*Biochimica et Biophysica Acta*, 601 (1980) 544—558


BBA 78933

# MEMBRANE FUSION INDUCED BY THE MEMBRANE MOBILITY AGENT, $A_2C$

## DIFFERENTIATION BETWEEN FUSIBLE AND NON-FUSIBLE CELLS

## TRANSFER OF FUSIBILITY

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(Received February 18th, 1980)
(Revised manuscript received May 21st, 1980)

*Key words: Mobility agent $A_2C$; Membrane fusion; (Red cell)*

## Summary

Red cells of different species respond differently to the treatment with the membrane mobility agent, $A_2C$, with respect to both the $A_2C$ interaction and the subsequent cell-cell interaction. Depending on whether both, one or neither of the processes are effective, some red cells (e.g., nucleated Leghorn hen red cells, rat red cells) fuse easily, some (human red cells) show morphological changes but do not fuse, and others (nucleated Rock hen red cells) show little or no response. Mixed fusion (i.e., between fusible cells of different species) is readily obtained, indicating that no species-specific recognition sites are required for $A_2C$-induced fusion.

The potential for fusion is a transferable characteristic. In the presence of fusible cells, $A_2C$ induces both heterologous and homologous fusion of otherwise 'non-fusible' cells.

Electron micrographs of fusing cells after treatment with $A_2C$ reveal 'onion-ring' structures ('whorls'), free of intramembranous protein particles but different from the smooth appearance of $A_2C$ particles. Whorls are considered to

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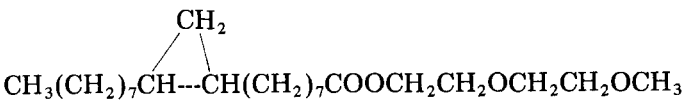
arise from fusion-potent membrane areas. Fusion is apparent at multiple sites along the contact line between apposed membranes. The postulated appearance of vesicle-like structures along the fusion line (Kosower, E.M., Kosower, N.S. and Wegman, P. (1977) Biochim. Biophys. Acta 471, 311—329) is confirmed by micrographs. The mechanism of this fusion process is discussed and compared to other types of fusion process.

## Introduction

Membrane fusion is one of the most ubiquitous cellular events, mediating such common biological phenomena as fertilization, phagocytosis and exocytosis [1—7]. Fusion can be induced by viruses or chemicals [8—14]. Membrane mobility agents are a class of lipid-related molecules, designed to promote lipid disorder. When added to cells, they increase the mobility of membrane components [15—17]. Their dispersion in aqueous media produces small particles which can be incorporated into the membrane. A member of this class, $A_2C$, promotes fusion for red cells in some strains of chicken [18,19].

$A_2C$



$CH_3(CH_2)_7CH\text{---}CH(CH_2)_7COOCH_2CH_2OCH_2CH_2OCH_3$

We now report a wide range of responses, after the treatment with $A_2C$, for cells of a variety of mammalian and avian species, some cells are fusible, others not. Moreover, mixed fusion, i.e., between fusible cells of different species, can readily be obtained indicating that species-specific recognition sites are not necessary for the fusion. Moreover, the potential for fusion is transferable: fusion can be induced in otherwise non-fusible cells treated in the presence of fusible cells.

Some of the morphological aspects of the fusion process have been investigated and are reported and interpreted in relation to the mechanism proposed for fusion processes, including $A_2C$-induced fusion [20].

## Materials and Methods

Heparinized blood from various species was centrifuged, the plasma and buffy coat removed and red cells washed twice with 150 mM NaCl and once with buffer (pH 5.7) containing sodium acetate (76 mM), NaCl (70 mM), $CaCl_2$ (2 mM) and Dextran T-70 (100 mg/ml). Cells were then resuspended in the same buffer to a hematocrit of 6—8% and mixed with an equal volume of $A_2C$ suspension (0.5 $\mu$l/ml) in NaCl (150 mM) (dispersion through sonication has been described previously [20]). Flomol F20C ($2 \cdot 10^{-3}$ $\mu$mol/$\mu$mol $A_2C$) in methanol was mixed with $A_2C$, the methanol removed with a stream of $N_2$, NaCl (150 mM) added, and the F20C-$A_2C$ suspension was used to follow the interaction of $A_2C$ particles with cells, using fluorescence microscopy.

Cell suspensions were incubated at 37°C and mixed gently every 5—10 min.

Aliquots were removed at intervals for observation by light, phase or fluorescence microscopy on wet preparations. Samples were also air-dried on glass slides, stained with Wright-Giemsa and used for photography. The percentage of cells hemolysed was determined by spectrophotometric measurement of hemoglobin concentration at 540 nm in the supernatants of centrifuged samples. Aliquots were fixed for electron microscopy (scanning, thin-section, and freeze-fracture) in a fixative containing 1% paraformaldehyde, 1% glutaraldehyde and 1% tannic acid in phosphate buffer (100 mM, pH 7.2).

For thin-sectioning, the cells were post fixed with 1% phosphate-buffered $OsO_4$, dehydrated, embedded in epoxy resin and sectioned. Sections were stained with uranyl acetate and lead citrate and studied with the electron microscope. For scanning electron microscopy, the cells were placed on a nucleopore filter membrane and similarly post-fixed with $OsO_4$. They were then dehydrated in graded alcohol, brought to critical-point drying with Freon 13, sputter-coated with AuPd and studied. For freeze-fracture, drops of fixed cell suspension were placed on a specimen holder, quickly frozen in Freon 22, and brought to the temperature of liquid $N_2$. They were fractuted in Balzers instrument at a pressure of $1 \cdot 10^{-6}$ Torr and a temperature of $-110°C$. They were then replicated with platinum and carbon, the replicas cleaned with Clorox and studied.

