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CRYSTAL-INDUCED INFLAMMATION: STUDIES OF THE MECHANISM OF CRYSTAL-MEMBRANE INTERACTIONS

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Abstract

Studies of the interactions of monosodium urate monohydrate (MSUM) crystals and calcium pyrophosphate dihydrate triclinic (CPPD) crystals with biomembranes have been reviewed. Crystal-membrane binding and crystal-induced membranolysis have been studied using human erythrocytes as a model membrane system. The extent of MSUM-membrane binding was determined by incorporating a hydrophobic, fluorescent probe into the membranes, centrifugation to separate free membranes from membranes with bound crystals and quantitation of free membranes by measuring the total fluorescence intensity. The ability of MSUM and CPPD to hemolyse red cells was used as a measure of the membranolytic potential of the crystals. Fluorescence polarization studies showed that MSUM-membrane binding resulted in fluidization of the membrane. Cross-linking of the membrane proteins of the erythrocyte or the presence of divalent cations in the incubation medium inhibited MSUM induced hemolysis. These findings were explained by hypothesizing a "pore" model mechanism for MSUM induced membranolysis as follows. Binding of crystals to membranes induces the redistribution of transmembrane proteins into clusters or aggregates leading to "pore" formation. The "pores" permit the leakage of low molecular weight soluble compounds and ions across the membrane which is followed by osmotic rupture of the membrane.

KEY WORDS: Crystal-induced arthritis, monosodium urate monohydrate, calcium pyrophosphate dihydrate crystal-membrane interaction, membranolysis, membrane fluidity, pore model.

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Introduction

Monosodium urate monohydrate (MSUM) crystals and calcium pyrophosphate dihydrate (CPPD) crystals produce the inflammatory reactions of acute gouty arthritis and acute pseudogout, respectively. Crystal-induced inflammation involves the interaction of the crystals with several cell types and inflammatory mediators and has been comprehensively reviewed by Gordon et al. (1988) and Terkeltaub at al. (1989). Of major importance is the interaction of the crystals with neutrophils and, based on the "rupture from within" hypothesis first proposed by Allison et al. (1966), the crystal-neutrophil interaction is thought to proceed as follows (Terkeltaub et al., 1989): Crystals with adsorbed proteins are phagocytozed by neutrophils and lie within phagosomes. Lysosomes fuse with the phagosomes to give phagolysosomes containing hydrolytic enzymes. The enzymes digest the protein coat on the crystal. There then follows a crystal-phagolysosomal membrane interaction resulting in membranolysis, cellular autolysis and inflammation.

The crystal-membrane interaction occurs in 2 consecutive steps, namely crystal-membrane binding followed by membrane lysing or membranolysis. An understanding of the mechanism of the crystal-phagolysosomal membrane interaction is of fundamental importance to understanding the process of crystal-induced inflammation. In this paper we have reviewed the studies of crystal-membrane interactions, including our recent data which provide some evidence for a "pore" model of crystal-induced membranolysis.

Membranolytic effects of MSUM and CPPD

Microcrystalline materials such as silica, asbestos, MSUM, CPPD, calcium oxalate and hydroxyapatite have the ability to cause hemolysis of erythrocytes and this has been used as a measure of the membranolytic potential of these crystals (Nash et al. 1966, Macnab and Harington 1967, Wallingford and McCarty, 1971, Weissner et al. 1986, Elferink 1986). Erythrocytes have been a very useful model system for our studies of the mechanism of crystal-membrane interactions.

