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Membrane Effects of Imidoesters in Hereditary Stomatocytosis

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The marked increase in cation (Na^+, K^+) permeability that results in swollen, cup-shaped red cells in the hereditary stomatocytosis syndrome can be corrected in vitro with a bifunctional crosslinking reagent, dimethyl adipimidate (DMA). ^{45}Ca influx in intact RBC, ^{45}Ca efflux in red ghosts, and ^{45}Ca retention in red ghosts are normal and not influenced by DMA. Endocytosis in resealed red ghosts is strikingly impaired but becomes normal if cells are first treated with 2 mM DMA. Protein kinase mediated phosphorylation of membrane proteins by $AT^{32}P$ – only 20–40% of normal control values in both short-term (5 min) and more extended (60 min) incubations – is not improved by DMA. After reaction of ^{14}C-DMA with stomatocytes, radiolabel is found associated with phosphatidyl serine and phosphatidyl ethanolamine and is also widely distributed among membrane proteins. Cation permeability of stomatocytes is corrected at DMA concentrations (1 mM) that result in barely detectable crosslinking of aminophospholipids or proteins, suggesting that either crosslinking of a minor component present in only small quantities or intramolecular (rather than intermolecular) crosslinking is responsible for the permeability effects. DMA, whose maximal crosslinking dimension is 7.3–9 Å, is the most effective bifunctional imidoester of those tested. Shorter (dimethyl malonimidate) or longer (dimethylsuberimidate) reagents are either less effective than DMA or totally without effect.

Key words: endocytosis; abnormality in stomatocytosis; hemolytic anemia, associated with hereditary stomatocytosis; stomatocytosis, hereditary; imidoesters, effects in stomatocytosis

Stomatocytes, or cup-shaped red cells, are the primary morphologic abnormality in the blood of individuals with hereditary stomatocytosis, a congenital and sometimes familial form of moderate to severe hemolytic anemia.

Although several earlier reports commented on the association between stomatocytes

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and hemolytic anemia, Zarkowsky and his co-workers were the first to describe this disorder in biochemical terms [1]. They, and others subsequently, found a near-complete reversal of the usual monovalent cation composition of the erythrocyte [1–4]. Such abnormalities have proved to be the result of remarkable increases in passive cation permeability. Potassium permeability is increased 3- to 20-fold, and sodium permeability is even higher. These increases are, to some extent, counterbalanced by equally remarkable increases in active transport supported by markedly accelerated erythrocyte glycolysis. However, when optimal conditions for glycolysis do not exist, the rate of active transport diminishes, the cation permeability lesion becomes predominant, and there may be rapid passive movement of sodium into the cell and efflux of potassium. Since the rate of sodium movement exceeds that of potassium, there is a net increase in cell cation content, leading to an obligate osmotic increase in cell water, cell swelling, and eventually cell lysis [2].

We have previously shown that the cation permeability defect can be repaired in vitro by chemical modification of stomatocytes with imidoesters [5]. In one patient with severe hemolytic anemia, reaction of stomatocytes with dimethyl adipimidate (DMA) reduced ouabain-associated potassium loss from 15 to 1.7 and sodium gain from 22 to 2.5 mEq per liter of red cells per hour. Red cell volume, cation concentration, and deformability, previously abnormal, rapidly became normal after stomatocytes were reacted with DMA. Instead of stomatocytes, normal red cells and target cells were noted on both light microscopy and scanning electron microscopy. The survival (half-time) of ^{51}Cr-labeled DMA-treated stomatocytes infused into rats rendered tolerant to human erythrocytes by pretreatment with ethyl palmitate and cobra-venom factor was double that of untreated stomatocytes. Thus, chemical modification of the defect in vitro allowed stomatocytes to regain many properties of normal erythrocytes and favorably influenced the subsequent survival of these cells in vivo.

The studies of endocytosis and calcium metabolism described in this report further characterize the effect of imidoesters on intact cells or membrane ghosts. In addition, attempts to determine the location and nature of the reaction between imidoesters and the stomatocytic erythrocyte membrane are described. These observations confirm the potential of imidoesters as chemical probes, of value in defining the nature of the membrane defect in hereditary stomatocytosis.

METHODS

After informed consent was obtained, heparinized blood was drawn from an eight-year-old splenectomized boy [2, 5] with hereditary stomatocytosis and persistent hemolytic anemia. Blood was also obtained from normal controls and patients with reticulocytosis due to sickle cell anemia, glucose phosphate isomerase deficiency, or glucose-6-phosphate dehydrogenase deficiency. The reaction between red cells and imidoesters was carried out as previously described [5]. Imidoester was added in dry powder form to heparinized, washed cells which were then incubated at 37° in Krebs-Henseleit buffer, pH 7.4, with added 10 mM glucose and 1% bovine serum albumin at a hematocrit of 5% for six hours. After incubation the cells were washed in isotonic Tris-Mg Cl_2, and red cell Na^+ and K^+ content were determined by flame photometry [5]. Shorter incubation periods were used in some experiments. Endocytosis was studied in red ghosts prepared from cells incubated with or without imidoester. The ghosts were resealed with 2.5 mM ATP, 2.5 mM Mg^{++}, and 1.0 mM Ca^{++} present in the hemolyzing solution [6]. After resealing, ghosts were incubated for 15 min and then fixed for transmission electron microscopy [6]. Calcium