To verify the entry of $A_2C$ into the cell membrane, red cells were incubated with F20C-$A_2C$, following which the cell suspension was analyzed in a Becton-Dickinson laser fluorescence-activated cell sorter operated at a laser power level of 400 mW and using a sample size of 200 000 cells. The cell size and fluorescence distribution were analyzed separately then combined.

## Results

The treatment of avian red cells with $A_2C$ under conditions suitable for fusion gave results which depended upon the genetic or species origin of the cell. The oval, nucleated red cells of Leghorn hens and of turkey became spherical within 20—40 min of the start of incubation, and fused within 50—70 min to form binucleated and multinucleated cells followed eventually by lysis of large multinucleated syncytia. Neither shape changes nor cell-cell fusion were observed in cells from Barred Rock hens under similar conditions. Observation of Rock hen red cells in the presence of F20C-$A_2C$ showed that the fluorescent particles approached the cells, either stuck to them or bounced off, and that those which remained attached had no further effect on the red cell, i.e., fluorescent red cells could not be seen. In contrast, Leghorn red cells appeared as fluorescent rounded cells after a similar treatment [20].

Incubation of non-nucleated mammalian cells with $A_2C$ led to pronounced morphological changes visible by light microscopy. Membrane investigations were followed by spherocytosis and clumping of cells. After 20—30 min, rat, mouse, and guinea-pig cells gave rise to fused cells, a process which continued until they eventually formed large syncytia and lysed. In contrast, human red cells underwent invagination and spherocytosis but did not readily clump or fuse, there being less than 5% fusion and little hemolysis after 60 min exposure to $A_2C$. That the morphological alterations of human cells resulted from the

incorporation of $A_2C$ was confirmed by the observation of fluorescent spherocytes after the exposure to F20C-$A_2C$. The fluorescent human cells gave a size distribution in the laser cell sorter identical to that expected for human red cells. The distribution of fluorescence, either separately or in combination with the red cell size distribution, gave a single peak, indicating that the F20C-$A_2C$ had been incorporated into the cell membrane.

Rabbit red cells exhibited a response to $A_2C$ treatment intermediate between rat and the human red cells, some morphological changes and a modest degree of fusion being noted within 60 min.

To determine whether or not the $A_2C$-induced fusion was species-specific, mixtures of fusible cells from different species were treated. Easily identifiable fusible avian cells were mixed with fusible mammalian cells and treated with $A_2C$. Both mixed and homologous fusions occurred in the mixtures of cells with almost equal frequency.

When mixtures of fusible cells and non-fusible cells were treated with $A_2C$, three fusion combinations were observed: fusible cells with fusible cells; fusible cells with non-fusible cells; and most remarkably, non-fusible cells with non-fusible cells. No fusions were observed upon treatment of mixtures of non-fusible cells with non-fusible cells.

The effects of $A_2C$ treatment on red cells from several species as seen by light microscopy are shown in Fig. 1, and the results are summarized in Tables I and II. The combined cell size and fluorescence distribution of human red cells, obtained with the laser cell sorter, are shown in Fig. 2.

Electron microscopy was used to obtain additional information about the membranes of fusing cells. Membrane changes were seen in cells undergoing homologous and heterologous fusion. Rough cell surfaces with distorted, drawn-out membrane connecting the fusing spherocyte cells were seen in scanning electron microscopy, especially in rat cells (Fig. 3). Portions of membrane protruded from the surface in regions not undergoing fusion. A probable

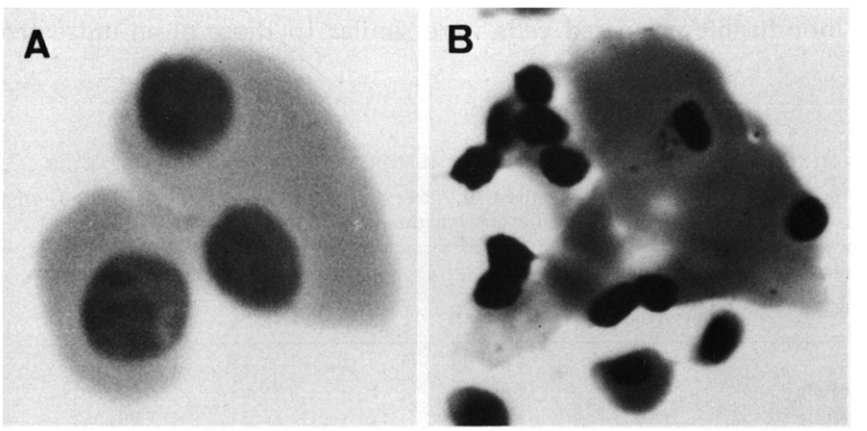



Fig. 1. Effects of $A_2C$ on avian cells, and on a mixture of avian and mammalian cells. (A) Three Leghorn hen red cells: fusion in early stages (×1500). (B) Rat red cells + Barred Rock hen red cells: mixed fusion is shown. Note the syncytium consisting of a cytoplasmic mass containing relatively few nuclei of the nucleated hen cells (×600).