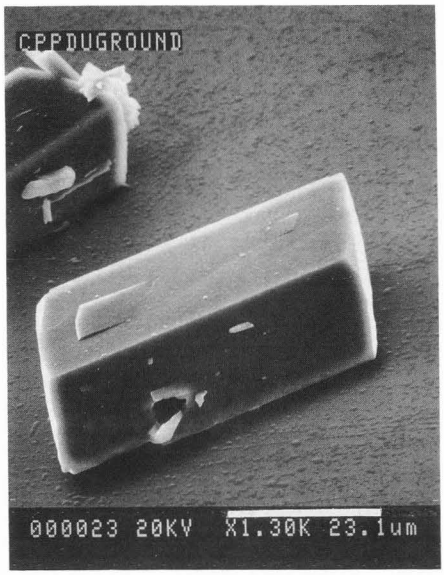

Figure 1. Scanning electron micrograph of CPPD crystal. Bar = 23.1 μm

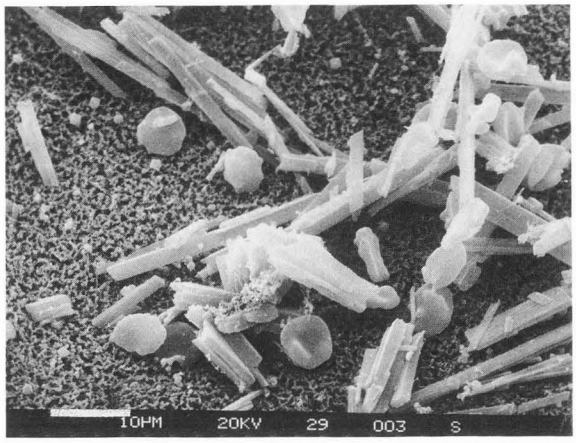

Figure 2. Scanning electron micrograph of MSUM-erythrocyte incubated at 37^{0}. Bar = 10 μm

Preparation and characterization of crystals

Crystals of MSUM were prepared by mixing sodium hydroxide and uric acid solutions at 55^{0} and pH 8.9 and leaving overnight (Denko and Whitehouse, 1976). Calcium acid pyrophosphate was synthesized using the method of Brown et al. (1963). Triclinic CPPD was prepared by mixing calcium acid pyrophosphate and calcium acetate and acetic acid solutions at 60^{0} and allowing the white gel to collapse and form crystals for 24 h (Burt and Jackson, 1987).

Large size fractions of MSUM and CPPD were obtained by sedimentation of crystal suspensions (Burt et al. 1989). Small size fractions of MSUM and CPPD were obtained by separating the cloudy supernatants following low speed centrifugation of MSUM and CPPD suspensions and recentrifuging the supernatants to collect the small crystals (Burt et al. 1989). Size distribution data for large and small size fractions were obtained using a computerized Leitz TAS Plus Image Analyzer (Burt et al. 1989).

The methods and results of MSUM and CPPD characterization using powder X-ray diffraction, differential scanning calorimetry and gas adsorption surface area analysis have been described by Burt et al. (1983) and Burt and Jackson (1987) respectively.

Figures 1 and 2 show the typical crystal habits of CPPD and MSUM crystals.

Membranolysis

Membranolysis experiments were carried out by incubating MSUM or CPPD crystals with red cell suspensions as previously described by Burt et al. (1983) and Burt and Jackson (1987).

MSUM crystals incubated with erythrocytes in 10 mM phosphate buffered saline were fixed in glutaraldehyde and 1% osmium tetroxide, dried by critical point drying, coated with gold and observed with a scanning electron microscope (Stereoscan 250T) (see Figure 2). Several of the erythrocytes show evidence of the initiation of echinocyte formation.

Both MSUM and CPPD crystals have been shown to cause significant hemolysis of erythrocytes (Burt et al. 1983, Burt and Jackson 1987). The maximal hemolytic effect of CPPD occurred between 8-11 h whereas maximum hemolysis took place in less than 2 h for MSUM crystals. The reason for the slower membranolytic effect of CPPD crystals is not known.

Wallingford and McCarty (1971) and Weissmann and Rita (1972) have suggested that crystal-membrane binding occurs through hydrogen bonding. Mandel (1976) studied the structure of MSUM and suggested that both a hydrogen bonding and an electrostatic interaction mechanism of MSUM association with membranes were theoretically possible. We have shown that MSUM and CPPD crystals possess highly negative surface potentials as evidenced by their high negative zeta potential values (Burt et al, 1983; Burt and Jackson, 1987) and have postulated that crystal-membrane binding may be mediated by the electrostatic mechanism first proposed by Mandel (1976).

