-cell fusions. Particularly pertinent points will be summarized in the succeeding subsections.

*Approach, contact and sticking.* Aside from obvious problems, such as ensuring that the elements destined for fusion occupy the same domain in the experimental system (raising the viscosity lowers the rate at which the lighter particles may escape from the domain of the red blood cells), we can ask why particles which encounter cells often stick to them. The observation that Flomol-loaded mobility agent particles do not initially insert any fluorescent probes into the membrane of the cell shows that sticking of the particles to the cells is an effect of simple contact. The most reasonable explanation is conveniently labeled as "hydrophobic bonding". The degree of organization of water at the surface of the particle and of the cell is diminished through contact of the two surfaces. Similar contact between two cells is apparently possible only

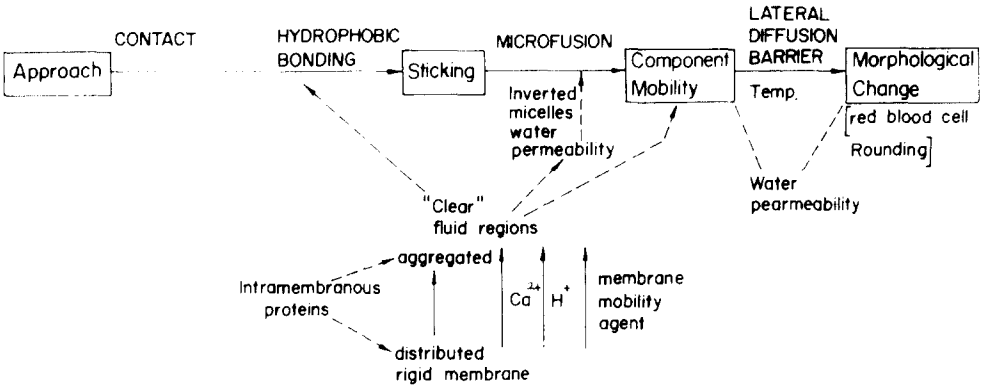



Fig. 6. A representation of the stages in fusion of elements, including particle-cell, vesicle-cell and cell-cell. The elements approach one another and make contact. Contact implies apposition of the phospholipid bilayers and thus requires in both elements "clear" fluid regions of sufficient size so that "hydrophobic bonding" can drive them together and make the combination stick together (see Fig. 7). The formation of "clear" regions may be promoted by various means including protons (which may lead to an aggregation of membrane proteins, those called intramembranous particles), membrane mobility agents, calcium and certain viruses. The apposed phospholipids (mobility agent-phospholipid apposition in the case of particle-cell fusions) give rise to inverted micelles within the inner region of the apposition (see Figs. 8 and 9). The ultimate result of the local changes at the apposition is microfusion. The components of the membrane are then free to move, although there is a barrier to lateral diffusion which is overcome by raising the temperature. The final stage of the fusion process is an accommodation of the combination of both elements to the new composition of the fused species. Given the physical properties which favor fusion, like fluidity, the fused species assumes a rounded form.

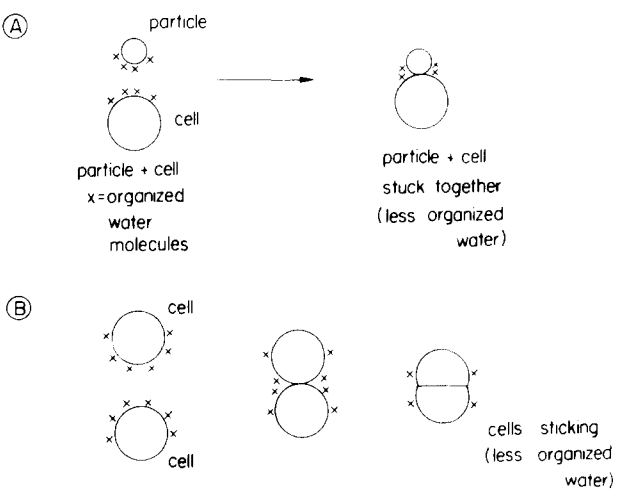



Fig. 7. A representation of the mechanism by which (A) particles and cells (B) cells and cells stick together. Organized water molecules are shown on the approaching portions of the elements which will make contact. The number of these organized molecules will be reduced after contact has been established. This argument is very similar to that used in describing "hydrophobic bonding" between two nonpolar fragments or species in water solution.

after the morphological change to spherical cells ("rounded forms") takes place following insertion of membrane mobility agent into cell membrane, (see below). A schematic illustration of particle-cell sticking and cell-cell sticking is shown in Fig. 7. Direct evidence for this interpretation may be found in the sticking of mitochondria to hydrophobic surfaces, a process which is easily reversed on lowering the temperature to 0°C, as expected for hydrophobic bonding [27].