TABLE I

$A_2C$-INDUCED MEMBRANE FUSION IN NUCLEATED AND NON-NUCLEATED RED CELLS

Percent of total cell population involved: 0, <0.5%; +, 10—25%; ++, 25—50%; +++, 50—75%; ++++, 75%. For details of morphological charges see text. Determinations of %lysis were made at 60 min and each figure represents two to five separate experiments.

| Species tested | Morphological + changes | Fusion | %lysis ($\mu$m and range) |
|---|---|---|---|
| Avian | | | |
| Leghorn hen | ++++ | ++++ | 91.1 (85.2—97) |
| Barred Rock hen | 0 | 0 | 8.15 (6.7—9.7) |
| Turkey (common barnyard, *Meleagris gallopavo*) | +++ | +++ | 62.5 (58—67) |
| Mammals | | | |
| Human | +++ | 0 | 10.6 (6.5—14.1) |
| Rat (Wistar) | ++++ | ++++ | 82.95 (80.58—85.3) |
| Mouse (Balb/c) | ++++ | ++++ | 80.5 (76—85) |
| Guinea-pig | +++ | +++ | 58.5 (48—69) |
| Rabbit (N.Z.) | ++ | + | 17.8 (15.6—20) |

correlate of these protrusions was the onion-ring structures (whorls) observed in the membranes of fusing cells in freeze-fractured replicas. The onion-ring structures appeared to be free of intramembranous particles (Figs. 4A and B). Micrographs of $A_2C$ particles obtained by freeze-fracture technique revealed that such particles lacked structure altogether, exhibiting a smooth appearance (Fig. 5).

The thin section of fusing cells indicated that membrane changes involved membrane loss through both internalization and externalization (Fig. 6). Internalized membrane fragments were seen as areas of multiple, small vesicles. Externalized membrane fragments were seen as densely stained, lipid-rich vesicles. The fragments were seen in areas not undergoing fusion (Fig. 6), and probably correspond to the onion-ring structures seen in freeze-fractured replicas (Fig. 4).

Micrographs of non-fusible cells did not show similar fragments. Sections of $A_2C$-treated non-fusible avian red cells were similar to those of an untreated

TABLE II

$A_2C$-INDUCED MIXED FUSION BETWEEN RED CELL MEMBRANES OF DIFFERENT SPECIES

Percent of cell population of each species involved: 0, <5%; +, 10—25%; ++, 25—50%; +++, 50—75%. Determinations of %hemolysis were carried out for one experiment at 60 min.

| Mixture of cells tested | Homologous fusion | | Mixed fusion | %Hemolysis |
|---|---|---|---|---|
| | Avian | Mammal | | |
| Leghorn + rat | +++ | +++ | ++ | 90 |
| Leghorn + guinea-pig | ++ | ++ | + | 50 |
| Leghorn + human | +++ | + | + | 60 |
| Barred Rock + rat | ++ | +++ | + | 80 |
| Barred Rock + guinea-pig | + | ++ | + | 40 |
| Barred Rock + human | 0 | 0 | 0 | 5 |

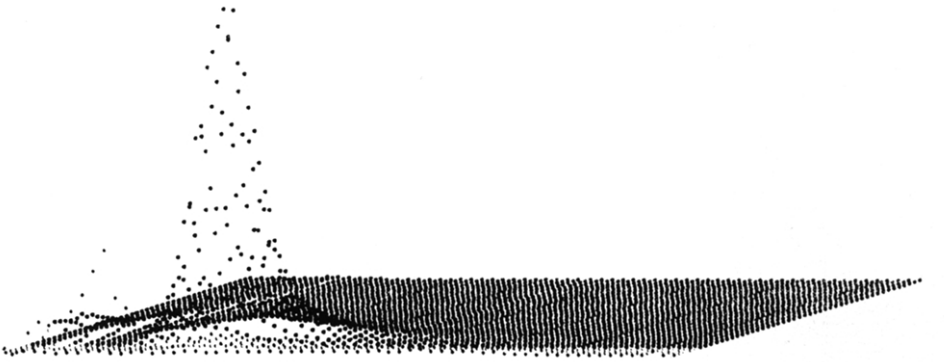


Fig. 2. Combined cell size and fluorescence distribution of human red cells incubated with F20C-$A_2C$ and studied with a laser cell sorter. The abscissa indicates the size distribution and the ordinate depicts the fluorescence distribution. The size distribution corresponds to that of normal human red cells. The single peak indicates the presence of fluorescent compound in the cells. A smaller rise seen on the left might well arise from the presence of $A_2C$ particles attached to but not yet incorporated into the cells.

cell. Sections of treated human cells revealed morphological alterations also evident in light microscopy (Fig. 7) but no fragments, either in the form of whorls or multivesiculated blebs, could be seen in freeze-fractured or transmission electron micrographs.