Spin Labelling Studies

Electron spin resonance is an extremely powerful tool in the study of biological membranes and membrane dynamics. MSUM crystals were incubated with intact erythrocytes, labelled

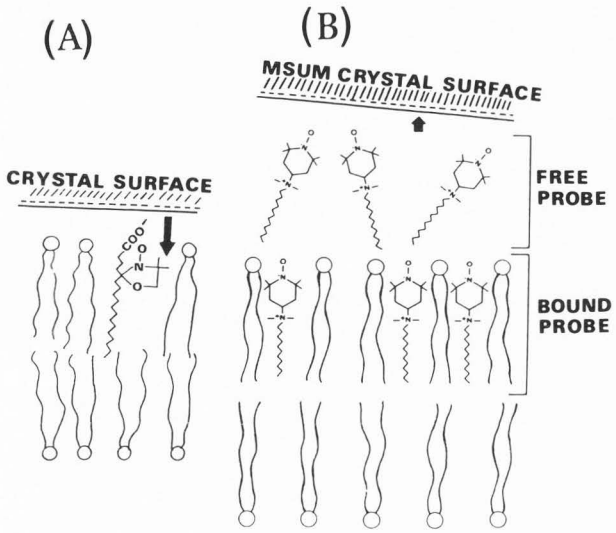

Figure 3. A schematic representation of the electrostatic interaction between the MSUM crystal surface and (A) 5-doxylstearate probe and (B) CAT_{12} probe. Arrows represent electrostatic repulsion between negatively charged MSUM and probe in (A) and electrostatic attraction between negatively charged MSUM and positively charged probe in (B).

with either a negatively charged 5-doxyl stearic acid probe or a positively charged, N-N-dimethyl-N-dodecyltempoylammonium bromide (CAT_{12}) probe and the ESR spectra recorded (Herring et al. 1986). There was an apparent increase in fluidity for doxylstearate labelled cells incubated with MSUM which was thought to be due to electrostatic repulsion between the negatively charged MSUM crystal surface and the anionic probe head group inducing a redistribution of probe from the outer more rigid layer to the more fluid inner leaflet of the bilayer via a flip-flop mechanism (see Figure 3A). MSUM also induced a change in the distribution of free (aqueous phase) and bound (membrane phase) CAT_{12} probe populations in erythrocytes. It was suggested that the increase in the % free probe observed was due to an electrostatic attraction between the negatively charged MSUM crystal surface and the membrane bound cationic probe inducing its redistribution into the aqueous phase (see Figure 3B). In support of Mandel's hypothesis (Mandel, 1976) we proposed that the mechanism leading up to membrane lysis was an electrostatic interaction of the surface of the negatively charged MSUM crystal with charged membrane components.

Fluorescence Polarization Studies

Membrane fluidity is the reciprocal of membrane viscosity and is a fundamental property of membranes, regulating enzyme activities, transport functions, cell fusion and many other processes (Houslay and Stanley, 1982). Based on evidence that alterations in membrane fluidity are triggered when membranes are stimulated by, or interact with, both physiologic perturbants (Shattil et al. 1977) and non-physiologic perturbants (Deliconstantinos et al. 1987, Berlin and Fera 1977) we felt that a study of fluidity changes in membranes bound to MSUM could increase our understanding of the mechanism of MSUM-induced membranolysis. The efficient fluidity probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was incorporated into erythrocyte ghost membranes and incubated with MSUM. Crystals bound to membranes were separated from unbound membranes by low speed centrifugation and analysed by fluorescence polarization to obtain fluorescence anisotropy values (Burt and Jackson, 1988). The problem of light scattering depolarization (decreased anisotropy values) from the suspension of membranes and crystals was addressed by using only membranes bound to crystals, resuspended at extremely low concentrations, where the effects on anisotropy values were negligible. The fluorescence anisotropy of ghosts with bound MSUM was significantly lower than for free ghosts, indicative of an increased membrane fluidity (Table 1). We proposed that this was the result of a redistribution of membrane proteins into clusters or aggregates and the partitioning of DPH into the more disordered and hence more fluid lipid regions associated with protein clusters. The following mechanism for MSUM crystal-induced membranolysis was hypothesized. Binding of MSUM crystals to membranes induces protein lateralization and the redistribution of membrane proteins into aggregates. The increased fluidity of phospholipids associated with the protein aggregates could confer an increased permeability, and disturbance of ion gradients across the membrane may be followed by osmotic rupture of the cell.

