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Ca^{2+}-induced fusion of avian erythrocytes

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SUMMARY

Fusion of avian erythrocytes can be induced by Ca^{2+} at pH 10.5. The addition of 40 mM Ca^{2+} causes agglutination of the erythrocytes at pH 10.5 in the cold. Fusion occurs when the agglutinated cells are transferred to 37°.

The mechanism of the process is discussed in relation to the virus-induced fusion of animal cells.

Fusion of biological membranes appears to be a basic phenomenon in the life of cells. Active processes such as pinocytosis, phagocytosis[1] and bulk transport[2] involve plasma membrane fusion either with itself or with the membrane of subcellular organelles. *In vitro* systems, in which fusion of mammalian cells was induced by enveloped viruses, were established by Enders and Peebles[3], Okada[4], Kohn[5] and others[6]. Although this kind of fusion has been studied quite eextensively during the last few years[7] and many of its characteristics have been described, very little progress has been made in the understanding of the biochemical reaction and the molecular changes taking place during the fusion reaction.

Because of its simplicity, fusion of erythrocyte plasma membranes might serve as a good system for the elucidation of the fusion mechanism. Erythrocytes have been shown previously to fuse with other cells such as lymphocytes and tumor cells, thus forming heteropolykaryons[8]. However, formation of virus-induced homopolykaryons by fusion of erythrocytes alone has not yet been achieved, since addition of enveloped viruses of the paramyxoviruses group to red blood cells causes agglutination followed by extensive lysis[9].

Recently we have reported that bivalent cations such as Mn^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} greatly reduced the virus-induced hemolysis of chicken erythrocytes. Under such conditions of limited lysis, fusion of chicken erythrocytes could be obtained[10]. Poole *et al.*[11] too have observed fusion of erythrocytes by addition of lysolecithin at low pH.

From the above observations it was therefore inferred that agglutination followed by controlled lysis might lead to fusion – even in the absence of viruses or any other reagent, thus enabling the study of this biological process in a simpler system. The

experiments described below show that preincubation of chicken erythrocytes at pH 10.5 and subsequent addition of Ca^{2+} causes extensive agglutination and fusion of the cells.

Red blood cells were collected from the necks of decapitated chickens into an erlenmeyer flask containing heparin (100 units/ml blood). The blood was stored at 4° and used within 5 days. The blood cells were washed 3 times in the cold with a buffered solution containing 140 mM KCl, 5.4 mM NaCl, 0.8 mM $MgSO_4$ and 40 mM glycine–NaOH buffer at pH 10.5. The final sediment was suspended in the above buffer to give 10% (v/v) concentration.

Chicken erythrocytes suspended at pH 10.5 were agglutinated in the cold by Ca^{2+}, only if they were preincubated at that pH at 37° before Ca^{2+} was added. Erythrocytes that had been preincubated at 0–4° at pH 10.5 for up to several hours failed to exhibit any agglutination upon addition of Ca^{2+}. When Ca^{2+}-agglutinated erythrocytes were transferred back from 4 to 37°, lysis occurred (Fig. 1). The degree of lysis was related to the length of the preincubation period (Fig. 1A) and to the Ca^{2+} concentration (Fig. 1B). As can be seen from Fig. 1A, Ca^{2+} induces as much as 40% hemolysis even without preincubation; neither agglutination nor fusion were obtained under these conditions. Some fluctuations in the degree of hemolysis were obtained by Ca^{2+} at pH 10.5 since different batches of erythrocytes were used in the present work. Bivalent cations other than