The electron micrographs indicated that fusion occurred at multiple points along the large contact area between fusing cells (Fig. 8A). At high magnifica-

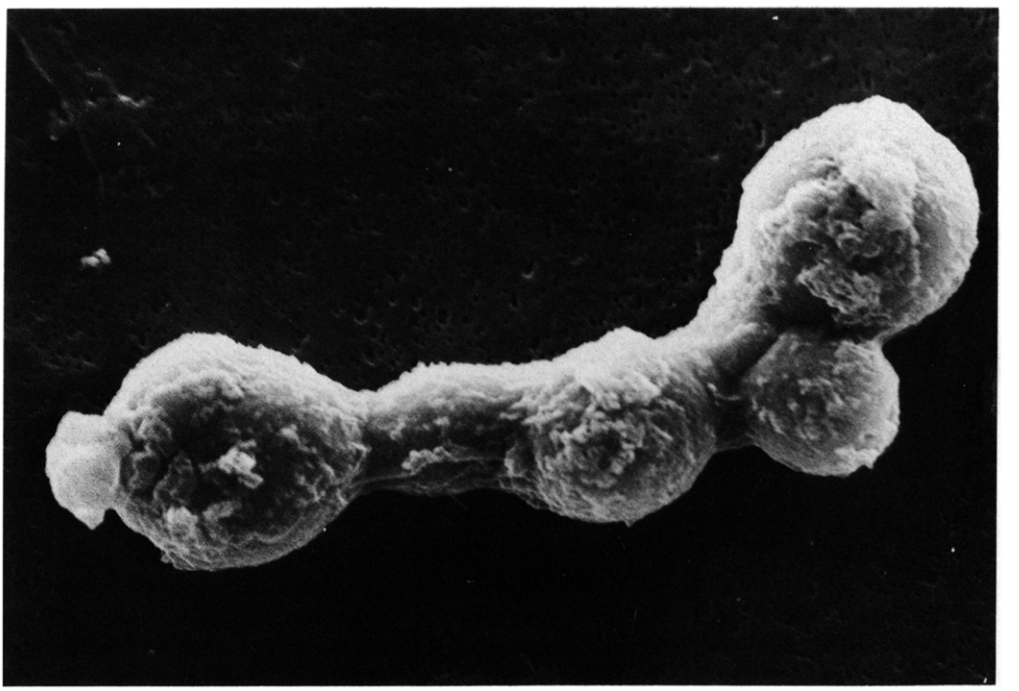


Fig. 3. Scanning electron micrograph of fusing rat red cells. Rough surfaces, drawn-out membrane connecting the fusing cells and membrane extrusions are all visible (×8220).

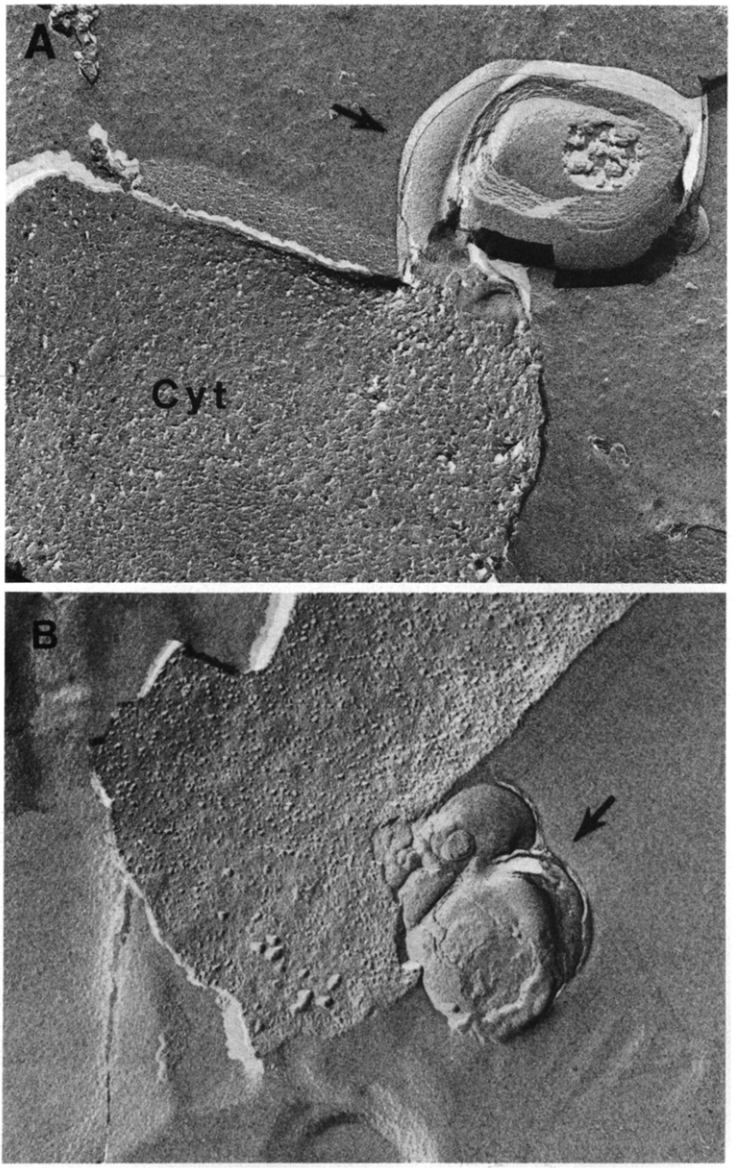



Fig. 4. Freeze-fractured replicas of cells fusing as a result of $A_2C$ treatment. (A) An onion-ring (whorl) structure is shown (arrow) being extruded from the membrane of a rat red cell which is cross-fractured through the cytoplasm (Cyt). (B) An intramembranous particle-free region (arrow) in the intramembranous particle-studded membrane of a Leghorn hen red cell (both X43 750).