influx (^{45}Ca) was measured in red cells that had been metabolically depleted by incubation for 90 min with 1 mM iodoacetate. The procedure used was that described by Wiley et al [7], except that 5 mM adenosine was substituted for 5 mM inosine in the initial metabolic depletion step and the final concentration of Ca^{++} was 1.0 mM instead of 1.5 mM. Efflux of ^{45}Ca was measured in resealed red ghosts, as described by Schrier and his co-workers [6]. At the conclusion (total incubation time, 20 min) of each efflux experiment, residual ^{45}Ca in the ghost pellet was also determined [6]. To evaluate the stability of the permeability effect of DMA, red cells were first incubated with imidoester (2 mM DMA) for 6 h (using the standard conditions described above) to restore normal cation and water content. As a control, cells were also incubated under identical conditions with sucrose (2 mM) instead of DMA. After incubation, the buffer was removed; the red cells were resuspended in fresh autologous plasma with added acid citrate dextrose (NIH formula "A" 0.6 cc/4 cc plasma), penicillin (12.5 μ/ml blood), and streptomycin (12.5 μgm/ml blood); and the cell-plasma mixture (hematocrit 20–25%) was stored at 4°C. At intervals, aliquots were removed for measurement of cell size, cation (Na^+, K^+) content, and water content [5]. At the end of two weeks of storage, hemolysis of normal red cells or stomatocytes was less than 1%.

To determine relative binding of imidoester to the cell membrane or to hemoglobin, red cells were incubated for 6 h with ^{14}C-DMA (specific activity 2.52 mCi/mM). After lysis, radioactivity present in ghosts or hemoglobin obtained from 1 ml of packed red cells was measured. Samples were thoroughly solubilized (Protosol) and bleached with H_2O_2 prior to analysis by B scintillation spectrometry.

Red cell ghosts were prepared using 5 mM phosphate buffer, pH 8.0, as described by Fairbanks and his associates [8]. For lipid analysis, ghosts were extracted with chloroform-methanol 1:1 v/v [9] and back extracted with 0.02 M KCl. The resulting extract was spotted on silica gel thin layer chromatography (TLC) plates. The usual developing solvent consisted of chloroform:methanol:ammonium hydroxide:water (35:15:2.5:1). In some experiments, another solvent, chloroform:methanol:acetic acid:water (50:15:4:1.5) was used. The location of each major phospholipid was determined by exposure of the TLC plate to iodine vapor. Comparisons were made with simultaneously chromatographed purified phospholipid standards obtained from bacterial membranes (Sigma Chemical Co). In addition, amidinated and crosslinked phospholipid standards were prepared with DMA for use in subsequent TLC analyses. Phosphatidyl ethanolamine and phsophatidyl serine were sonicated with Hepes-salt buffer (pH 7.84) in separate tubes. The resulting emulsions were transferred to tubes containing dry 1,6-^{14}C-DMA dihydrochloride (ICN Pharmaceuticals) and resonicated. The DMA concentration was 5 mM and the DMA to phospholipid molar ratio was 3.7 to 1. The lipid was extracted with 2:2:1.8 chloroform–methanol–0.02 M KCl as described by Bligh and Dyer [33] and then concentrated under a stream of dry nitrogen. The identity of amidinated or crosslinked phospholipids was verified by determining the molar ratio of DMA:phosphorus after elution and concentration of each abnormally migrating phospholipid found after reaction with DMA. Total lipid phosphorus was determined by the method of Lowry et al [34], and the amount of DMA present was calculated from its known specific activity after suitable corrections for counting efficiency. Molar ratios obtained were: PE-DMA = 1.01, PE-DMA-PE = 2.07, PS-DMA = 0.78. PS-DMA-PS could not be determined because it migrated with cold PS in the systems we used. In some experiments, measured segments of the silica gel were removed from the TLC plate by scraping, and the amount of radioactivity in each segment was determined by B scintillation spectrometry.

Red cell ghosts were solubilized in 0.5% SDS plus 0.5 mM EDTA and 0.02 M dithiothreitol, incubated 1 h (37°), then analyzed by 4% polyacrylamide gel electrophoresis, as described by Fairbanks et al [8]. The resulting gels were stained for protein with Coomassie blue and for glycoproteins with PAS [8]. Alternatively, the gels were sliced (2-mm segments), heated to 60° with 30% H_2O_2 for 1–2 h, then analyzed for radioactivity using B scintillation spectrometry.

The phosphorylation of membrane proteins was evaluated in ghost preparations using methods described by Greenquist and Shohet [11]. The incorporation of ^{32}P from $AT^{32}P$ was determined after both 5 and 60 min of incubation. In some experiments, casein (10 mg/ml) was added to the system in order to detect the rate of phosphorylation of an exogenous (nonmembrane) protein substrate by membrane protein kinase [12].

Imidoesters were obtained from Pierce Chemical Company, $^{45}Ca\ Cl_2$ from New England Nuclear, and ^{14}C-DMA from ICN Pharmaceuticals, Inc. $AT^{32}P$ was prepared according to the method described by Glynn and Chappell [13]. Precast 4% polyacrylamide gels were supplied by Bio Rad.