Table 1. Anisotropy values for ghosts bound to MSUM compared to free ghosts at different temperatures.

Temp (^{o}C)	Anisotropy of ghosts bound to MSUM	Anisotropy of free ghosts
25	0.234 ± 0.005	0.266 ± 0.002
30	0.217 ± 0.005	0.250 ± 0.006
33	0.210 ± 0.007	0.242 ± 0.007
37	0.190 ± 0.007	0.234 ± 0.13

Membrane Protein Aggregation

When erythrocyte membranes are freeze-fractured along their hydrophobic interior, characteristic intramembrane particles (IMP) are

seen which are primarily formed by the integral membrane proteins of erythrocytes, band 3 and glycophorin. The IMP of human erythrocytes may be aggregated by various treatments and many earlier studies have addressed the effects of pH, ionic strength, glutaraldehyde cross-linking, neuraminidase digestion and spectrin depletion on IMP aggregation in erythrocyte membranes (Pinto da Silva 1972, Nicolson 1973, Elgsaeter and Branton 1974, Elgsaeter et al. 1976).

The aggregation of protein components in membranes has also been implicated in the stimulation of and secretion by cells interacting with various membrane perturbants. Jacques and Ginsberg (1982) studied MSUM-induced platelet secretion and showed that MSUM bound certain membrane glycoproteins in platelets. MSUM-induced platelet secretion could be inhibited by either removing the glycoproteins from the cell surface or using anti-glycoprotein antibodies. They hypothesized that the crystal interaction with membrane proteins triggers cells by binding to membrane proteins and causing a clustering of proteins in the plane of the membrane. The bee venom polypeptide, mellitin, causes disruption and lysis of erythrocyte membranes. Demin (1978) showed by freeze-fracture that mellitin induced significant aggregation of IMP. Subsequent work has confirmed that mellitin and other polyvalent cationic species cause aggregation or clustering of the major red cell integral protein, band 3, in the plane of erythrocyte membranes (Dufton et al. 1984a, Clague and Cherry 1988, Clague and Cherry 1989). Dufton et al. (1984b) demonstrated a correlation between the capacity of mellitin, polylysine and other synthetic peptides to aggregate band 3 protein in erythrocyte membranes and their ability to stimulate the release of 5-hydroxytryptamine from mast cells. They proposed that the peptides trigger secretion from mast cells by aggregation of protein components within the membrane.

Several investigators studying crystal-membrane interactions have proposed that membrane proteins may play a role in crystal-induced membranolysis (Weissner et al. 1986, Kozin et al. 1982, Elferink 1986, Harington et al. 1975, Leyko and Gendek 1985). Harington et al. (1975), proposed a mechanism for asbestos fibre-induced hemolysis where the fibres bound electrostatically with erythrocyte membrane sialoglycoproteins resulting in the formation of protein clusters in the membrane. These protein-rich regions of the membrane would have an increased permeability to small ions and a Donnan redistribution of ions could lead to osmotic rupture of the cell.

Red Cell Hemolysis and the "Pore" Model

A "pore" model has been invoked to describe the action of agents such as mellitin and polypeptides which possess the ability to aggregate band 3 protein and hemolyse red cells. Formation of protein aggregates in the membrane provides a site for increased leakage of ions across the membrane (i.e. a "pore") and mellitin-induced hemolysis occurs by osmotic rupture of the cell (Bashford et al. 1988).