tion, areas of apposed membrane in tight contact could be seen (Fig. 8B). Within these areas, regions which correspond to channels of communication between the cells were noted, and these contained disordered membrane remnants (Fig. 8B). Adjacent to the channel regions and between the apposed membranes of the fusing cells were extracellular spaces, which corresponded in size to 'vesicles' (Fig. 8B). At later stages of fusion, transmission electron micro-

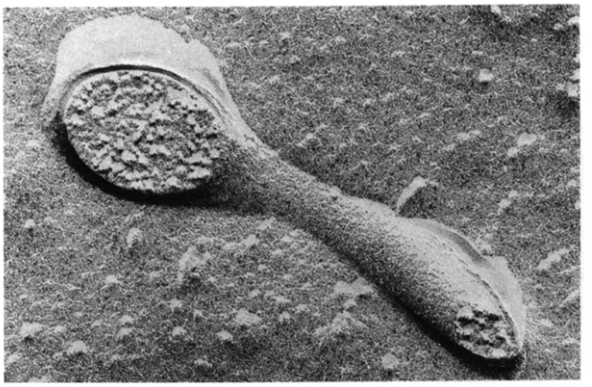


Fig. 5. Freeze-fracture replica of an $A_2C$ particle. Note the absence of structure similar to that of a membrane or liposome (×9200).

scopy showed many points of communication between the cells, with many vesicles between these points (Fig. 9). These membrane vesicles disappeared at a later stage of fusion or possibly were left as small, heavily stained remnants (Fig. 10).

## Discussion

In this study, we have shown a considerable but consistent heterogeneity in red cell response in different species to treatment with $A_2C$. Cells from some

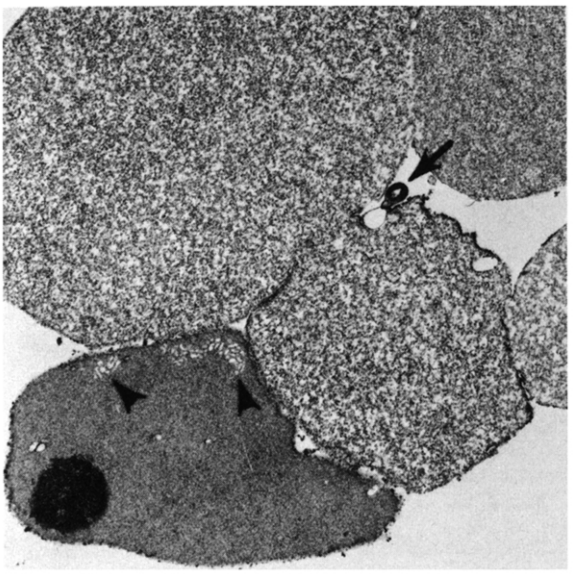


Fig. 6. Transmission electron micrograph of a sample taken from an $A_2C$-treated mixture of rat red cells and Barred Rock hen red cells. An area of fusion between two rat red cells is illustrated. Internalization (arrow-heads) and externalization (arrow) of membrane are seen in areas not undergoing fusion. The heavily stained externalized fragment (arrow) is a lipid-rich, multilayered vesicle (×8500).

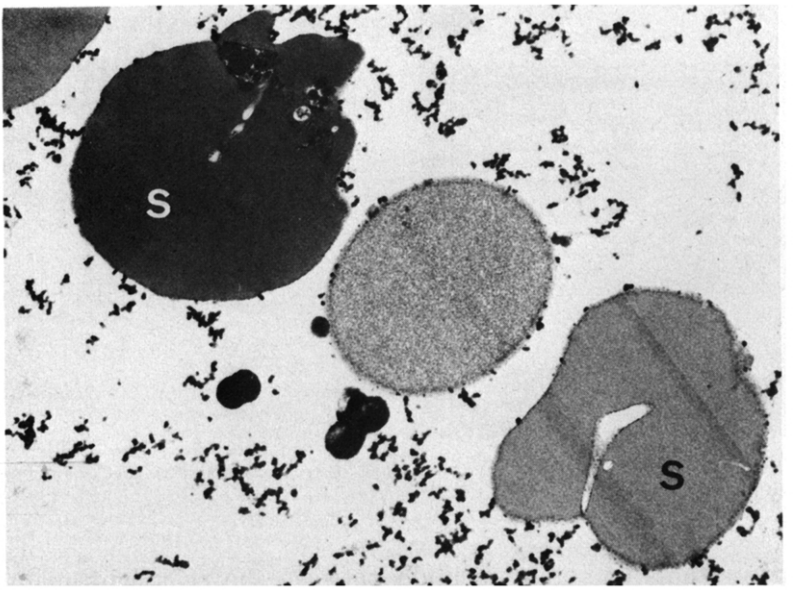



Fig. 7. Transmission electron micrograph(s) of human red cells treated with $A_2C$. Two stomatocytes with deep, enclosed invaginations are shown (×6600).