RESULTS

Endocytosis and Calcium Metabolism

Endocytic vacuole formation induced in resealed red ghosts by ATP in the presence of calcium and magnesium is shown in Figure 1. Endocytosis was virtually absent in stomatocyte ghosts. If stomatocytes were treated with 2 mM DMA, intense, rather than impaired, endocytosis was subsequently noted in ghost preparations. The active endocytosis present in untreated normal control ghosts was not altered by DMA.

Because alterations in the incorporation of calcium into red cell membranes or in Ca^{++} efflux may modify endocytosis [6], several aspects of calcium metabolism were evaluated. As shown in Table I, ^{45}Ca uptake by metabolically depleted stomatocytes was twice that observed in normal cells and was further increased slightly in DMA-treated stomatocytes. The efflux of $^{45}Ca^{++}$ from resealed red ghosts was slightly higher in stomatocytes than in normal control cells, consistent with the large number of reticulocytes present [14]. DMA had no effect on calcium efflux of either normal or stomatocytic red cells. Measurement of residual $^{45}Ca^{++}$ within the ghosts at the conclusion of the efflux study revealed increased Ca^{++} in stomatocytes, once again probably consistent with reticulocytosis [14]. DMA did not affect residual $^{45}Ca^{++}$ in normal cells or stomatocytes.

Duration of the Effect of DMA Upon Stomatocyte Cation Permeability

Under the incubation conditions employed, uptake of ^{14}C-DMA was rapid and was virtually complete after 30 min of incubation [15]. The continued presence of imidoester in the incubation medium after 30 min was not required for the complete correction of the permeability defect in stomatocytes.

The duration of the effect upon permeability was evaluated in red cells stored at 4° in acid citrate dextrose (Fig 2). Under these conditions, normal erythrocytes gradually gained sodium. In contrast, stomatocytes rapidly gained sodium, reaching an equilibrium value within the first day of incubation. However, if stomatocytes were first treated with DMA and allowed to reestablish normal levels of potassium and sodium, the subsequent gain of sodium by such cells after two weeks of storage was no greater than that observed

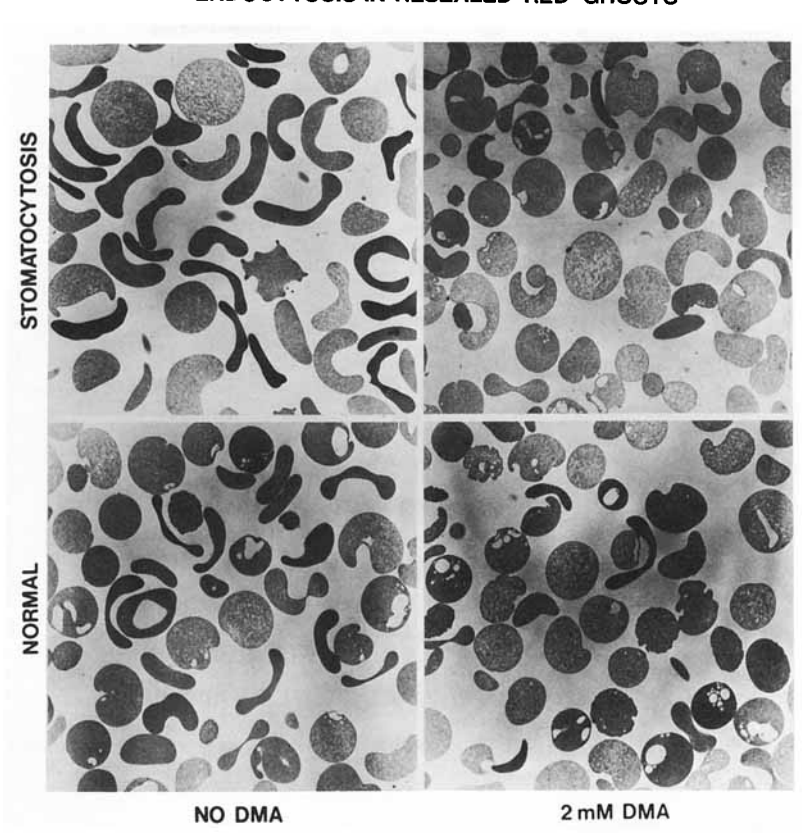

Fig 1. Electron micrographs of erythrocyte ghosts hemolyzed with 2.5 mM ATP, 1.0 mM Ca, and 2.5 mM Mg and then incubated for 15 min at 37° [6]. Top left – stomatocytes, no DMA; top right – stomatocytes + 2 mM DMA; bottom left – normal red cells, no DMA; bottom right – normal red cells + 2 mM DMA. (original magnification, × 1,900).

TABLE I. Calcium Metabolism in Stomatocytosis

	No DMA	2 mM DMA
^{45}Ca Uptake[a] by red cells		
Stomatocytosis	5.59	9.95
Normal control	2.61	–
^{45}Ca Efflux[b] from resealed red ghosts		
Stomatocytosis	98	102
Normal control	75	68
Residual ^{45}Ca[c] in resealed red ghosts		
Stomatocytosis	77	62
Normal control	22	20

[a]Nanomoles Ca/ml RBC/2 h.
[b]Nanomoles Ca/10^{10} ghosts/min
[c]Nanomoles Ca/10^{10} ghosts (after 20 min)