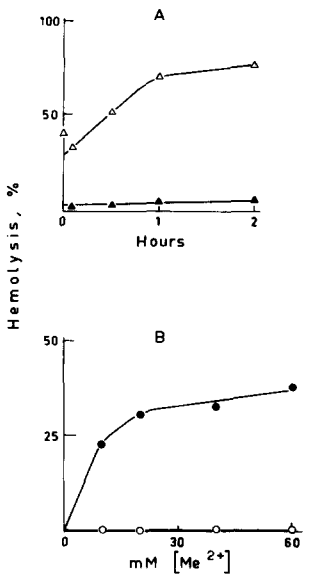

Fig. 1. Ca^{2+}-induced hemolysis of chicken erythrocytes at pH 10.5. 0.5 ml of 5% washed cells suspended in a buffered salt solution at pH 10.5 (see text) were incubated for 30 min at 37° with gentle shaking (100 rev./min in a New Brunswick shaker). At the end of 30 min the cells were cooled in an ice basket whereupon 40 mM of $CaCl_2$ and a buffered salt solution at pH 10.5 were added to give a final volume of 1 ml. The suspension was kept 20 min in the cold to allow agglutination and then transferred to 37°. The cells were shaken for 10 min at 37°, removed and immediately centrifuged at 500 × *g* for 10 min in the cold. The clear supernatant was diluted with 4 vol. of the buffered medium and read at 540 nm[12]. A. Hemolysis as a function of the preincubation time at 37°. △—△, 40 mM $CaCl_2$; ▲—▲, without $CaCl_2$ B. Hemolysis as a function of $CaCl_2$ and $MgCl_2$ concentration. ●—●, $CaCl_2$; ○—○, $MgCl_2$.

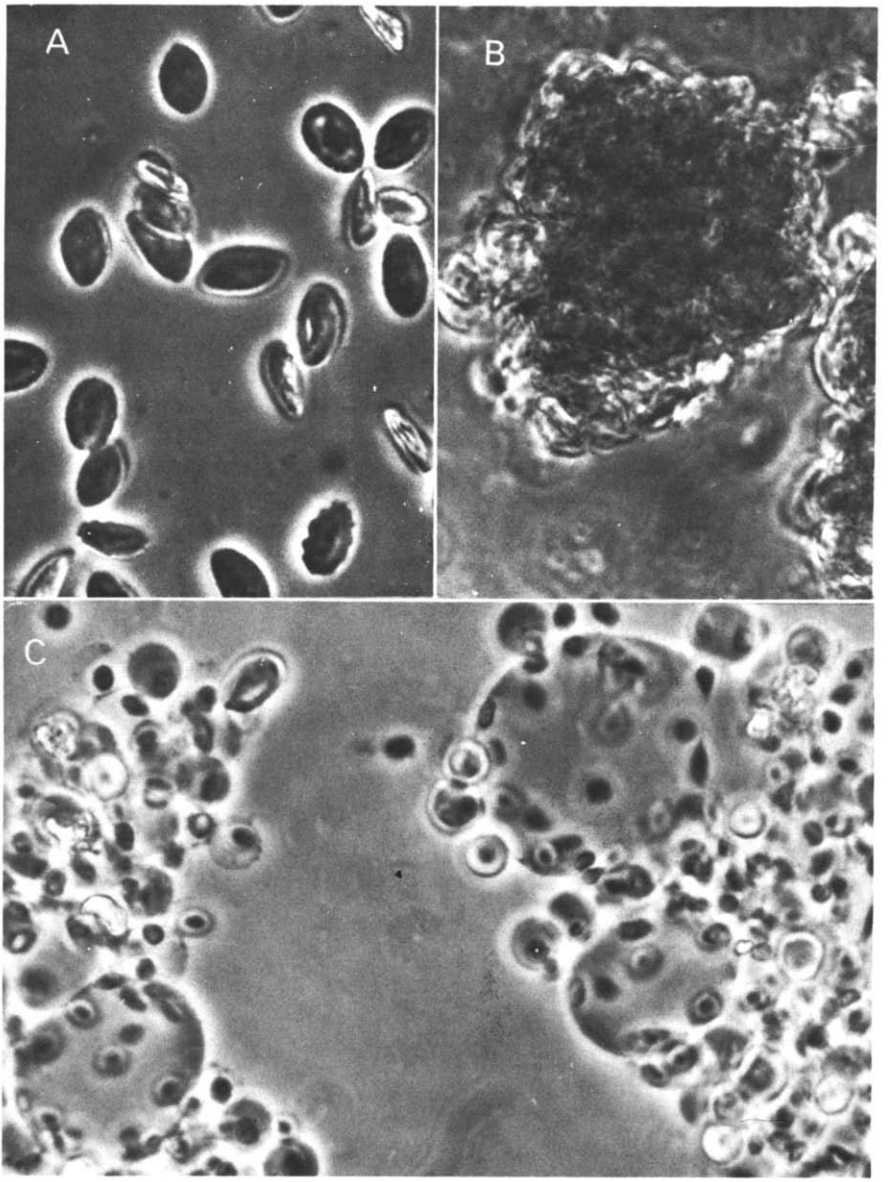

Fig. 2. Photomicrographs of Ca^{2+}-induced fusion of chicken erythrocytes. (Phase optics with final magnification of x 880.) Experimental conditions as described in Fig. 1. After the preincubation at 37° and the agglutination in the cold, Samples A and C were shaken for 15 min at 37°, removed and immediately photographed. Sample B was removed after 20 min in the cold and photographed. A. Control of chicken erythrocytes in which Ca^{2+} was omitted. B. Ca^{2+}-induced agglutination of chicken erythrocytes. C. Polynucleate cells formed at pH 10.5.

