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# Electron microscopic study of the calcium phosphate-induced aggregation and membrane destabilization of cytoskeleton-free erythrocyte vesicles

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**Cytoskeleton-free vesicles derived from human erythrocytes were treated with trypsin, chymotrypsin, or neuraminidase followed by calcium, phosphate, or combined calcium/phosphate treatments in order to study the roles of cell surface proteins and glycoproteins in calcium/phosphate-induced cell aggregation and fusion. Vesicle aggregation (a necessary pre-cursor to membrane fusion) and subsequent membrane destabilization (an essential component of fusion) were examined by freeze-fracture electron microscopy. Enzymatic treatment alone had no effect on the morphology of the cytoskeleton-free vesicles. Neither did separate calcium nor phosphate treatments, although the treatment of the cytoskeleton-free vesicles with calcium did reduce their size slightly. Enzymatic pretreatment had no effect on the calcium-induced size changes. In contrast, the combination of calcium and phosphate drastically disrupted the membrane integrity of aggregated cytoskeleton-free vesicles at pH 7.8, although the effect was reduced at lower pH values. The extent of this membrane destabilization was independent of enzyme treatment. Our results indicate: (1) that the cell surface proteins and glycoproteins have only secondary effects on calcium/phosphate-induced cell aggregation and membrane destabilization, (2) that these processes primarily depend on the reaction between calcium and phosphate ions at the membrane surface, and (3) that cytoskeletal elements probably play no active (positive) role in the $Ca^{2+}/PO_4^{3-}$ induced erythrocyte membrane fusion process, apart from maintaining cell shape.**

## Introduction

The fusion of biological membranes is a complicated process, the molecular mechanism (s) of which is still not well understood. Previous studies on biological membrane fusion have led to a number of proposed molecular mechanisms, some involving the lipid components [1–6], and others involving membrane proteins [7 8] and cytoskeletal elements [9].

Recently, a model system consisting of large (0.5–1.0 $\mu$m) cytoskeleton-free membrane vesicles derived from intact human erythrocytes has been obtained [10]. These vesicles enable one to study membrane phenomena isolated from the effects of a cytoskeletal network. In a recent study of membrane aggregation induced by $Ca^{2+}/PO_4^{3-}$ treat-

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ment of cytoskeleton-free vesicle suspensions, it was found that membrane lipids played a pivotal role in $Ca^{2+}/PO_4^{3-}$ induced membrane vesicle aggregation, whereas membrane proteins affected the process to a much smaller degree [5,11]. However, it still remains to be determined whether calcium/phosphate induces the lateral aggregation of the proteins within the plane of the membrane during this process, or whether protein aggregation precedes the calcium/phosphate–lipid interactions. In addition, it is necessary to differentiate between the products of various $Ca^{2+}/PO_4^{3-}$ treatments of the cytoskeleton-free vesicles and to ascertain whether the contact area between the membranes is indeed protein-free. Such data are required to help elucidate the mechanism of $Ca^{2+}/PO_4^{3-}$-induced membrane aggregation and fusion.

To examine these questions, cytoskeleton-free vesicles were studied under various treatment conditions by freeze fracture electron microscopy. The effects of both calcium/phosphate, calcium alone, as well as in combination with trypsin, chymotrypsin, or neuraminidase treatment on the morphology and aggregation of the vesicles were assessed. Variations in the calcium/phosphate effect as a function of small pH changes (7.4–8.0) were also examined.

## Materials and Methods

*Preparation of cytoskeleton-free erythrocyte vesicles*

Fresh human blood was supplied by the Veteran's Administration Hospital, Buffalo, NY. The blood was used within 2–3 hours and not cooled below room temperature before use.