Membrane Protein Cross-Linking Studies

Glutaraldehyde pretreatment of erythrocytes has been shown to prevent acidic pH induced IMP aggregation (Pinto da Silva 1972, Nicolson 1973). Since glutaraldehyde affects the ability of membrane proteins to aggregate and presumably therefore inhibits protein mobility, we felt that an investigation of the effects of protein cross-linking agents on MSUM-induced hemolysis could provide information on the role of membrane proteins in the mechanism of crystal induced membranolysis (Burt et al. 1990, Burt and Jackson 1990). Three membrane permeable cross-linking agents were used, glutaraldehyde, dimethyl adipimidate hydrochloride (DMA) and dimethyl suberimidate hydrochloride (DMS). Washed, intact, human erythrocytes (1.5×10^8 cells /mL) were added to glutaraldehyde in phosphate buffered saline (0.001-0.015%), DMA in isotonic Tris buffer, pH 9.2 (0.5 - 3mM) or DMS in isotonic Tris buffer, pH 9.8 (1-10mM). Following incubation at 25^o for 45 min, cells were washed and membranolysis experiments carried out as previously described (Burt and Jackson, 1987) with a red cell concentration of 2.8×10^7 cells/mL and MSUM concentration of 20 mg/mL. The percent hemolysis values for cross-linker pretreated cells and untreated cells were calculated and the results expressed as the percent inhibition of lysis of cross-linker pretreated cells relative to untreated cells. The effect of the pretreatment of cells with different cross-linking agents on the extent of binding of MSUM crystals to membranes was determined using the fluorescence method of Burt and Jackson (1988). The binding results were expressed as % ghosts bound to 10 mg MSUM calculated from 100 - (% ghosts remaining in the supernatant).

Figure 4a and 4b show that there was a concentration dependent inhibition of lysis for all 3 cross-linking agents. There was no resistance of the pretreated cells to distilled water lysis over the entire range of cross-linker concentrations and no difference in binding of membranes to MSUM for cross-linker treated versus untreated membranes. Gel electrophoresis studies (Burt et al. 1990, Burt and Jackson 1990, Ji 1973, Steck 1972) have shown that glutaraldehyde, DMA and DMS result in cross-linking of erythrocyte cytoskeletal proteins and cross-linking of the cytoskeleton with other proteins such as band 3. We have proposed that as the concentration of cross-linking agent used to pretreat the cells is increased, membrane protein cross-linking becomes more extensive, the degree of lateral mobility of band 3 and possibly also glycophorin becomes greatly reduced and the MSUM crystal-membrane binding induced formation of protein aggregates in the membrane is inhibited. Thus the formation of "pores" in the membrane is inhibited and this would then result in an inhibition of lysis since the "pores" are believed to be sites of increased ion flow across the membrane which ultimately leads to osmotic lysis of the cell.