*Microfusion.* The central step in the overall process of cell-particle or cell-cell fusion is that which fuses the outer layers together. Lucy [6] recognized the problem and formulated a detailed scheme in which normal phospholipid micelles were generated in an unspecified way by a "perturbing agent" as critical intermediates within the bilayer. The micelles in different bilayers could then interdigitate to give membrane fusion. Neither the mechanism of formation of such normal micelles nor how they might be responsible for fusion is clear.

Our mechanism for the critical step is based on a careful consideration of what happens on a molecular level when a particle and a bilayer are brought together. A contact region is illustrated in Fig. 8. We can regard the contact region as a concentrated solution of head group ions. (The energy for the concentration process was provided by the bulk of the solution through "hydrophobic bonding"). Bilayers are permeable to water. The half-life for exchange for the water within a red blood cell and that outside is 15 ms [28]. It thus appears inevitable that water will migrate from the cytoplasm of the cell into the contact region (Fig. 8B). The arrangement of molecules in the contact region and the presence of extra water can either (a) lead to the separation of the particle and the cell (contact region too small) or (b) cause the molecules in the excess water region to form inverted micelles (for review, see Fendler, ref. 29). In the latter case, a region of instability is created, in which the normally stable bilayer arrangements are disrupted. Resolution of the instability (Fig. 8C) would lead in the case of the particle-cell combination to the insertion of molecules from the particle along with phospholipid into the inner half of the bilayer (Fig. 8D). On the basis of the ideas of Sheetz and Singer [30,31], we would expect this mechanism to give rise to cup-shaped cells in the early stages before rounding obscured this shape. We have in fact obtained clearly defined cups soon after the addition of membrane mobility agent $A_2C$ to human erythrocytes.

To distinguish the joining of the particle of the cell from the overall particle-cell fusion process, we have called this step microfusion. We believe that the microfusion step during cell-cell fusion follows a course similar to that of particle-cell fusion. The region of instability probably takes longer to form or resolve because the phospholipid can transfer water in both directions. Thus, cell-cell fusion includes a stage in which a large area of contact is developed between the two fusing cells before the actual fusion takes place. Disappearance of the "fusion line" follows resolution of the instability which predisposes the remaining double bilayer to further re-arrangement, formation of water channels and, finally, separation of the "fusion line" as vesicles within the binary cell. An illustration of these ideas is given in Fig. 9.