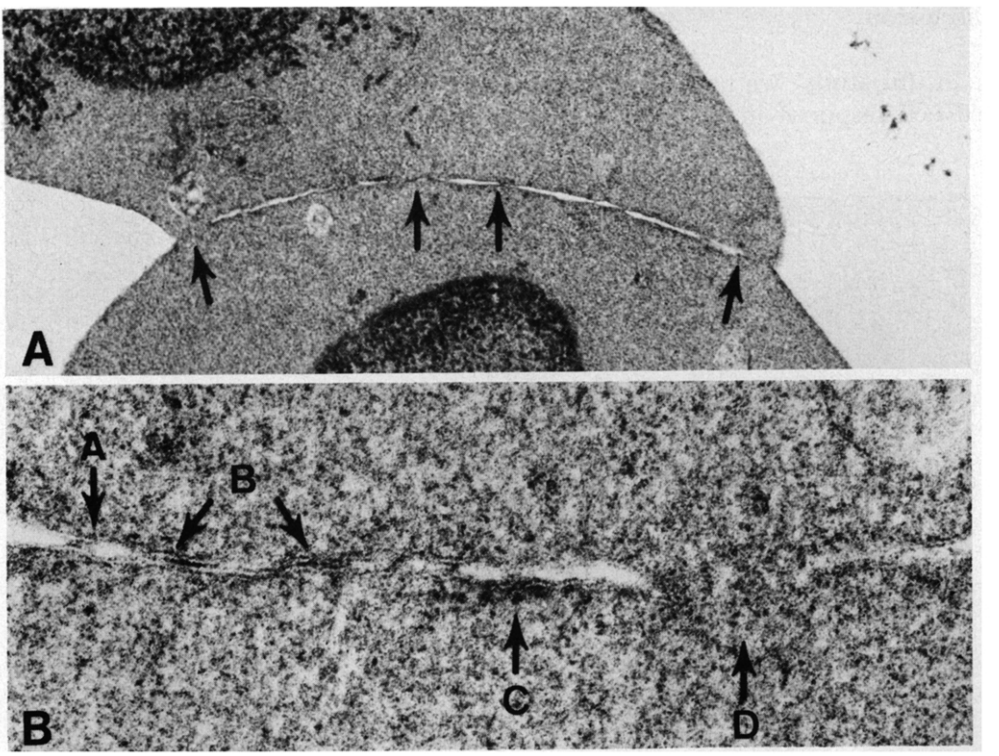



Fig. 8. Transmission electron micrographs of Leghorn hen red cells during fusion. (A) Apposition of membranes at multiple points (arrows) is illustrated (×13 500). (B) A fusing region is shown under high magnification. Arrow A indicates an apposition between the two membranes. Arrows labeled B show a tight apposition between the two bilayers and the beginning of channel formation between the cells. Note the 'pentalaminar' structure in which the tightly apposed outer layers of the two membranes give the appearance of a dense middle 'layer' Arrow C points at a delimited space ('vesicle') between two fusing areas. Arrow D indicates a communicating channel in the process of formation, with membrane remnants still present (×150 000).

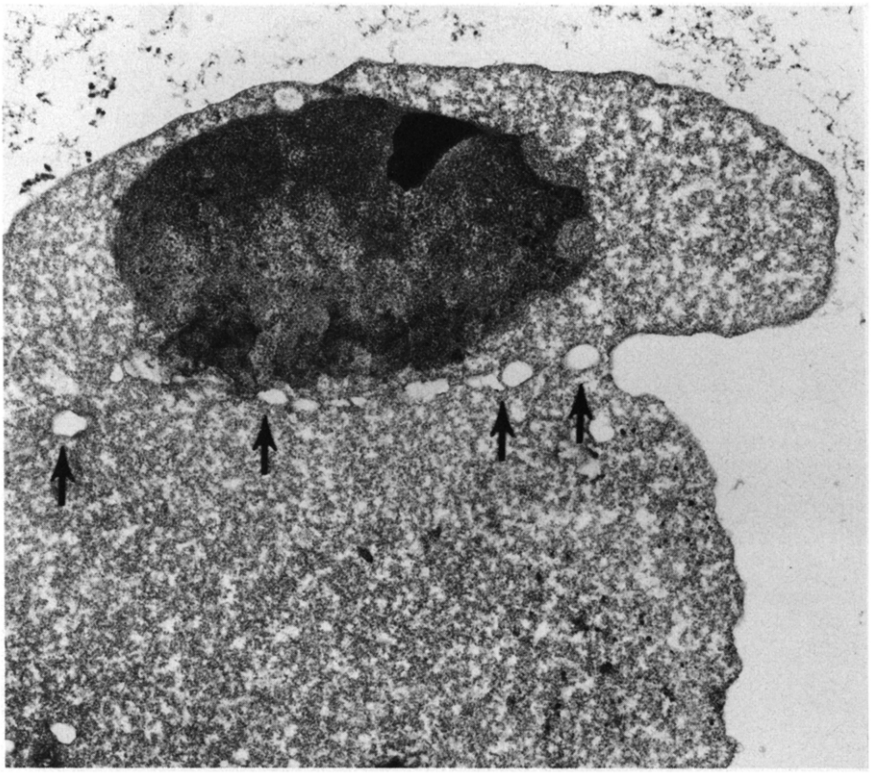


Fig. 9. An electron micrograph of the fusing region between a non-nucleated rat red cell and a nucleated Barred Rock hen red cell at a late stage of fusion. Multiple points of fusion are visible along a large contact area between the two cells with an array of vesicles (arrows) indicated (×11 000).

species fuse easily, in others they show only shape changes but no fusion, in yet another group they show no change at all. Mixed fusion between fusible cells of different species is easily obtained and in addition, '$A_2C$-fusible' cells can promote the fusion of '$A_2C$-non-fusible' cells in the presence of $A_2C$. To gain more insight into fusion and non-fusion, the phenomenon was studied morphologically. Strong evidence was obtained in support of a phenomenological description and molecular theory of membrane (and cell) fusion advanced a few years ago [20]. We shall briefly review certain features of that description and theory in order to provide a proper background for the present discussion.