Ca^{2+}, such as Ba^{2+} and Mn^{2+}, may serve as agglutination agents and lysis inducers at high pH, although to different extents. Unlike Ca^{2+}, Ba^{2+} and Mn^{2+}, Mg^{2+} neither serves as an agglutination agent nor as a lysis inducer (Fig. 1B). Between the bivalent cations tested only Ca^{2+} promoted fusion concomitant with the lysis of the agglutinated cells.

Samples of erythrocytes taken from the lysis media contain many polynucleate cells as observed by phase microscopy (Fig. 2C). The fusion process of chicken erythrocytes was initiated about 5 min after the cells were transferred to 37°, a temperature at which the fusion and the lysis are taking place. The polynucleate cells were stable for about an hour at 37° but later they burst and most of the fields contained membrane's fragments. If, after the polykaryons have been formed, the incubation temperature is lowered to 4° and the pH to 7.4, the fused cells are more stable and remain intact for several hours. Survey of the fields revealed that about 30–50% of the erythrocytes had fused during the incubation at 37°. Measurements of fusion indices[10] were impossible in the present system since the homopolykaryons remained strongly agglutinated. However, it seems that the amount of the fusion obtained was not directly related to the degree of lysis. The system is sensitive to the pH of the reaction medium. While complete lysis with very little fusion takes place above pH 11.5 (Fig. 3), no agglutination or lysis occurs below pH 10.0. Electron micrographs revealed cells containing 2–6 nuclei (Fig. 4). The ultrastructural changes taking place during the Ca^{2+}-induced lysis and fusion at pH 10.5 are under investigation.

In summary, in order to induce agglutination lysis and fusion of chicken erythrocytes by Ca^{2+}, the following steps are required: (1) preincubation of the cells at high pH at 37°; (2) addition of the bivalent cations in the cold in order to cause agglutination; (3) incubation of the agglutinated cells at 37°.

The mechanism by which cations and especially Ca^{2+} induce agglutination in the cold is not known yet. It is reasonable to assume that the Ca^{2+} and other bivalent ions bind to sites unmasked after preincubation at pH 10.5, and so allow contact and agglutination of the erythrocytes.

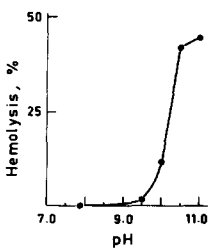

Fig. 3. Ca^{2+}-induced hemolysis of chicken erythrocytes as a function of pH. Experimental conditions as described in Fig. 1. At pH 7.8, 40 mM of Tricine–NaOH were used while at all higher pH 40 mM of glycine–NaOH served as the buffer solution.

Fig. 4. Electron micrographs of fused chicken erythrocytes. Fused cells were removed after 10 min from the 37° incubation bath and centrifuged at 500 x *g* for 10 min. The pellet obtained was fixed with 2% OsO_4 in a final concentration of 0.2 M sucrose, overnight in the cold. The fixed cells were centrifuged, dehydrated by transfer to increasing concentrations of ethanol and embedded in Epon as described by Luft[13]. The sections were cut on an LKB Ultrome III and stained with uranyl acetate and lead citrate as described by Reynolds[14]. Electron micrographs were obtained with Philips E.M. 300. A. Polynucleate erythrocyte (magnification x 7470). B. Polynucleate ghosts (magnification x 3780).


A
B

Of interest may be the observation that under similar conditions, Ca^{2+} also induced fusion of human erythrocytes (J. Laster and A. Loyter, unpublished results).

The agglutination and the lysis caused by Ca^{2+} are probably two separate phenomena. The agglutination could be mediated by changes in the negative surface charges caused by one of the several bivalent ions. The lysis might be caused by the saponification of the membrane phospholipids at high pH. Such saponification could form some lysophospholipid molecules at point of Ca^{2+} localization thus promoting lysis. At these points where erythrocyte membranes are already in contact and lysis is initiated, fusion could take place. Such a possibility fits the observation of Poole *et al.*[11] that lysolecithin can serve as a fusion inducer. A similar process might occur during the virus-induced fusion of mammalian cells. It seems to us that the present system in which fusion takes place in the absence of added virus might serve as a good model for elucidation of the molecular changes taking place within the membranes during the fusion process.

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