*Component mobility.* That membrane mobility agent motion and membrane

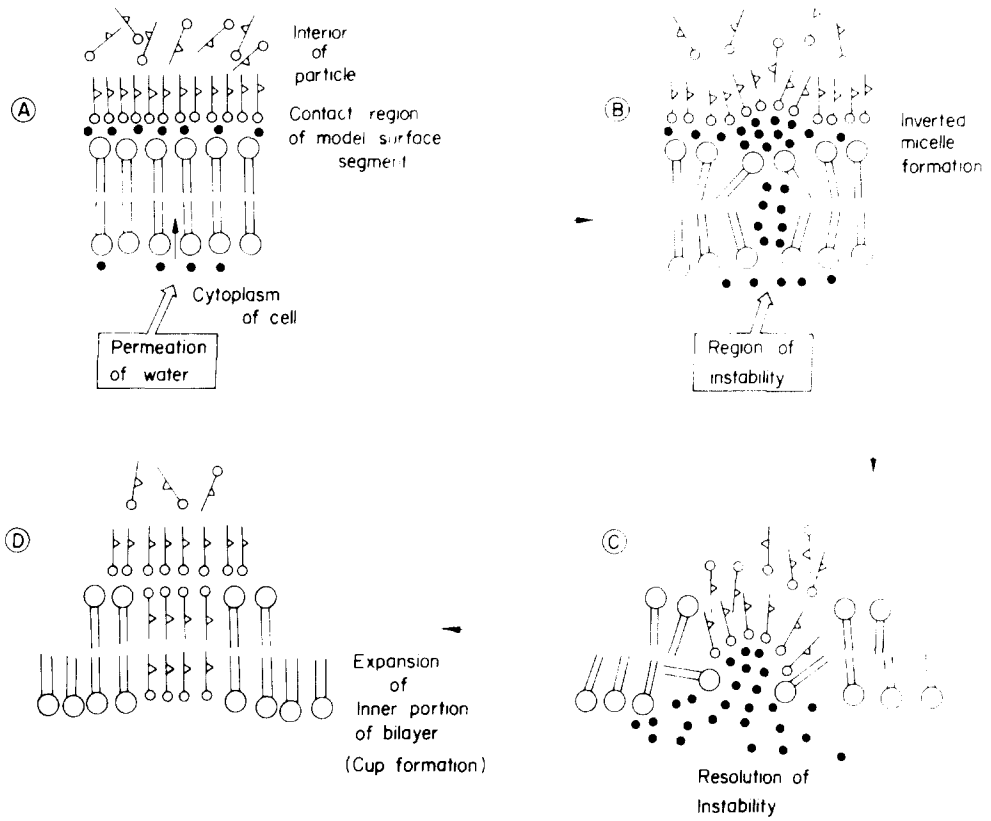



Fig. 8. A detailed representation of molecular dispositions in the model surface region during progress of fusion of a particle with a cell. In A, the contact region between the particle and the cell bilayer is shown in part. Water molecules permeate the bilayer, entering the contact region to produce the structure illustrated in B, an inverted micelle. An inverted micelle forms when the polar solvent is surrounded by polar (hydrophilic) portions of the amphiphilic molecules of the contact region. The formation of an inverted micelle disturbs the stability of the bilayer (and perhaps the surface structure of the particle) and leads to a region of instability. The instability can be resolved either through return to the original arrangement or by a rearrangement as indicated in C, in which molecules of the particle, being more mobile and greater in number than those present in the original bilayer, are forced into the bilayer region. It appears from the drawings (see D) that the inner portion of the bilayer will be expanded preferentially, at least in the initial stages. From the proposals of Sheetz and Singer [30], a development along these lines should produce a cup-shaped cell. In the case of human red cells, cup formation has been observed under these circumstances.

component mobility, a necessary step in the fusion process as depicted in Fig. 6, is based on our studies of the agents and on published results for virus-cell fusion.

We have previously shown that cap formation is promoted by $A_2C$, which thus enhances membrane component mobility [1,32]. In the present work, we have found that the contents of an $A_2C$ particle eventually occupy the whole cell membrane after entry at a local site. In addition, we have found a two phase temperature dependence for the fusion process. The "activation energy" estimated for the high temperature range (10 kcal/mol) is what might have been expected for a process involving diffusion through a lipid bilayer or the lipid portion of a membrane. The "activation energy" derived for the low tem-