Cytoskeleton-free vesicles were produced and isolated from human erythrocytes according to the method of Leonards and Ohki [10]. Briefly, erythrocytes were repeatedly washed by centrifugation at room temperature in isotonic solution (150 mM NaCl) buffered with 10 mM *N*-(Tris(hydroxylmethyl) methyl) glycine (Tricine) ($NaT_r^-$, pH 7.4) followed by washing in Na-Tricine buffer containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF) ($NaT_r^+$, pH 7.4). The erythrocyte pellets (5–6 ml) were then diluted with $NaT_r^+$ to 40 ml and incubated at 45 °C. The large cytoskeleton-free vesicles were produced from the incubating erythrocytes by 'budding'. This was accomplished by titrating the cell suspensions with ethylenediaminetetraacetic acid (EDTA) and $CaCl_2$. The budding and vesiculation process was monitored microscopically. The titration procedure involved the addition of EDTA and $CaCl_2$ in increments, separated by 15-min incubation periods. Once approx. 50% of the cells were observed to be forming buds, EDTA and $CaCl_2$ addition were stopped, and the cell suspension was incubated another 15 min. At this point, the buds had been pinched off from the cells ('mother cells') and were free as large vesicles in the suspension. The vesicles could then be easily separated from the remaining intact cells, other cells, etc. by sucrose gradient centrifugation.

Enzymatic modification of the erythrocyte vesicle membrane proteins was carried out with (i) chymotrypsin (EC 3.4.21.1) (Sigma, Type II) by using the procedure of Siegel et al. [12], (ii) trypsin (EC 3.4.21.4) (Sigma, Type XI) according to the procedure of Elgsaeter and Branton [13], and (iii) neuraminidase (EC 3.2.1.28) (Sigma, Type V) as described by Nigg et al. [14]. Quantitative analysis of protein components of the membrane treated by various enzymes have been described in the previous papers. Incubation times and concentrations of $PO_4^{3-}$ (2 mM) and $Ca^{2+}$ (10 mM) followed the 'standard conditions' developed previously [5].

*Freeze-fracture electron microscopy*

All samples were rapidly frozen without cryoprotectant using the sandwich method of Boni and Hui [15]. An aliquot (0.1 $\mu$l) of the sample was placed between two thin copper plates, and the sandwich was accelerated and plunged into liquid propane. The samples were stored in liquid nitrogen until fractured. Freeze-fracturing was carried out at −120 °C in a Polaron E7600 unit evacuated by a Perkin-Elmer Ultek ultrahigh vacuum apparatus equipped with an ion pump, at vacuums of better than $8 \cdot 10^{-7}$ torr. Platinum-carbon was evaporated at 45 °C. Carbon was evaporated at 90 °C to the specimen surface. Electron microscopy was carried out using a Siemens 101 electron microscope at 80 kV. For vesicle size distribution analysis, about 50 vesicles were measured from each group of randomly chosen micro-

graphs. Sizes were measured by the maximal diameters which scale sterologically to the mean diameter by random sampling [16].

*Turbidity measurements for cytoskeleton-free vesicle aggregation*

$Ca^{2+}/PO_4^{3-}$-induced vesicle aggregation was monitored turbidimetrically. For a detailed discussion concerning the turbidity assay, its limitations, and the optimization of the experimental conditions for studying the aggregation of these vesicles, see Leonards and Ohki [5]. In brief, an aliquot of the stock erythrocyte vesicles was suspended in 0.15 M NaCl, 0.05 mM EDTA, and 2 mM phosphate of various pH values. After the vesicles were incubated for 10 min in the vesicle suspension solution at 30°C, a concentrated $CaCl_2$ solution (1 M) was added to the sample to give a final $Ca^{2+}$ concentration of 10 mM. The time-dependent turbidity changes were monitored continuously at 400 nm ($A_{400}$) with a double-beam spectrophotometer (Hitachi 100-60), equipped with a temperature-controlled cell housing connected to a water bath/pump (Neslab Instruments). The experimental temperature was kept at 30°C throughout.