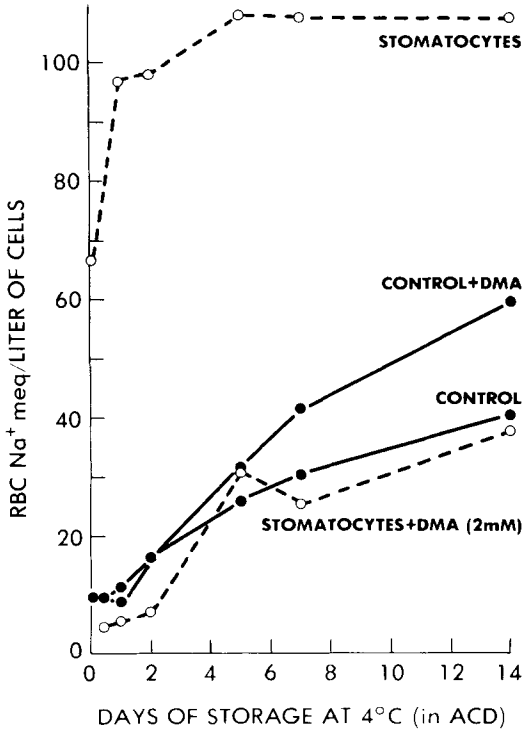

Fig 2. Stability of DMA effect on Na^+ permeability. o- - -o Stomatocytes. ●—● Control.

for normal cells. Favorable effects of DMA on potassium and water content, as well as on cell size, were also stable over a two-week period of storage. The prolonged duration of these effects is consistent with the expected formation of stable covalent bonds between imidoester and membrane-free amino groups.

Sites of Reaction of DMA With the Erythrocyte Membrane

It has previously been shown that DMA rapidly enters the erythrocyte and reacts with free amino groups on hemoglobin, resulting in intermolecular crosslinking and the formation of high molecular weight hemoglobin aggregates [16, 17].

In stomatocytes, at the concentrations of DMA that alter permeability, considerably more radioactive DMA is bound to hemoglobin than is bound to the membrane. The ratio of binding to hemoglobin and to membrane (calculated from total radioactivity associated with each component/ml of packed red cells) varies from 69.4:1 at 5 mM DMA to 44.3:1 at 0.5 mM DMA. At the lower DMA concentration, 4.26 nmoles of DMA are bound per milligram of membrane protein.

Under the reaction conditions we employed, approximately 80% of all membrane-associated ^{14}C-DMA was associated with lipid (stomatocytes 80%, normal cells 82%) and approximately 20% with protein. The lipid extracts obtained from ghosts prepared from red cells reacted with DMA (1–10 mM) were analyzed by TLC. In the absence of DMA, no difference between the phospholipid pattern of stomatocyte or normal red cell membranes was detected. Crosslinked and amidinated aminophospholipids, prepared by react-

ing purified phosphatidyl ethanolamine or phosphatidyl serine with DMA, were used to define the position of imidoester reaction products on TLC. As shown in Figure 3, both amidinated and crosslinked aminophospholipids were evident in membranes prepared from red cells reacted with DMA. To determine the extent to which aminophospholipids had reacted with DMA, the amidinated and crosslinked products were removed from the TLC plate by scraping. Their phosphate content was then determined and compared to the total amount of lipid phosphorus applied to the plate. Approximately 30% (range 28–31%) of membrane aminophospholipids reacted with imidoester at a DMA incubation concentration of 10mM. At lower (2mM) DMA concentrations no visable amidination or crosslinking was evident on TLC. However, with ^{14}C-DMA it was possible to detect trace amounts of both amidinated and crosslinked aminophospholipids. Calculations based on the known specific activity of ^{14}C-DMA indicated that less than 3% of the total membrane phospholipid (8% of membrane aminophospholipid) had reacted with DMA, assuming that all