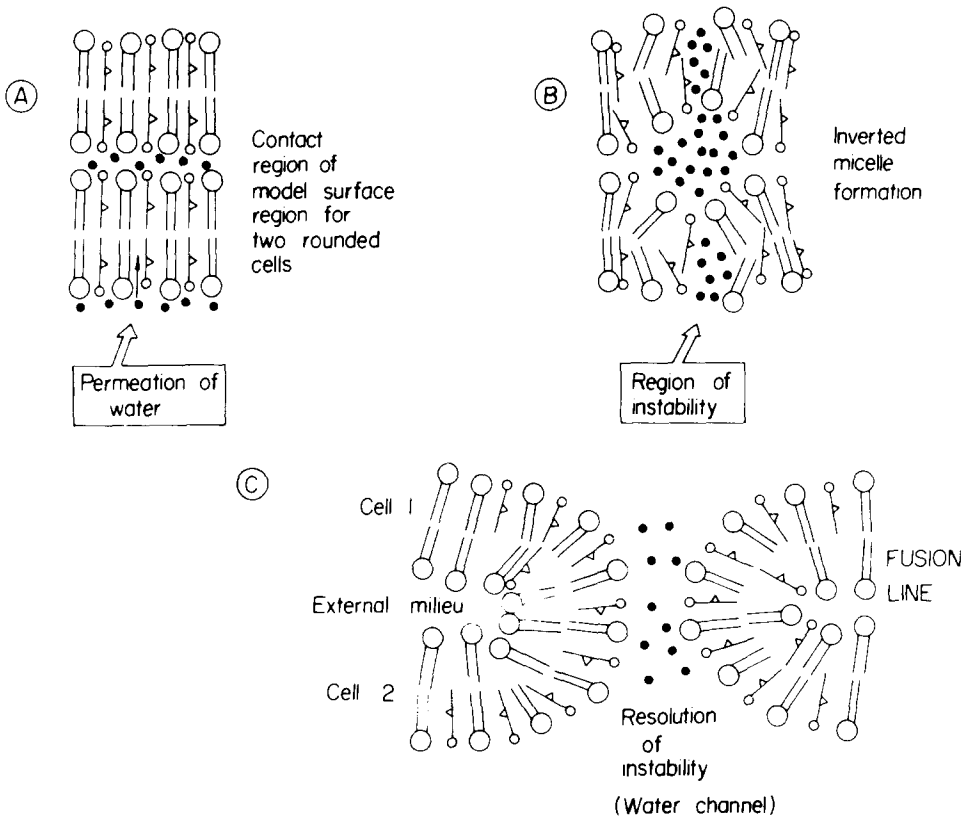



Fig. 9. A detailed representation of the molecular dispositions of the phospholipid bilayers during fusion of two cells. In A, a model surface region for the contact between the two cells is shown. Permeation of water can now occur from the cytoplasms of both cells, but inverted micelle formation should be less frequent than in the case of particle-cell fusion, (see Fig. 8) because (i) water can easily move in either direction and (ii) rearrangement into an inverted micelle involves phospholipid molecules on both sides (these molecules are bigger, thus bound more effectively in the original bilayer than membrane mobility agent molecules). Thus, maximum contact regions can be developed, (see Fig. 7) with direct evidence from Flomol-loaded cells that the "fusion line" between cells is persistent. (See Fig. 2d.) If an inverted micelle does develop (B) a region of instability is created which can be resolved through the formation of an aqueous channel connecting the two cells. Our model suggests that little or no protein need be present in the fusion line, but evidence on this point is still lacking.

perature range (19—26 kcal/mol) suggests that a barrier to lateral mobility (of agents and membrane components) other than the viscosity of the lipid bilayer, is present. A barrier of this type has been proposed by Webb [33]. The barrier may consist of a complex of intramembranous particles and phospholipid and may or may not include cytoskeletal components. That such a barrier plays a role in other fusion processes is implied by the findings of heat-promoted fusion [34], of two phase temperature dependence for the transfer of spin-labeled phospholipid from Sendai virus into human red blood cells [35] and for virus-promoted cell fusion [7].

Component mobility could clear proteins from an area of membrane sufficiently large to produce effective bilayer-bilayer contact, a process which would be facilited by an increase in temperature, by $Ca^{2+}$ and by lowering the pH.

The temperature jump experiments complemented nicely the information we had obtained in other ways about the stages of fusion. Although an $A_2C$-red cell suspension shows no obvious changes when incubated at 17°C for a long time, changing the temperature abruptly to a higher temperature (37°C) reveals that the cells had been "prepared" in some way, but could not proceed through the stages of component mobility and morphological change.

The temperature jump experiments have parallels in the interaction of HVJ virus with cell membranes. Okada and his coworkers [7,8] have shown that the HVJ virus becomes attached (i.e., "stuck") to the cell membrane at 0°C at a particular site. Warming the virus-cell combination to 37°C leads to very rapid fusion (just as in the case of the $A_2C$-activated chicken erythrocytes after incubation at 17°C) and the virus proteins are dispersed within the cell membrane. A reversible alteration in the ionic permeability of the cell membrane occurs at about this time, the membrane becoming much more permeable to ions. In other words, the virus acts at a local site at low temperature, and then promotes changes like those seen in $A_2C$-membrane spreading at higher temperature. The behavior of HVJ virus in promoting cell fusion may thus follow the scheme of Fig. 6.

*Morphological change.* Treatment of hen erythrocytes with suspensions of membrane mobility agents leads to a change from the native oval shape to a rounded form. The latter must represent a spherical cell, with a volume of approximately 240—260 $\mu m^3$, the volume of the native cell being 127 $\mu m^3$. The increase in membrane volume as judged from the membrane area of the spherical cell in comparison to the area of the oval cell, is probably a small fraction (3—8%) of the original membrane volume. The change in red cell shape and volume would therefore be due to increased membrane flexibility and permeability, and reflects the effect of $A_2C$ on the structural components of the membrane. As noted above, the large area of contact which precedes cell-cell fusion is probably promoted by "clearing" of membrane proteins. Large areas of "clear" membrane are apparent in mucocyst release (see discussion below) and in mast cell release [36].