Agent-promoted cell-cell fusion consists of two distinct processes: (a) introduction of the inducing agent into the cell membrane and; (b) the fusion of the membranes of two cells. In the case of $A_2C$, fusion of the dispersed particles (microdroplets) of $A_2C$ with the cell membrane proceeds in the following stages. (1) Particles approach the cell and adhere to the cell membrane. (2) The agent enters and diffuses throughout the membrane. (3) The cell undergoes morphological changes such as spherocytosis. Cell-cell fusion, the second process, follows a parallel course: (1) approach and adherence of a cell to another; (2) membrane fusion; (3) morphological changes.

*Agent entry into cell membrane.* Direct evidence for the entry of the agent

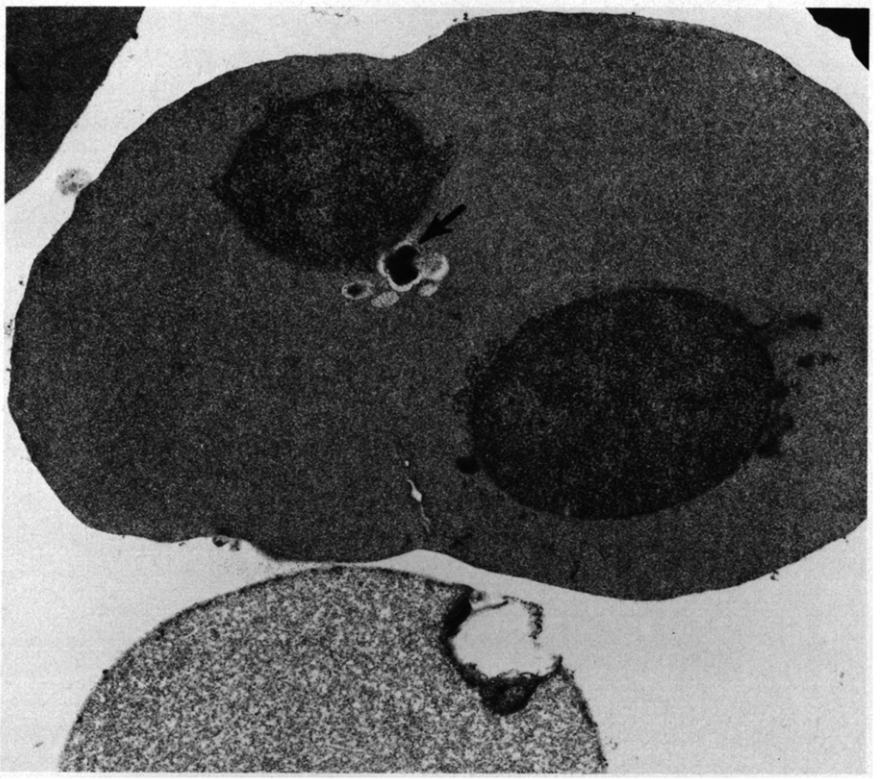


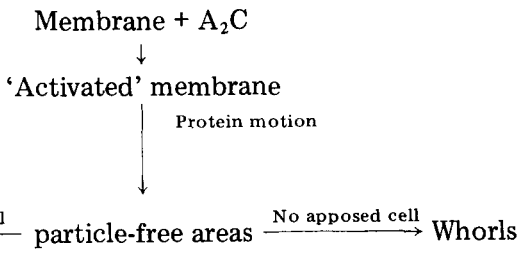
Fig. 10. The end stage of cell-cell fusion between two Leghorn hen red cells in which some membrane remnants (arrow), some heavily stained, are seen along with a vesicle along the line of fusion (×30 000).

into the cell membrane has previously been obtained by the use of a fluorescent probe (F20C) loaded into the $A_2C$-particles [16,17,20,21]. In the present study, loading was detected in a cell sorter which showed a single fluorescent peak corresponding to the size distribution of red cells. Fluorescent cells could be seen under the microscope directly.

*Mobility of membrane components.* By use of the freeze-fracture technique, the normal red cell membrane shows a reasonably uniform distribution of particles that are known as 'intramembranous particles' and are considered to consist of membrane proteins. Particle-free areas have been reported in association with fusion processes such as exocytosis of synaptic vesicles, degranulation of mast cells, extrusion of trichocysts, virus-induced cell fusion, myoblast formation, etc. It has been suggested [20] that mobility of membrane components is enhanced by fusion-promoting agents and that the enhanced mobility augments the rate of formation of particle-free areas suitable for fusion. In this manner, the membrane is 'activated' and becomes fusion-potent.

We observed these particle-free areas in fusing red cells. They appear as whorls or onion-ring structures in areas in which membranes are not apposed. These particle-free areas have been noted infrequently but have not been assigned a direct role in fusion [5]. We now propose that these whorls represent

a further stage in the evolution of unutilized fusion-potent areas. This is classified in the following scheme.




These whorls have a layered, 'onion-skin' structure resembling liposomes, but distinct from $A_2C$ particles. The whorls, therefore, must consist of phospholipid and $A_2C$ but no visible protein particle.