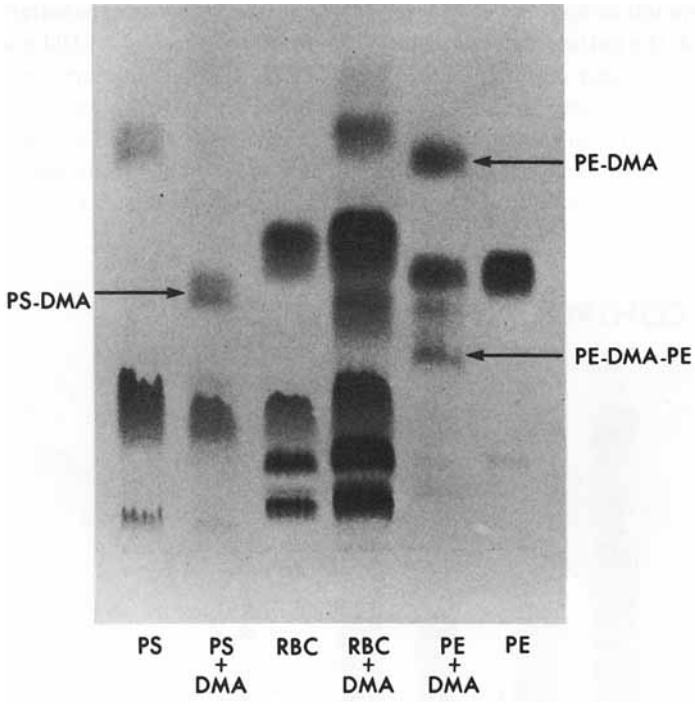

Fig 3. Effect of DMA on RBC membrane phospholipids. A membrane lipid extract obtained from red cells treated with 10 mM DMA or from untreated cells was subjected to thin layer chromatography on silica gel using chloroform:methanol:acetic acid:water (50:15:4:1.5) as the developing solvent. Aminophospholipid standards (PE = phosphatidyl ethanolamine, PS = phosphatidyl serine) were prepared (see Methods) to identify the positions of amidinated (PE-DMA, PS-DMA) and crosslinked (PE-DMA-PE) phospholipids. The slower migration of PE standard when compared to RBC PE was attributed to differences between bacterial and human PE. Crosslinked PS (PS-DMA-PS) was also probably present since ^{14}C-DMA radioactivity was found in the region of normal PS (in the DMA treated specimen), but the TLC system used did not separate PS from PS-DMA-PS. The impurities migrating ahead of and behind untreated PS standard were also usually seen in DMA-treated PS standard specimens. Although their migration was slightly altered by DMA, they did not contribute to the region labeled PS-DMA.

reactions represented crosslinking (ie, 1 mole of DMA equivalent to 2 moles of crosslinked phospholipid). The actual amount of reacted phospholipid was even lower, however, since a portion of the radioactive aminophospholipid was amidinated (1 mole of DMA equivalent to 1 mole of phospholipid) rather than crosslinked. The minimal effective concentration of DMA that corrects the membrane permeability defect (0.05 mM) is far lower than the concentration used in these studies. At these lower concentrations of DMA one would expect little or no amidination or crosslinking of aminophospholipids to occur.

No major differences between stomatocyte and control membrane proteins were detected on SDS-PAGE, although it was noted that band 7 was considerably less intense in stomatocyte membranes than in normal (Fig 4). SDS polyacrylamide gel electrophoresis of membranes prepared from erythrocytes treated for 1 h with 2 mM DMA (stained with Coomassie blue for proteins) revealed the presence of small amounts of high molecular weight aggregates and of globin. The remainder of the pattern was identical to that of the untreated specimen. Incubation of erythrocytes for 6 h instead of 1 h did not appreciably increase the amount of high molecular weight aggregates. If higher concentrations of DMA were employed, the pattern was considerably more altered. Carbon-14 DMA was used in an attempt to locate any preferential reaction of DMA with certain membrane proteins. However, there was no selective accumulation of radioactivity in any one component. Instead, small amounts of radioactivity were dispersed among the various bands. Gels were also stained with PAS to detect glycoproteins. The glycoprotein pattern was normal in stomatocytosis and was not altered in membranes prepared from cells reacted with 2 mM DMA.

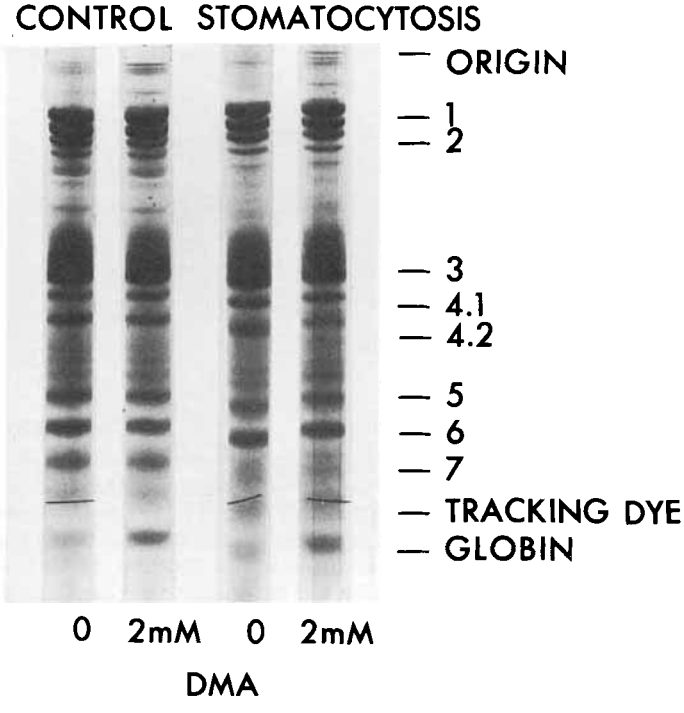

Fig 4. Effect of DMA on RBC membrane proteins.

The extent of phosphorylation of membrane proteins was evaluated in ghost preparations (Fig 5) after 1 h of incubation with $AT^{32}P$. The results were compared to those obtained with normal ghosts and ghosts from hereditary spherocytosis red cells. Phosphorylation of band 2 in hereditary stomatocytes was only 20% of normal, and treatment with 2 mM DMA had no favorable effect upon phosphorylation. The addition of cyclic AMP failed to normalize membrane protein phosphorylation in stomatocytes. The extent of phosphorylation of band 3 was also reduced in stomatocytes and was not improved by treatment with DMA.

Membrane protein phosphorylation was also evaluated in short-term incubations (Table II). The phosphorylation of an endogenous membrane protein, spectrin, in stomatocytosis was 79 pmoles per mg per 5 min, a value only 40% that of a simultaneously run control. Prior incubation of stomatocytes with 2 mM DMA had little influence on these results. In contrast, phosphorylation of an exogenous substrate, casein, by membrane protein kinase was similar in control and stomatocytic red cells. Again, DMA had little influence upon the rate of phosphorylation.