*Other fusions.* There are a number of other fusion processes of particular biological importance which may be understood in terms of the mechanism proposed in this paper. These include the fusions which lead to neurotransmitter release and mucus release, both of which are type IIA fusions (exocytosis). Satir [37] cites a number of other cases which are presumably similar. We might also cite virus-cell fusion, a type IIB fusion. The general features of these fusions may be summarized as follows: 1. Approach of vesicle to specific site on cell membrane (for neurotransmitter, the synaptic vesicle binds at a vesicle release site [38]; for mucus, the mucocyst binds at a fusion rosette; for virus fusion, the virus particle binds at an attachment site. 2. Sticking of vesicle or virus particle to attachment site. 3. Microfusion (water permeation and accumulation, inverted micelle formation). 4. Component mobility (accepted for all three cases and demonstrated for protein components of virus coat [7,9]). 5. Morphological change ("crystalline" state of internal vesicle contents is transformed by water into a solution with a considerable expansion in volume, resulting in explosive discharge).

The main question which might arise in connection with the central role

assigned to water entry through a bilayer membrane, is whether or not the rates of such entry are compatible with the overall timing for the various fusion processes. The answer is yes, provided (a) entry of a relatively small fraction of the volume of the vesicle is sufficient to commit the vesicle irreversibly and (b) the rate is somewhat non-linear, in that initial entry of water into the intravesicle space expands the target bilayer in the region of instability (see Figs. 8 and 9) and makes further entry of water easier and faster.

Several different methods have been used to measure the exchange of water between the interior of red blood cells and the external milieu. Tritium exchange and a rapid flow method [39,40] lead to a half-life of 7.6 ms at 25°C. Several different magnetic resonance techniques ($T_2(^1H)$, $T_1(^{17}O)$ and $T_1(^1H)$) at temperatures between 25 and 37°C yield half-lives from 11.5 to 15 ms at the lower temperature and 5.7—6.4 ms at the higher temperature [28,41,42]. No clear decision between "pores" and single molecule diffusion has yet been made for the diffusion of water through lipid membranes [43], but the latter seems to us more likely. Thus, through the 40% of the red blood cell surface, which is covered by bilayer, one may calculate a rate of water entry of 380 $Å^3/Å^2$/ms. The relevant contact area for entry of water into the vesicle-cell contact region is difficult to determine, although the size of the fusion rosette (600 Å diameter, or 500 Å clear area [37]), and the vesicle release site for acetyl choline-containing synaptic vesicles (25 Å—75 Å radius or more? for contact zone) [44] gives us a fair idea. Rosette-like particle arrangements have been found at release sites in the neurohypophysis. The overall diameter was approx. 400 Å [45]. The volume of the synaptic vesicle is about $10^{-6}$ of a red blood cell but the contact area may be as much as $10^{-5}$ of the bilayer area of the red blood cell membrane. Thus, the half-time for exchange could be as little as 1.5 ms. If only 10% of the water entry occurs at the red blood cell rate (and subsequently becomes faster) and we realize that the highly unsaturated character of the acyl groups in synaptic membranes makes those membranes more fluid and more permeable to water, we can understand a "time to extrusion of contents" of 0.2—0.3 ms. We may conclude that water permeation is fast enough to serve as trigger for the fusion of vesicles to cell membranes. Additional consequences, like the dissolution of the vesicle contents in the entering water (acetylcholine and adenosine triphosphate in the synaptic vesicle; mucus in the mucocyst) and explosive release of contents would presumably also readily fit this time scale.

*Further comments on Table II*

The separation of oil-in-water dispersions corresponds to a particle-particle fusion process [46]. The demonstration of a direct relationship between the electrical events at the neuromuscular end plate and what is seen in the morphology of the synapse through the electron microscope has proven very difficult to achieve, but there are strong indications that exocytosis occurs in localized zones (ref. 49 and Heuser, J.E. and Reese, T.S., personal communication). No specific mechanism has as yet been formulated for myotube formation, a cell-cell fusion process [55].

*Conclusions*

Membrane mobility agents, especially in conjunction with Flomols, have

proved to be valuable agents for the study of the important biological process of cell-cell fusion. Through their use, we have been able to recognize clearly a number of stages in membrane fusion, and then evolve a general scheme for fusion. Our scheme for fusion sets the stage for further and more quantitative investigations into various aspects of the fusion process.

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