## Results

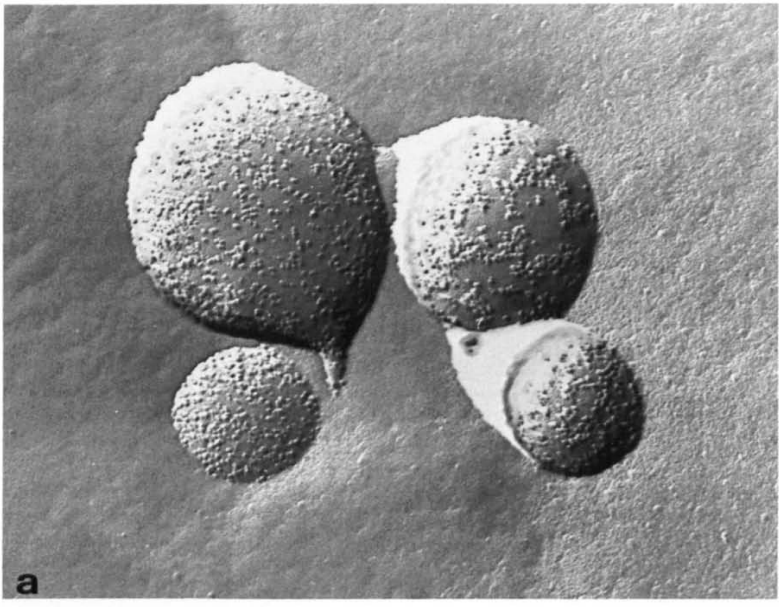
Freeze-fracture electron microscopy of cytoskeleton-free erythrocyte vesicles was carried out to study the effects of various treatment conditions on vesicle size distribution, intramembranous particle distribution, and aggregation and membrane integrity of these vesicles. Alteration of the vesicles's surface was accomplished by enzymatic treatment, specifically by modifying the membrane proteins, band 3 and glycophorin A. Chymotrypsin is able to attack both band 3 and glycophorin A at cleavage sites exposed on the exterior surface of the vesicle membrane. Enzymatic attack by trypsin is restricted to glycophorin A because these enzymes do not enter the vesicle [5]. Previous work on the enzyme treatments in this system confirmed the modifications of the vesicle surface as shown by SDS ($NaDodSO_4$) gel electrophoresis [5]. To investigate whether the carbohydrate portions, in particular the sialic acid residues of glycoprotein and glycolipids, modify the characteristics of the cytoskeleton-free vesicles, the vesicles were also treated with neuraminidase and compared to the controls.

Freeze-fracture electron microscopy revealed no alteration of the vesicle size distribution or intramembranous particle distribution upon treatment with chymotrypsin (Fig. 1b), as compared with the controls (Fig. 1a). Trypsin and neuraminidase treatments (micrographs not shown) also showed no differences in vesicle size distribution or intramembranous particle distribution from control vesicles. No difference was observed in the extent of vesicle aggregation between untreated vesicles in the absence of calcium/phosphate.

Phosphate and combined phosphate/enzyme treatments in the absence of calcium were compared. There were no modifications of vesicle size distribution, intramembranous particle distribution, or vesicle aggregation in phosphate-treated vesicles with respect to untreated vesicles. Also, there were no differences between phosphate alone and phosphate/chymotrypsin, phosphate/trypsin, and phosphate/neuraminidase treatments observed (micrographs not shown).

Analogous experiments were done with calcium in place of phosphate. An overall reduction in size was detected in calcium-treated and calcium/enzyme-treated cytoskeleton-free erythrocyte vesicles. The intramembranous particles in these vesicles were more aggregated. Fig. 1c shows calcium/chymotrypsin-treated vesicles. The vesicle size distributions of chymotrypsin-treated and calcium/chymotrypsin-treated vesicles were counted and are shown in Fig. 2a and b. A shift to smaller size vesicles upon calcium treatment is apparent with a mean diameter shifting from 0.40 to 0.35 $\mu$m. Similar vesicle size changes were observed upon calcium treatment irrespective of enzyme treatment. However, there was no evidence of vesicle aggregation or membrane destabilization under these conditions.

