J. Membrane Biol. 20, 181–190 (1975)


# Membrane Proteins and Urea and Acetamide Transport in the Human Erythrocyte

Michael A. Kaplan*, Richard M. Hays and Olga O. Blumenfeld

Department of Medicine, Department of Biochemistry and Unit for Research in Heart, Lung, and Ageing, Albert Einstein College of Medicine, New York, N.Y. 10461

Received 1 July 1974; revised 20 September 1974

*Summary.* Previous studies have shown that urea and acetamide traverse the erythrocyte membrane by way of facilitated diffusion. The nature of this selective pathway is unknown. The present studies investigate the effects of proteolytic enzymes and crosslinking agents on amide transport. Cleavage of the erythrocyte membrane surface by pronase or trypsin had no effect on urea and acetamide permeability or inhibition by phloretin. These findings suggest that the sialoglycopeptide segment of the sialoglycoproteins is not critical to urea and acetamide transport. In addition, extensive crosslinking of membrane proteins with glutaraldehyde had no effect on amide transport in the absence or presence of phloretin.

The transport of urea across several membranes appears to occur by facilitated diffusion. In the human erythrocyte this conclusion is based on the fact that urea moves faster than its more lipid-soluble analogue, acetamide, and the permeability of both these amides is decreased by phloretin (Macey & Farmer, 1970; Kaplan, Hays & Hays, 1974). A similar conclusion is suggested by Wieth and associates (1974) on the basis of flux studies.

It has been suggested that the mechanism for urea transport involves a "selective pathway". This pathway may include a protein constituent of the cell membrane (Hunter, George & Ospina, 1965).

In the present studies, we have investigated the effects of membrane protein modification on amide permeability in the intact erythrocyte. Sodium metaperiodate was used to selectively oxidize the surface sialoglycoproteins, trypsin and pronase to hydrolyze the cell surface proteins, and tannic acid and glutaraldehyde to crosslink membrane proteins. Our

* *Correspondence and reprint requests:* Michael A. Kaplan, M.D., Dept. of Medicine, U.S.A.F. Regional Hospital, Maxwell Air Force Base, Montgomery, Alabama 36112.

findings suggest that under the conditions employed, the sialoglycopeptide segment of the sialoglycoproteins of the erythrocyte membrane play no apparent active role in facilitated diffusion of urea and acetamide. In addition, extensive crosslinking of membrane proteins and thereby, presumably, major alterations in structure have no effect on amide permeability.

## Materials and Methods

Freshly collected blood was obtained in heparinized syringes from peripheral veins of normal laboratory personnel. Erythrocytes were washed and erythrocyte membranes prepared in isotonic sodium phosphate buffer at pH 7.4 (Dodge, Mitchell & Hanahan, 1963) as previously described (Blumenfeld, Gallop & Liao, 1972).

Urea and acetamide permeability was measured by the method of osmotic hemolysis. This method has been used extensively to indirectly measure the permeability of various nonelectrolyte solutes (Whittam, 1964). The theory of osmotic hemolysis has recently been reviewed (Rabinowitz & Gunther, 1973). At zero time a 0.1 ml aliquot of packed erythrocytes was pipetted into test tubes kept in an ice bath and containing 8 ml of a 2 M urea or acetamide solution buffered with 3 mM sodium phosphate at pH 7.0. Each tube also contained either ethanol, or phloretin ($6 \times 10^{-4}$ M) dissolved in ethanol. Final ethanol concentration was 0.6%. After addition of the cells, the tube under study was inverted and replaced in the ice bath for a specified length of time. It was then centrifuged (International Clinical Centrifuge, International Equipment Co., Needham Heights, Mass.) for 45 sec and slowly brought to a stop by hand pressure over a period of 15 sec. A 2-ml sample of the supernatant was carefully removed with a Pasteur pipette, transferred to a $10 \times 75$ mm tube, and the absorbance determined at 510 nm in a Coleman Junior Spectrophotometer. The blank (no hemolysis) was the supernatant from an aliquot of cells suspended in isosmolar NaCl. Complete hemolysis (100%) was determined by suspending a 0.1 ml aliquot of cells in distilled water. The percent hemolysis of any given sample was determined by comparing its absorbance with that of the complete hemolysis tube.