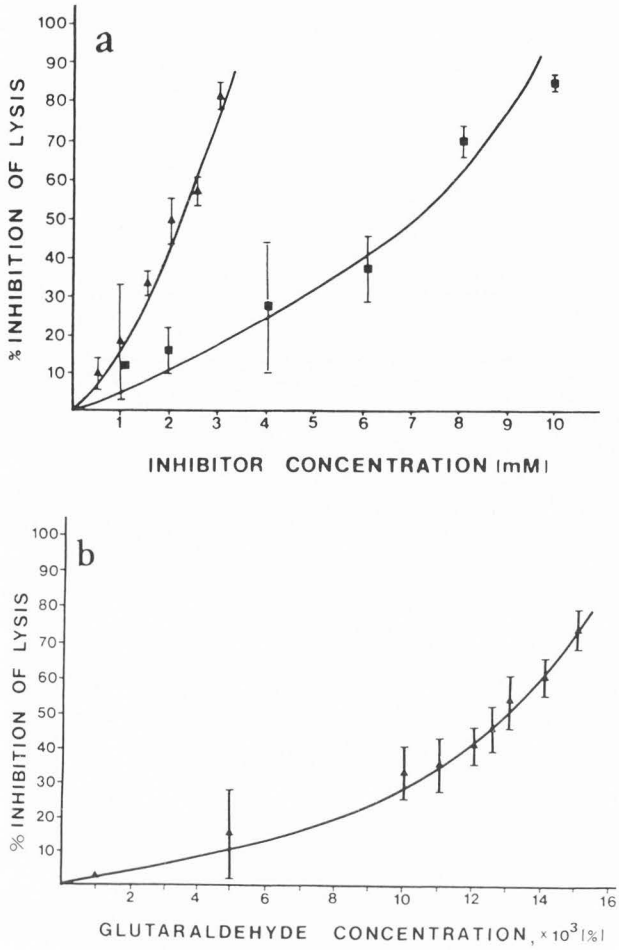

Figure 4. Effect of pretreatment of erythrocytes with cross-linking agents on MSUM-induced hemolysis. a. Effect of increasing inhibitor concentration on the percent inhibition of lysis for DMA pretreated erythrocytes (▲) and DMS pretreated erythrocytes (■). b. Effect of increasing glutaraldehyde concentration on the percent inhibition of lysis.

Mechanism of MSUM-Induced Hemolysis: a "Pore" Model Hypothesis

Our "pore" model hypothesis describing the mechanism of MSUM crystal-induced membranolysis can be stated as follows: binding of crystals to membranes induces the redistribution of transmembrane proteins into clusters or aggregates leading to "pore" formation. The "pores" permit the leakage of low molecular weight soluble compounds and ions across the membrane which is followed by osmotic rupture of the membrane.

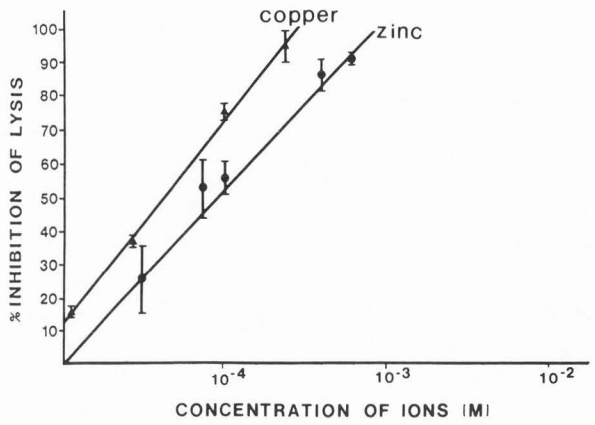

Figure 5. Effect of increasing concentrations of Cu^{2+} and Zn^{2+} on the percent inhibition of lysis.

Effect of Divalent Cations on MSUM-Induced Hemolysis

Bashford et al. (1986, 1988) found that mellitin and polylysine induced red cell lysis could be inhibited by divalent cations. At concentrations less than 10^{-4}M, zinc, cadmium and copper inhibited polypeptide induced hemolysis. Although the mechanism remains unclear, they suggested that the divalent cations may bind to negatively charged sites in the membrane protein aggregates, reducing the repulsive forces between the proteins and thus effectively reducing the "pores" to a non-functional size where no leakage could occur, therefore inhibiting hemolysis.