$Ca^{2+}/PO_4^{3-}$ treatment affected the morphology and integrity of the vesicles in a highly pH dependent manner (Fig. 3a, b). This is consistent with previous observations of human erythrocyte ghosts and intact chicken erythrocytes using phase contract microscopy [17,18]. A series of experiments were undertaken to investigate the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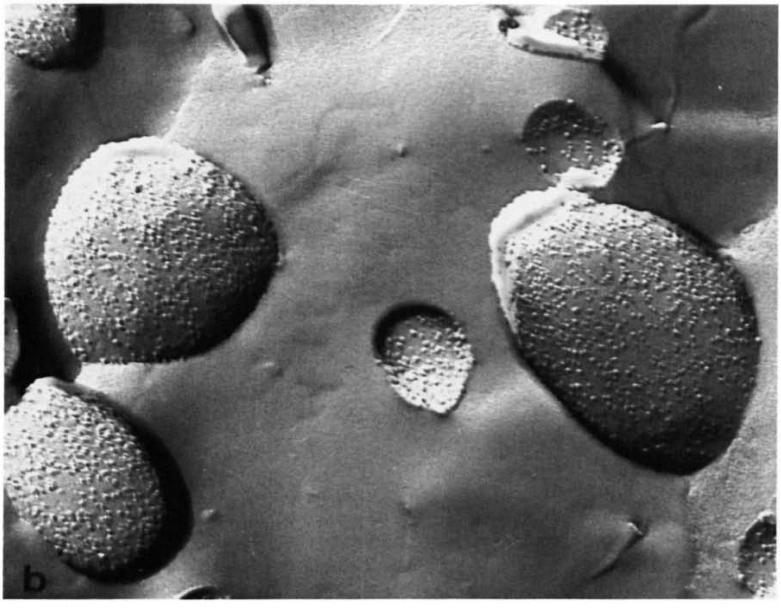
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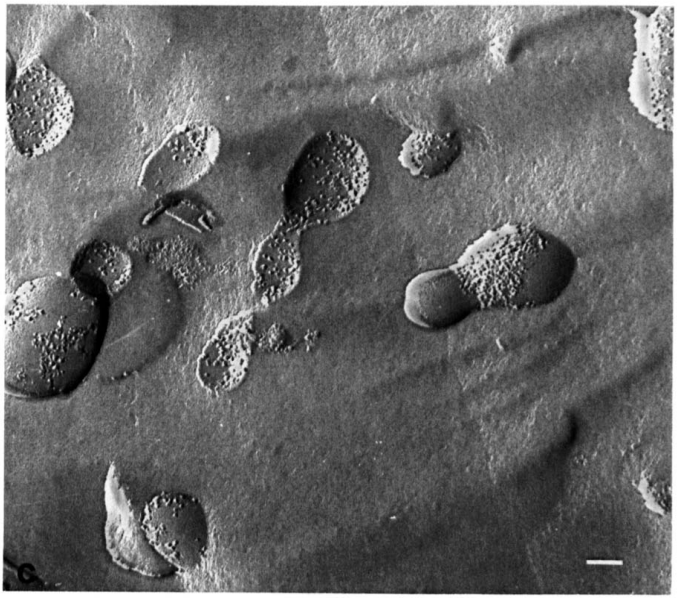


Fig. 1. Freeze-fracture electron micrograph of cytoskeleton-free vesicles (a) without prior treatment, (b) with chymotrypsin, and (c) with chymotrypsin and calcium (10 mM). Magnification bar equals 0.1 μm.