A variety of both monofunctional and bifunctional imidoesters was used in an attempt to determine whether crosslinking is a prerequisite for the effect on stomatocyte cation permeability or whether, alternatively, the effect is due to chemical modification alone (Table III). If no imidoester were employed, stomatocytes remained high in sodium and low in potassium after 6 h of incubation. Use of as little as 0.1 mM DMA, a bifunctional reagent, converted these cells into high-potassium, low-sodium cells, and a partial effect was noted at the even lower concentration of 0.05 mM. In contrast, the mono-

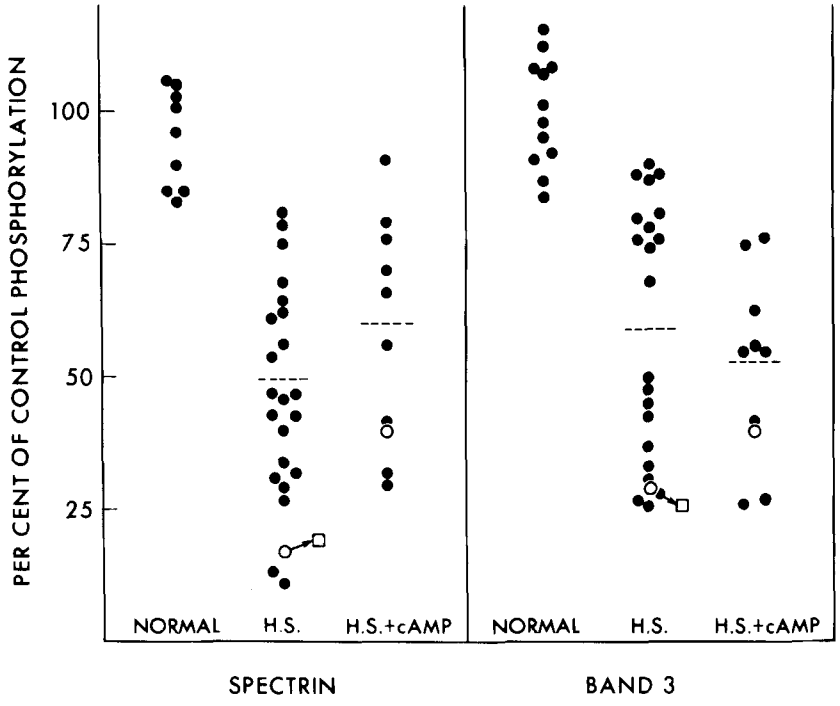

Fig 5. Membrane protein phosphorylation in hereditary stomatocytosis. ● – Normal or hereditary spherocytosis (H.S.). [Data reprinted from Greenquist, A., and Shohet, S.G., Blood Cells 3:122, 1977.] ○ – Stomatocytosis. ■ – Stomatocytosis + 2 mM DMA.

TABLE II. Protein Phosphorylation in Red Cell Ghosts

	Spectrin[a]	Casein[b]
Control	197 ± 4	19.2 ± 1.2
Stomatocytosis	79 ± 9	17.8 ± 0.2
Stomatocytosis + 2 mM DMA	101 ± 9	17.3 ± 1.3

[a]Picomoles/mg/5 min
[b]Nanomoles

TABLE III. Correction of the Abnormal Cation Content of Stomatocytes by Monofunctional and Bifunctional Imidoesters

Reagent	Concentration (mM)	RBC Cation Content[a] K^+	Na^+
None	–	28.9	99
Bifunctional			
Dimethyl adipimidate	0.1	107	10.1
Dimethyl adipimidate	0.05	84.1	34.1
Monofunctional			
Methyl acetimidate	5	20.2	118.6
Ethyl acetimidate	5	25.7	101.8
Ethyl acetimidate	20	48.6	45.7
Methyl butyrimidate	5	45	83.6
Methyl butyrimidate	20	72.8	26.7
Isothionyl acetimidate	5	14.3	124.9
Normal values	–	90–110	6–12

[a]mEq/liter Cells after 6 h incubation in vitro with indicated reagent. T 37°, pH 7.4, Hct 5%.

functional reagents methyl acetimidate and ethyl acetimidate were without effect at 5 mM, although, at a concentration of 20 mM, a partial correction was seen with ethyl acetimidate. The most effective monofunctional agent evaluated was methyl butyrimidate but, on a concentration basis, this reagent was 400 times less effective than the bifunctional reagent DMA. Isothionyl acetimidate, a monofunctional imidoester that reacts primarily at the external surface of the erythrocyte membrane, had no influence upon stomatocyte cation permeability.

Using imidoesters of differing chain length allowed a tentative estimate to be made of the distance between the crosslined free amino groups responsible for alternation in cation permeability (Table IV). A short imidoester, dimethyl malonimidate, whose maximal crosslinking dimension is approximately 3.7–5 Å, was without effect on cation permeability. The maximal effect on permeability was noted with dimethyl adipimidate, and a slightly lesser effect was seen with the larger imidoester dimethyl suberimidate.