We determined the effect of copper and zinc ions on MSUM induced hemolysis. Solutions of each metal ion were prepared from its salt in isotonic Hepes buffer at concentrations between 5 x 10^{-5} to 8 x 10^{-4} M Zn^{2+} and 2.5 x 10^{-6} to 4 x 10^{-4} M Cu^{2+}. Membranolysis experiments were carried out as previously described (Burt and Jackson, 1987) with a red cell concentration of 2.8 x 10^{7} cells/mL and MSUM concentration of 25 mg/mL. The results were expressed as % inhibition of lysis of cells in the presence of divalent cations relative to lysis in the absence of cations. The effect of the presence of Zn^{2+} and Cu^{2+} on the extent of binding of MSUM crystals to membranes was determined using the fluorescence method of Burt and Jackson (1988). Zn^{2+} and Cu^{2+} in solution both significantly increased the binding of MSUM crystals to membranes. However, despite the increased binding, Figure 5 shows that Zn^{2+} and Cu^{2+} inhibited MSUM induced hemolysis over the concentration ranges studied. There was no significant difference between the percent inhibition of lysis values for the two cations. The mechanism of Zn^{2+} and Cu^{2+} inhibition of MSUM-induced hemolysis may be similar to that proposed by Bashford et al. (1986, 1988) for the divalent cation inhibition of pore forming agent induced membrane damage. Clague and Cherry

(1986) suggested that a mechanism for the inhibitory effects of metal ions such as Zn^{2+} could involve the competition of metal ions with the lytic agent to aggregate integral membrane proteins into two different conformations, only one of which is a "pore".

We believe our results are consistent with a "pore" model hypothesis for MSUM induced membranolysis.

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Discussion with Reviewers

R. Tawashi: The authors observed evidence of transformation of erythrocytes to echinocytes (Fig. 2) when the erythrocytes were incubated with MSUM in phosphate buffer. How can the authors explain this transformation? Is it due to the MSUM crystals or to the SEM preparation of the specimen?

Authors: There are many conditions which may induce echinocyte formation. For example, agents such as fatty acids, bile acids and some anionic drugs, ATP depletion and increased intracellular calcium are all factors which can cause the transformation of discocytes into echinocytes. Control specimens (cells in buffer alone) also contained some echinocytes. Therefore we believe that echinocyte formation is probably induced by physicochemical or physiological stresses and not by SEM specimen preparation.

Reviewer I: The new data presented in this study suggest that certain agents that crosslink protein groups in membranes (including glutaraldehyde) decrease hemolysis to monosodium urate but not to distilled water. These experiments need to be more carefully controlled (for example, using Triton X-100, activated complement etc.).

Authors: The purpose of lysing the cells in distilled water was to obtain the 100% lysis absorbance value so that the percent hemolysis values could be determined. Distilled water lysis for crosslinker treated cells was not decreased and therefore the pretreatments of cells with crosslinking agents have not affected the ability of the cells to respond to conditions of hypotonic stress. The percent lysis values for untreated and crosslinker treated cells could be compared because the 100% lysis absorbance values for untreated and treated cells were the same and control absorbances (MSUM absent) for all incubations were less than 3% of the absorbances of 100% lysed cells.

Reviewer I: The authors show that copper and zinc ions decrease MSUM-induced hemolysis of red blood cells but do not decrease binding of the crystals to the red blood cells. These studies need to be controlled as Bashford's studies were. In addition, before concluding that the mechanism of hemolysis might be similar to that advanced by Bashford, the authors should demonstrate that the order of potency of divalent cations is similar with both controls and urate crystals and that the rate of hemolysis demonstrates similar changes by changing the ionic strength of the external medium.

Authors: The major difference between Bashford's studies using various hemolytic agents and our studies using crystals, is that initially the crystals must bind to the red cell membrane which is then followed by disruption of the membrane. This is a variable not encountered in Dr. Bashford's studies. The extent of crystal-cell membrane binding and hence the extent of crystal induced lysis are strongly influenced by univalent and divalent cations and by ionic strength. We found that Cu^{2+} and Zn^{2+} decreased hemolysis even though binding was increased. Due to the variable effects of different cations and changing ionic strength on the extent of crystal-red cell binding, we cannot compare crystal induced lysis with other agent induced hemolysis, nor can we determine the order of potency of

different cations or the effect of ionic strength on crystal induced hemolysis.