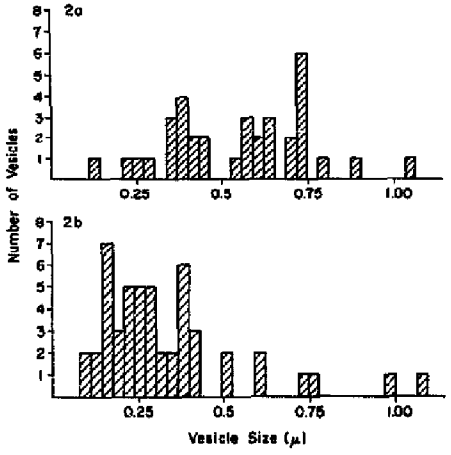
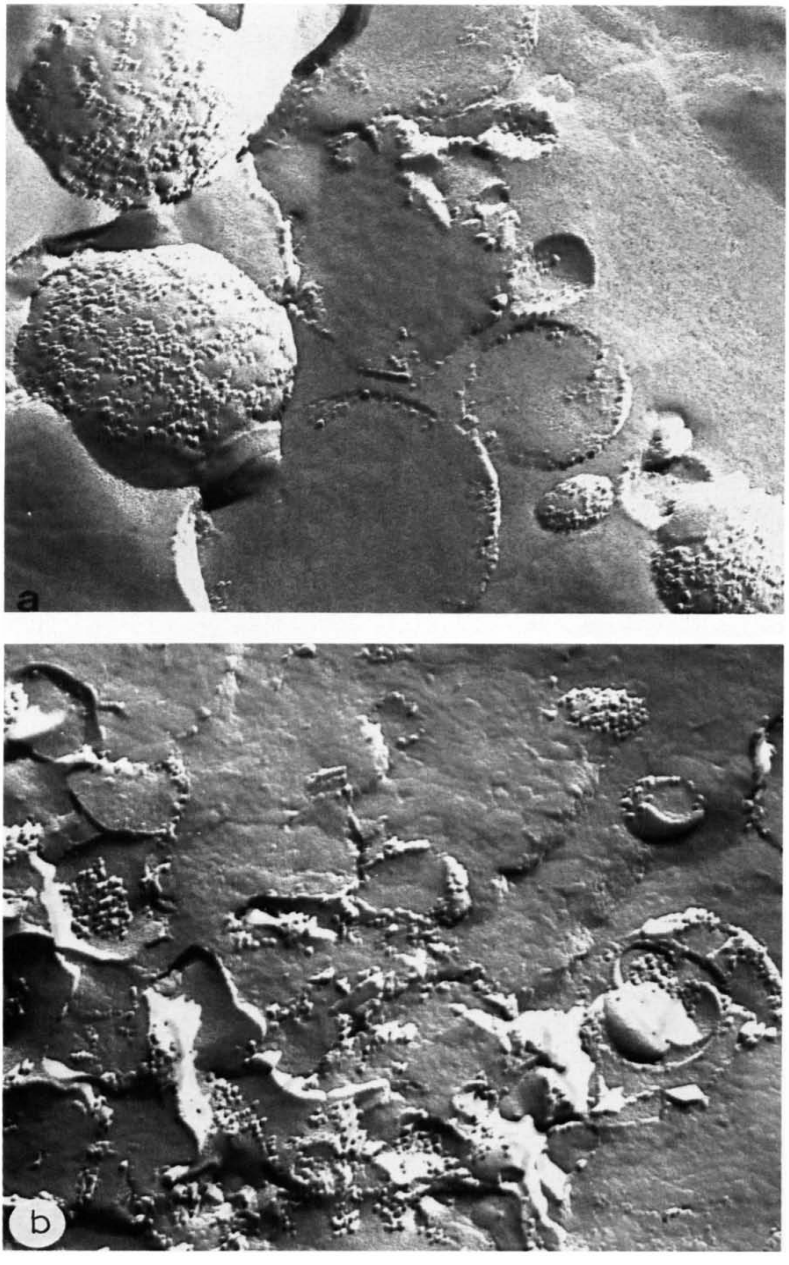



Fig. 2. Cytoskeleton-free vesicle size distribution of (a) chymotrypsin-treated and (b) calcium/chymotrypsin-treated samples.

effect of varying pH upon $Ca^{2+}/PO_4^{3-}$ effects on ultrastructural changes. Vesicle suspension (with pH values ranging from 7.4 to 8.0 in 0.2 increments) were prepared for analysis by freeze-fracture. A freeze-fracture electron micrograph of $Ca^{2+}/PO_4^{3-}$ treatment at pH 7.4 is shown in Fig. 3a. The shape of the vesicles is preserved though aggregation is occurring. Some degree of intramembranous particle aggregation is also apparent. Preparations at pH 7.6 are similar to those of pH 7.4, with respect to the extent of vesicle aggregation and disruption. Fewer intact vesicles can be seen at pH 7.8 (Fig. 3b). At pH 8.0, extensive disintegration and loss of vesicle integrity is observed (micrographs not shown). This is again consistent with the disintegrations of erythrocyte ghosts at pH 8.0 observed by others [17,18].

Vesicle size distributions were counted for the pH 7.4 and 7.8 suspensions, and are shown in Fig.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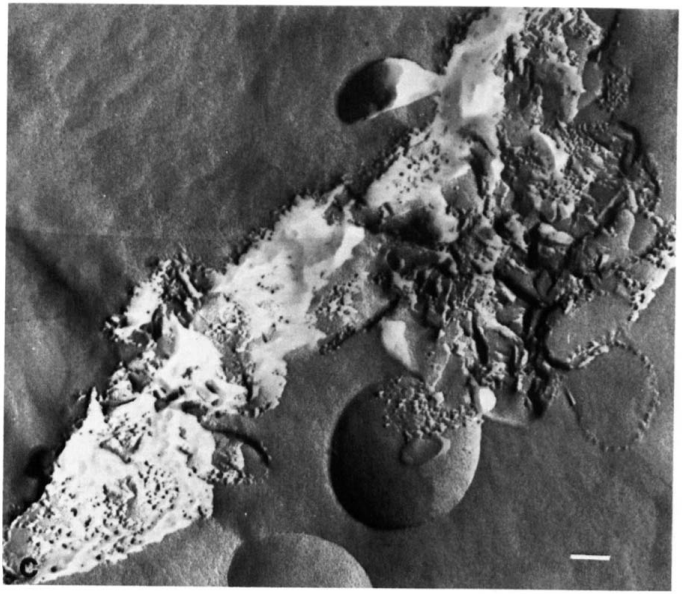