*Cell-cell adherence and the progress of fusion.* In the course of fusion, cells approach one another closely. Pontecorvo et al. [22] have illustrated a case in which fusion appears to occur via the fusion of microvilli, avoiding in a sense, gross contact between the cells. The initial contact between the particle-free areas is probably at one localized area but, promoted by 'hydrophobic bonding' [20], this can extend to multiple areas. Permeation of water through the contact areas leads to the formation of inverted micelles, creating a membrane instability which is resolved through fusion of the bilayers and the formation of aqueous channels through which the internal contents of the two cells are in communication [20]. Multiple channels may develop in a single contact region. We have now documented these multiple connecting channels, which were separated by vesicles arranged in a linear array along the fusion line. In thin sections, these vesicles are two-dimensional representations of the regions in which the extracellular fluid has been trapped between the membranes adjacent to the sites of fusion.

*Similarity to other fusion processes.* Okada et al. [23—26] have shown that the virus-induced fusion may be divided into two stages, the virus-cell fusion and cell-cell fusion. The adherence of virus to the cell membrane at low temperature (approx. 4°C) is followed by rapid virus cell fusion on warming the combination to 37°C. Viral components are incorporated and dispersed into the cell membrane. Intermixing of viral and cell phospholipids was shown by a spin-label study to be a necessary step in virus-cell fusion [27]. Mobility of membrane components is promoted by the fusion [28,29] and the redistribution of intramembraneous particles in the red cell membrane induced by Sendai virus is a prerequisite for cell-cell fusion [30,31]. Cell-cell fusion proceeds at multiple points along the contact area [28,32]. Hence, the $A_2C$-induced fusion is similar to virus-induced fusion.

The rearrangement of membrane components and the formation of particle-free phospholipid regions are alos associated with various 'natural' fusion events [3—5,33—37]. Particle-depleted membrane areas with a fine, fuzzy coating, or multilamellar whorls or multivesiculated blebs have been observed in degranulating mast cells [4,5]. The whorls are similar to those described herein. Clear, smooth bulges have been found in the spermatozoa undergoing the acrosomal reaction, and are seen especially easily in the case of sperm activation in a capacitating medium of liposomes [37].

The general parallelism in the steps discerned in various kinds of fusion indicates similarity in mechanism with only the details varying. One such detail involves cell-cell recognition. Mixed fusion between cells of different species, described here, implies that recognition is not critical to this type of fusion, although virus-induced fusion may require specific receptors.

*Heterogeneity in cell response and transfer of fusibility.* The sequence of events associated with fusion may be interrupted or diverted. The absence of shape changes in non-fusible hen cells in the presence of $A_2C$-particle adherence implies that the agent does not enter the cell. Lack of entry precludes any further consequences. In human cells, $A_2C$ does enter the cell, and shape changes ensue without the evolution of whorls. We attribute this behavior to the relative immobility of membrane proteins in human red cells. Ordinary molecular motion leads to 'excess' lipid (phospholipid + $A_2C$ in treated cells) in localized regions. If the proteins surrounding this 'excess lipid area' are mobile, the area can grow in size laterally leading to the expansion of membrane, but if the proteins are immobile, the excess lipid area can only grow in the 'vertical' direction (i.e., perpendicular to the cell membrane) leading to extrusion or invagination. Intermediate cases may also exist in which cell-cell fusion may depend upon the effect of external conditions on protein mobility. In the case of human red cell, therefore, the $A_2C$-promoted fusion process seems to be inhibited at the stage of membrane component mobility.

Heterogeneity in cell response is also observed in response to treatment with Sendai virus. Mutant KB cells show a diminished fusion response with Sendai virus, apparently because of a membrane defect, preventing viral envelope-cell membrane fusion [39,40]. Thus, the fusogenic agent does not enter the cell. Later stages of virus-induced fusion may also be interrupted, with saccharides inhibiting motion within the cell membrane [25], and cytochalasin D inhibiting a still later stage of fusion [26].

Finally, we have shown that fusibility may be transferred, so that fusion may be induced in otherwise non-fusible cells by a factor introduced into the system by fusible cells. The nature of this factor is as yet unknown. It is clear that differences in membrane composition and structure are responsible for the heterogeneity in red cell response. The sodium dodecyl sulfate gel electrophoretic patterns of red cell membrane protein are similar among several species (human, rat, mouse) [41,42], suggesting a gross parallelism between the polypeptides of the major protein fractions of the membrane [41]. Yet, certain differences between the proteins composition of various species have been demonstrated, including the property of aggregation to proteins of high molecular weight, the charges on the proteins and the topography of the glycoproteins [41—43]. In addition, differences in lipid composition have been found among various species [44]. The relationship, if any, of these variations to the differences in fusibility and transfer of fusibility is unknown. It is interesting that the transfer of lipids from F glycoprotein-deficient influenza virus to human red cell membrane is promoted by Sendai virus which does contain the F glycoprotein [45]. It may be hoped that specific factors may be identified in the case of other types of fusion.

## Acknowledgements

This work was supported by NIH grants AM 25510, AM 70551 and DOE-EV00899 to M.T. N.S.K. has been the recipient of a United States-Israel Binational Science Foundation (BSF) grant. E.M.K. was a J.S. Guggenheim Fellow, University of California, San Diego and University of California, Berkeley 1977—1978. M.T. is a recipient of Research Career Development Award for National Institute of Arthritis, Rheumatism and Digestive Disease.

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