Reviewer I: In MSUM-induced hemolysis, mechanisms may be coming into play that are not accounted for by the hypothesis advanced. For example, urate crystals may simply insert into and physically distort the plasma membrane, or possibly activate small amounts of phospholipases present in red cell membranes, generating lysophospholipids with detergent-like effects. The authors should consider dealing with such possibilities with more detailed experimentation.
Authors: Previous studies have shown that crystal-induced hemolysis of red cells was not merely due to the mechanical impact of crystals on red cells because there were no differences in the lysis produced by a tumbling or a stationary system (text references, Burt et al 1983 and Burt and Jackson 1987). In addition, hemolysis is almost completely abolished by coating crystals with serum proteins and yet this does not interfere with the physical interaction of the crystals with cell membranes.

Other mechanisms which could explain our data showing the inhibition of lysis by protein crosslinking agents could include inactivation of enzymes such as phospholipases, as the reviewer suggests, or a disruption of ion transfer functions of the major integral membrane proteins. Work on these aspects is in progress.

K.P.H. Pritzker: Although there appears to be differences between calcium pyrophosphate and monosodium urate in their ability to make membranes more permeable, the authors have not attempted to utilize scanning microscopy to demonstrate the changes. Comment on the feasibility of observing the changes by scanning electron microscopy.
Authors: We have had great difficulty showing representative crystal-cell membane interactions using SEM which we believe is probably due to disruption of the interaction during specimen preparation. We are currently attempting to use freeze fracture techniques and transmission electron microscopy to visualise the integral proteins of the membrane. However, again, we are experiencing difficulty with specimen preparation for membranes with bound crystals.

K.P.H. Pritzker: The authors' findings, important as they are, are limited by the methods of using crystals the surfaces of which are devoid of other substances. Within the body, the crystal surfaces will almost certainly have adsorbed substances.
Authors: It is known that the crystals adsorb proteins and other components from synovial fluids, serum etc. and that their surface properties are altered. Adsorbed proteins greatly reduce MSUM induced hemolysis and for our studies with crosslinking agents, Cu^{2+}, Zn^{2+} etc., we required extensive hemolysis of cells by the crystals so that we could determine the extent of inhibition of crystal induced lysis by the inhibitory agents. In future work, we will be addressing the effect of proteins on the crystal-membrane interaction.

K.P.H. Pritzker: In the review, the authors correctly state that cell membrane disruption begins within the phagolysosome. However, initially crystals are phagocytozed into phagosomes. At this stage membrane disruption does not occur but presumably the quality of crystal-membrane interaction should not be different between the phagosome and the phagolysosome. Can the authors account for the differences?
Authors: Following phagocytosis, formation of the phagosome and subsequent fusion of lysosomes with the phagosome, it has been hypothesized that the proteins adsorbed to the crystals may be digested by lysosomal enzymes in the phagolysosome, allowing an interaction to take place between the uncoated crystal and the phagolysosomal membrane. Thus in the phagosome, the phagosomal membrane may be "protected" from the lytic effect of the crystals by the presence of adsorbed proteins on the crystal surface.

We also agree with the reviewer that an alternative hypothesis which would account for the specificity of reaction in the phagolysosome as opposed to the phagosome is that enzymatic release of substantial amounts of sodium from MSUM or calcium from CPPD may provide a high concentration of ions which then leads to osmotic rupture.

P. Halverson: Is there any biomolecular evidence of cell activation through membrane protein aggregation i.e. inositol phosphate, cyclic AMP etc?
Authors: Independent of our crystal-membrane interaction studies using erythrocytes, we are currently investigating neutrophil activation by crystals, monitoring increases in calcium concentration, inositol phosphate concentrations etc.

P. Halverson: Are similar data obtained with protein coated crystals?
Authors: This will be the subject of future work.

P. Halverson: Are there differences in membrane protein aggregation found with crystals that are not associated with much inflammation eg. hydroxyapatite crystals?
Authors: Other investigators have shown hydroxyapatite to be strongly membranolytic following incubation of hydroxyapatite crystals with red cells. However we have not, as yet, extended our studies to include hydroxyapatite.