Fig. 3. Freeze-fracture electron micrographs of cytoskeleton-free vesicles under (a) $Ca^{2+}/PO_4^{3-}$ treatment at pH 7.4, (b) $Ca^{2+}/PO_4^{3-}$ treatment at pH 7.8, and (c) $Ca^{2+}/PO_4^{3-}$ and chymotrypsin treatment at pH 7.8. Magnification bar equals 0.1 $\mu$m.

4a and b. There is a slight increase in vesicle size from a mean diameter of 0.37 to 0.39 $\mu$m as the pH is raised, resulting in a bimodal distribution at pH 7.8. This suggests that some fusion among vesicles may have occurred. The small number, however, of intact vesicles observed at pH 7.8 limits the accuracy of the statistical results. Freeze-fracture electron microscopy revealed no differences with or without additional enzymatic treatments and between different enzyme treatments (Fig. 3c).

To augment the above electron microscopy observations, the turbidities of $Ca^{2+}/PO_4^{3-}$-treated vesicle suspensions were measured as a function of pH. The turbidity of $Ca^{2+}/PO_4^{3-}$-treated vesicle suspensions increased as the pH was raised over small increments (pH 7.0–8.0). Fig. 5a shows the time course of the net turbidity increase due to possible vesicle aggregation; the net turbidity equals the total turbidity of $Ca^{2+}/PO_4^{3-}$-treated vesicle suspension minus the turbidity of

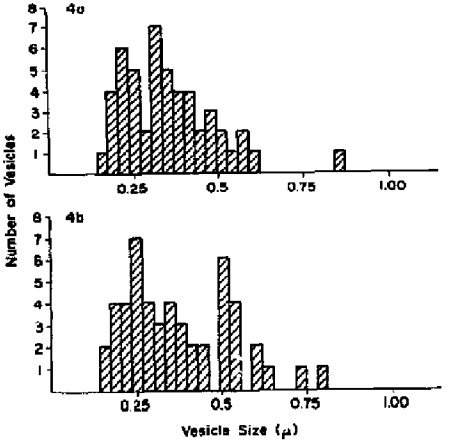


Fig. 4. Size distribution of calcium/phosphate-treated cytoskeleton-free vesicles at (a) pH 7.4 and (b) pH 7.8. Note that only a limited number of intact vesicles could be found for pH 7.8.

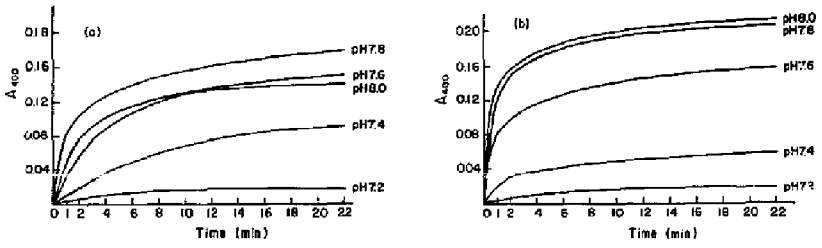


Fig. 5. Turbidity ($A_{400}$) of cytoskeleton-free vesicle suspensions in 2 mM $PO_4^{3-}$ + 10 mM $CaCl_2$ at different pH values as a function of time. (a) The net turbidity due to vesicle aggregation which is the total turbidity of $Ca^{2+}/PO_4^{3-}$-treated cytoskeleton-free vesicle suspensions minus the turbidity of $Ca^{2+}/PO_4^{3-}$ solution without cytoskeleton-free vesicles at the same experimental conditions. (b) The turbidity of $Ca^{2+}/PO_4^{3-}$ solution mentioned above. The experimental procedure is given in the Materials and Methods.