DISCUSSION

The impaired endocytosis noted in resealed red ghosts prepared from stomatocytes highlights the difference between stomatocytosis and other membrane disorders such as spherocytosis [20] or pyropoikilocytosis [21] where ghost endocytosis is normal. Endocytosis is a complex and incompletely understood process variably involving energy, Ca^{++},

TABLE IV. Effect of Chain Length on the Correction of Stomatocyte Cation Content by Crosslinking Reagents

Reagent	Maximal crosslink dimension (Å)	RBC Cation content[a] K^+	Na^+
None	–	28.9	99
Dimethyl malonimidate	3.7–5.0	28.9	83.9
Dimethyl adipimidate	7.3–9.0	87.2	14.4
Dimethyl suberimidate	9.7–11	51.2	58.5
Normal values	–	90–110	6–12

[a] mEq/liter Cells after 6 h incubation in vitro with indicated reagent (at a concentration of 5 mM). T 37°, pH 7.4, Hct 5%.

and the membrane processes of invagination and fusion [6, 14, 20]. Although the specific defect responsible for impaired endocytosis in stomatocytes has not yet been defined, an increase in membrane rigidity would be consistent with previous observations on the abnormal rigidity of intact red cells [5]. The restoration of both whole cell deformability [5] and ghost endocytosis to normal by DMA would then be the consequence of a reduction in membrane rigidity induced by the imidoester. Because of the well-known relationships between red cell rigidity and membrane calcium [22], as well as those between Ca^{++}, Ca^{++}–Mg^{++} ATPase, and endocytosis [6], we evaluated several aspects of Ca^{++} metabolism. The severalfold increases in calcium influx into metabolically depleted stomatocytes, calcium efflux from ghosts, and calcium retention by red ghosts are all consistent with the high reticulocyte content of the blood sample used for analysis [7, 14]. Wiley and co-workers [23] have also found normal calcium uptake by metabolically depleted cells, normal membrane calcium content, and a normal rate of calcium extrusion in the red cells of other individuals with the hereditary stomatocytosis syndrome who, in some respects, resemble the patient we studied. The lack of any effect of DMA on ^{45}Ca efflux or ^{45}Ca retention make it unlikely that the enhanced endocytosis induced by DMA is a consequence of altered calcium metabolism. Furthermore, if the favorable effect of DMA on endocytosis involved calcium, one would expect a reduction in calcium influx rather than the increase actually observed.

The dramatic effects of DMA on stomatocytes led us to investigate the nature and location of the reaction between imidoesters and the stomatocytic erythrocyte membrane. The persistence of normal cation permeability during several weeks of storage of imidoester-treated stomatocytes is consistent with the expected formation of stable covalent bonds between imidoester and membrane-free amino groups. In view of the ubiquity of free amino groups on proteins, glycoproteins, and aminophospholipids, imidoesters would be expected to react with a variety of membrane constituents. In normal red cell membranes, reactions have been demonstrated with proteins [18], glycoproteins [19], and aminophospholipids [9]. The present study has confirmed that such reactions also occur in stomatocyte membranes and has attempted to define which reaction is responsible for the correction of cation permeability.

A number of investigators have failed to demonstrate any major abnormality in stomatocyte membrane lipids. Total membrane lipids are increased on a per cell basis, reflecting the larger size and increased membrane surface area of such cells [1, 2, 4, 24]. However, calculated per liter of cells, the amount of membrane lipids is normal in stomatocytes. There is no abnormality in the distribution of phospholipids [2, 4] and no undue

loss of phospholipids on incubation for periods of up to 24 h [1]. Pathways of membrane lipid renewal also appear to be normal [1].

On the other hand, several observations suggest that there may be an abnormality of membrane proteins in stomatocytosis. First, amino reactive reagents can completely correct the permeability defect in vitro [5]. Second, Bienzle and co-workers [25] have tentatively identified an abnormal membrane protein with a molecular weight of about 25,000 daltons in red cells from one patient. Although we have not yet subjected the membrane proteins from our case of stomatocytosis to analysis by the two directional electrophoretic techniques employed by Bienzle, the appreciable diminution in band 7 (mol wt = 29,000 daltons [32]) we noted on SDS-PAGE (Fig 3) certainly underscores the possibility of an abnormality in one or more low molecular weight membrane proteins. Finally, the defective phosphorylation of spectrin and band 3 in the subject of the current study implies a general disturbance in regulatory mechanisms important in the maintenance of normal cell shape (Fig 5) [11, 27]. That this disturbance is not unique to stomatocytosis is evident from previous reports of similar defects in hereditary spherocytosis [11] and in sickle cell anemia [26]. Furthermore, any inference that membrane protein phosphorylation is defective in intact stomatocytic red cells would be premature, since the measurements were made in ghosts and not in whole cells. In this regard it is important to note, as recently shown by Wolfe and Lux [31], that in hereditary spherocytosis membrane protein phosphorylation, although abnormal in ghost preparations, is normal in intact cells. In stomatocytosis, the abnormal phosphorylation seems to involve an altered interaction between the substrate proteins and the protein kinase, rather than a defect in protein kinase, since protein kinase-mediated phosphorylation of an exogenous substrate, casein, by ghost preparations is normal. Abnormal phosphorylation is not repaired by imidoesters, but it is conceivable that they may stabilize membrane proteins in an optimal conformation, perhaps substituting, in this regard, for a process normally requiring phosphorylation by protein kinase.