$Ca^{2+}/PO_4^{3-}$ suspension without vesicles under the same condition. The increase in vesicle aggregation (or turbidity) (Fig. 5a) parallels the turbidity increase of the $Ca^{2+}/PO_4^{3-}$ suspension which is due to $Ca^{2+}/PO_4^{3-}$ complex formation (Fig. 5b). It is noticed that the turbidity of the vesicle suspensions decreased slightly at pH 8.0 (Fig. 5a).

## Discussion

In this study, the effects of various treatments with $Ca^{2+}/PO_4^{3-}$ and proteolytic enzymes on the size distribution, intramembranous particle distribution, and aggregation, and membrane destabilization of cytoskeleton-free erythrocyte vesicles were evaluated using freeze-fracture electron microscopy. Our results closely correlate with previous turbidometric measurements of cytoskeleton-free vesicles [5], with the exception of the combined neuraminidase/ calcium/ phosphate treatment. When vesicles were incubated with either chymotrypsin or trypsin, no effect on the extent of vesicle aggregation induced by $Ca^{2+}/PO_4^{3-}$ was observed. However, neuraminidase treatments reduced both the rates and levels of aggregation of vesicle suspensions by 20% [5]. This reduction of vesicle aggregation was slight and may not be easily ascertained via the freeze-fracture microscopic observation.

Human and chicken erythrocytes and their ghosts have been induced to aggregate and fuse by combined phosphate/ calcium treatments [6,17,18]. Our results agree with their findings indicating some intramembranous particle aggregation and consequent production of smooth particle free area on the fracture face, which is also consistent with models of cell fusion [3]. Our results showing production of a bimodal vesicle size distribution at pH 7.8 in the presence of $Ca^{2+}/PO_4^{3-}$ are suggestive of vesicle fusion. Other authors [17,18] using phase contrast microscopy directly observed intact cell or ghost cell fusion at pH 7.5. In addition, Zakai et al. [17] and others [18] observed ghost cell disintegration shortly after fusion at pH 8.0. Both our results and theirs indicate that small pH changes lead to drastic alterations in vesicle integrity. Aggregation of vesicles was only observed at pHs higher than 7.0 (see Fig. 5). This pH dependency parallels those of $Ca^{2+}/PO_4^{3-}$ complex formations (see Fig. 5), indicating that the $Ca^{2+}/PO_4^{3-}$ complex formation is the key factor in vesicle aggregation and in the disruption of membranes. In our cytoskeleton-free vesicle system, it reduced the strength and stability of the membrane since there is no cytoskeletal protein. This may have caused the destabilization of the vesicle membrane observed at higher pH values. The membrane destabilization could significantly limit the number of intact vesicles available for the statistical analysis and mask the direct observation of membrane fusion.

The pH dependency of the freeze fracture results confirms the correlations of increased pH and turbidity measurements with the extent of vesicle aggregation (Fig. 5). Previous authors [6,17–19] have suggested that calcium/phosphate acted by modifying the cytoskeletal proteins of erythrocytes, and consequently induced vesicle ag-

gregation and fusion. Leonards and Ohki [5,10] and Ohki and Leonards [11] instead have suggested that vesicle aggregation and disruption are directly induced by the interaction of the $Ca^{2+}/PO_4^{3-}$ complex at the membrane surface without cytoskeletal proteins. A third alternative is that the cytoskeleton-free vesicles are permeable to complexed $Ca^{2+}/PO_4^{3-}$, but not $Ca^{2+}$ alone. Once inside the vesicle, the $Ca^{2+}$ or $Ca^{2+}/PO_4^{3-}$ complex would be able to interact with the inner monolayer surface of the membrane, inducing lateral phase separations and thus intramembranous particle aggregation as demonstrated by Gerritsen et al. [20]. All three mechanisms may result in producing particle free areas for membrane fusion, since we observed some intramembranous particle aggregation upon calcium/phosphate treatment in the absence of cytoskeletal proteins. Extensive alterations of the vesicular surface via enzymatic modification of the glycoproteins which are present in our system, band 3 and glycophorin A, and the glycolipids had no effect upon the calcium/phosphate-induced vesicle aggregation or intramembranous particle aggregation. This suggests that neither the cytoskeleton nor the proteinaceous components of membrane proteins and carbohydrate components of glycolipids and glycoproteins modifiable by chymotrypsin, trypsin, or neuraminidase play a major role (if any) in the phenomenon occurring under these experimental conditions.

The cytoskeleton-free vesicles maintained their structural integrity under treatment with either phosphate or calcium alone. However, notable vesicle size distribution changes were observed even with calcium treatment alone. Treatment with 10 mM $Ca^{2+}$ did break up some vesicles and induce the formation of intramembranous particle free areas. The changes, however, are minor, and the size change is opposite to the result of membrane fusion. In the absence of cytoskeletal proteins, this tendency for vesicle break-up with calcium is interesting. Since this applies to all samples with and without enzyme treatment, the mechanism is likely to be a calcium–phospholipid interaction.

No differences in vesicle size distribution were observed with phosphate treatment alone. Although binding of phosphate to vesicle membrane lipids is suspected to be the major component of calcium/phosphate-induced membrane aggregation [5,10,11], we observed no obvious effect of phosphate on the morphology of the vesicles. Alterations in the distribution of intramembranous particle by phosphate were not observed. This agrees with the suggestions previously made that any phosphate–membrane protein interactions are secondary and indirect [5], and are thus not revealed by freeze-fracture electron microscopy.

Based on morphological data presented here, cytoskeleton-free vesicle aggregation and membrane destabilization and therefore vesicle fusion occurs only with the combined action of $Ca^{2+}$ and $PO_4^{3-}$. Moreover, these processes are independent of membrane protein integrity and the cytoskeleton which is absent. If phosphate binding to the membrane is a precursor to the subsequent calcium/phosphate reaction, membrane proteins, as represented by the intramembranous particle distribution, do not seem to be altered by this phosphate binding. The pH sensitivity of membrane disruption by calcium/phosphate is closely linked to the precipitation characteristics of this salt. Thus, the final reaction is likely to be a reaction between calcium, phosphate, and membrane phospholipids [5,11].

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## References

1 Akhong, O.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature 253, 196–197.
2 Cullis, P.R. and Hope, J.J. (1978) Nature 271, 672–675.
3 Hui, S.W., Stewart, T.P., Boni, L.T. and Yeagle, P.L. (1981) Science 212, 920–922.
4 Nir, S., Bentz, J., Wilschut, J. and Düzgüneş, N. (1983) Progr. Surface Sci. 13, 1–124.
5 Leonards, K. and Ohki, S. (1984) Biochemistry 23, 2718–2725.
6 Zakai, N., Kulka, R.G. and Loyter, A. (1977) Proc. Natl. Acad. Sci. USA 74, 2417–2421.
7 Hong, K., Düzgüneş, N. and Papahadjopoulos, D. (1982) Biophys. J. 37, 297–305.
8 Morris, S.J., Sudhoff, T.C. and Haynes, D.H. (1982) Biophys. J. 37, 117–118.

9 Lalazar, A. and Loyter, A. (1979) Proc. Natl. Acad. Sci. USA 76, 318–322.
10 Leonards, K. and Ohki, S. (1983) Biochim. Biophys. Acta 728, 383–393.
11 Ohki, S. and Leonards, K. (1984) Biochemistry 23, 5578–5581.
12 Siegel, D.L., Goodman, S.R. and Branton, D. (1980) Biochim. Biophys. Acta 598, 517–527.
13 Elgsaeter, A. and Branton, D. (1974) J. Cell Biol. 63, 1018–1030.
14 Nigg, E.A., Gahmberg, C.G. and Cherry, R.J. (1980) Biochim. Biophys. Acta 600, 636–642.
15 Boni, L.T. and Hui, S.W. (1983) Biochim. Biophys. Acta 731, 177–185.
16 Weibel, E.R. and Bolender, R.P. (1973) in Principles and Techniques of Electron Microscopy (Hayat, M.A., ed.), Vol. 3, pp. 237–296, Van Nostrand-Reinhold, New York.
17 Zakai, N., Kulka, R.G. and Loyter, A. (1976) Nature 263, 696–699.
18 Majumdar, S. and Baker, R.F. (1980) Exp. Cell Res. 126, 175–182.
19 Baker, R.F. and Kalra, V.K. (1979) Biochem. Biophys. Res. Commun. 86, 920–928.
20 Gerritsen, W.J., Verkleij, A.J. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 555, 26–41.