Whether chemical modification or crosslinking of free amino groups is required for the permeability effects is not yet completely clear. When red cells were reacted with 2 mM DMA, intermolecular crosslinking of membrane aminophospholipids was limited to a maximum of 8% of the available PE & PS. A barely detectable amount of high molecular weight material, evident on SDS gel electrophoresis, was the only indication of intermolecular crosslinking of membrane proteins. These results suggest that, if crosslinking is essential, it must involve a minor membrane component, present in low concentration, or be predominantly intramolecular rather than intermolecular. The experiments with bifunctional reagents of differing chain length support the premise that crosslinking is required for the permeability effects observed in stomatocytes. If chemical modification, rather than crosslinking, of free amino groups were required to alter permeability, the entire family of bifunctional reagents employed should be equally effective, since they differ only in chain length. The clear superiority of DMA, compared to larger or smaller bifunctional reagents, suggests that crosslinking is essential and that the dimensions of the critical crosslink are approximately 7–9 Å.

It is of interest that isothionyl acetimidate, a monofunctional imidoester that reacts primarily at the external surface of the erythrocyte membrane, has no influence upon stomatocyte cation permeability, implying that the effect of imidoesters upon permeability may occur deeper within the membrane. Although the effects of monofunctional reagents seemingly demonstrate that chemical modification also can influence permeability, these effects may, in fact, be the result of crosslinking, since Browne and Kent have noted that,

at the pH range we employed for these studies, monofunctional reagents may form transient intermediates that have crosslinking capabilities [28]. That monofunctional reagent are, indeed, capable of crosslinking reactions in red cells and other tissues has been confirmed by several investigators [29, 30]. The relatively small amount of such transient intermediates available for crosslinking would then explain the much larger concentration requirement for monofunctional reagents noted in the present study.

The potential of imidoesters as probes, useful in defining the nature of the membrane abnormality in stomatocytosis, is apparent from the studies described here. It can be anticipated that their continued use will provide additional insight into the nature of these fascinating cells.

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REFERENCES

1. Zarkowsky HS, Oski FA, Sha'afi R, Shohet SB, Nathan DG: N Engl J Med 278:593, 652, 1968.
2. Mentzer WC, Smith WB, Goldstone J, Shohet SB: Blood 46:659, 1975.
3. Bienzle U, Niethammer D, Kleeberg U, Ungefehr K, Kohne E, Kleihauer E: Scand J Haematol 15:339, 1975.
4. Schroter W, Ungefehr K: "Membrane and Disease." New York: Raven Press, 1976, p 95.
5. Mentzer WC, Lubin BH, Emmons S: N Engl J Med 294:1200, 1976.
6. Schrier SL, Bensch KG, Johnson M, Junga I: J Clin Invest 56:8, 1975.
7. Wiley JS, Gill FM: Blood 47:197, 1976.
8. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
9. Marinetti GV, Baumgarten R, Sheeley D, Gordesky S: Biochem Biophys Res Commun 53:302, 1973.
10. Marfey SP, Tsai KH: Biochem Biophys Res Commun 65:31, 1975.
11. Greenquist A, Shohet S: Blood 48:887, 1976.
12. Greenquist A, Wyatt J, Guatelli J, Shohet S: "Erythrocyte Membranes: Recent Clinical and Experimental Advances." New York: Alan R Liss. In press.
13. Glynn Chappell: Biochem J 90:147, 1964.
14. Schrier SL, Bensch KG: "Membranes and Disease." New York: Raven Press, 1976, p 31.
15. Lubin B, Bradley T, Mentzer W, Messer M, McRae J: INSERM 44:291, 1975.
16. Lubin BH, Pena V, Mentzer WC, Bymun E, Bradley TB, Packer L: Proc Natl Acad Sci USA 72:43, 1975.
17. Waterman MR, Yamaoka K, Chuang AH, Cottam GL: Biochem Biophys Res Commun 63:580, 1975.
18. Ji TH: Proc Natl Acad Sci USA 71:93, 1974.
19. Wang K, Richards FM: J Biol Chem 249:8005, 1974.
20. Schrier SL, Ben-Bassat I, Bensch K, Seeger M, Junga I: Br J Haematol 26:59, 1974.
21. Walter T, Mentzer W, Greenquist A, Schrier S, Mohandas N: Blood 50:98, 1977.
22. Weed RL, LaCelle PL, Merrill EW: J Clin Invest 48:795, 1969.
23. Wiley JS, Ellory JC, Shuman MA, Shaller CC, Cooper RA: Blood 46:337, 1975.
24. Wiley JS: "Membranes and Disease." New York: Raven Press, 1976, p 89.
25. Bienzle U, Bhadki S, Knufermann H, Niethammer D, Kleihauer E: Klin Woschr 55:569, 1977.

26. Beutler E, Guinto E, Johnson C: Blood Cells 3:135, 1977.
27. Shohet SB, Greenquist AC: Blood Cells 3:115, 1977.
28. Browne DT, Kent SBH: Biochem Biophys Res Commun 67:126, 1975.
29. Chao TL, Berenfeld MR, Gabuzda TG: FEBS Lett 62:57, 1976.
30. Packer L, Bymun EN, Tinberg HM, Ogunmola GB: Arch Biochem Biophys 177:323, 1976.
31. Wolfe LC, Lux SE: Blood 48:963, 1976.
32. Steck TL: J Cell Biol 62:1, 1974.
33. Bligh EG, Dyer WJ: Can J Biochem Physiol 37:911, 1959.
34. Lowry OH, Roberts NR, Leiner K, Wu ML, Fan AL: J Biol Chem 207:1, 1954.