Periodate oxidation and borohydride reduction of sialic acid residues were performed as previously described (Blumenfeld *et al.*, 1972). Sodium metaperiodate was added to an aliquot of packed erythrocytes at a final concentration of $2 \times 10^{-3}$ M. After 10 min at room temperature with frequent agitation the oxidation reaction was terminated by 1:1 dilution with isotonic phosphate buffer and addition of glucose on a 1:1 molar basis with the added periodate. The cells were then washed 3 times in isotonic phosphate buffer, tested for amide permeability, and membranes prepared.

A tritium label was introduced into the sialoglycoprotein of the erythrocyte membrane after periodate oxidation by reduction with tritiated sodium borohydride (New England Nuclear Corp., Boston, Mass.). To 10 ml of periodate-treated erythrocytes was added 0.7 mg of $[H]^3$ $NaBH_4$. The reduction was allowed to proceed at pH 7.4 for 20 min, at room temperature, with frequent agitation. The reaction was terminated by 1:1 dilution with isotonic phosphate buffer and washing 3 times. An aliquot of these labeled cells was lysed for membrane preparation and the rest used for subsequent experiments.

Labeled erythrocytes were diluted 1:1 with isotonic phosphate buffer and then treated with either pronase, B grade (Calbiochem., Los Angeles, Cal.) or trypsin-TPCK (Worthington Biochem Corp., Freehold, N. J.). Incubation with 0.7 mg/ml of pronase or 0.4 mg/ml of trypsin was allowed to proceed for 60 min at room temperature at which

time the cells were washed 5 times with isotonic phosphate buffer[1], tested for amide permeability, and membranes prepared. Radioactivity of an aliquot of membranes was determined before and after incubation to evaluate the extent of proteolysis.

Both the instantaneous and the irreversible protein crosslinking effects of gallnut tannic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) were investigated in separate experiments. The direct effect was studied by adding normal cells to tubes containing tannic acid $10^{-4}$ M in addition to the urea or acetamide solution. The irreversible effect was examined by incubating labeled cells in tannic acid $10^{-4}$ M for 60 min at room temperature. These cells were then washed 3 times in isotonic phosphate buffer, tested for amide permeability, and membranes prepared.

Membrane crosslinking was produced by incubating labeled erythrocytes in 0.15% glutaraldehyde (0.015 M) (Union Carbide Corp., New York, N.Y.) at 4 °C for 60 min. The cells were then washed 3 times in isotonic phosphate buffer, tested for amide permeability, and membranes prepared. At higher concentrations of glutaraldehyde (0.30%) the intact erythrocyte could no longer be hemolyzed after incubation.

Polyacrylamide gel electrophoresis was carried out on membranes from each experiment according to Fairbanks, Steck and Wallach (1971). It was performed on parallel gels; one was stained with Coomassie stain for proteins, the other with the Schiff stain for carbohydrates. Radioactivity in the Coomassie stained gel was determined using the procedure of Tishler and Epstein (1968). The gels were sliced promptly since the modified sialic acid which contains the radioactive label is labile in acetic acid.

## Results

Fig. 1 shows the osmotic hemolysis curves obtained for urea and acetamide permeability in erythrocytes treated with periodate. It can be seen that urea permeability is substantially more rapid than acetamide permeability and that both are inhibited by phloretin. These curves are similar to our previous observations (Kaplan *et al.*, 1974). Oxidation of the surface sialoglycoproteins by periodate appears to have no effect on either baseline amide flux or the ability of phloretin to inhibit this flux. Similar curves were plotted for all subsequent experiments and in Table 1 selected time points are shown for each set of these studies.

Incorporation of the tritium label by borohydride reduction had no adverse effect on amide permeability (Table 1). Fig. 2*A* shows the gel pattern of these labeled erythrocyte membranes. As shown previously (Blumenfeld *et al.*, 1972) the gel pattern appears normal and the radioactivity corresponds to the sialoglycoprotein. It can be concluded that oxidation

1 Complete removal of proteolytic enzymes was of importance to prevent subsequent proteolysis of membranes during isolation and preparation for gel electrophoresis. We established, in the case of pronase, that at least five washings were essential for removal of the enzyme from the erythrocytes. Thus, after extensive washing, gel patterns over a time course of 5 to 60 min remained unchanged; incomplete washing resulted in gel patterns suggesting extensive proteolysis of membrane components.

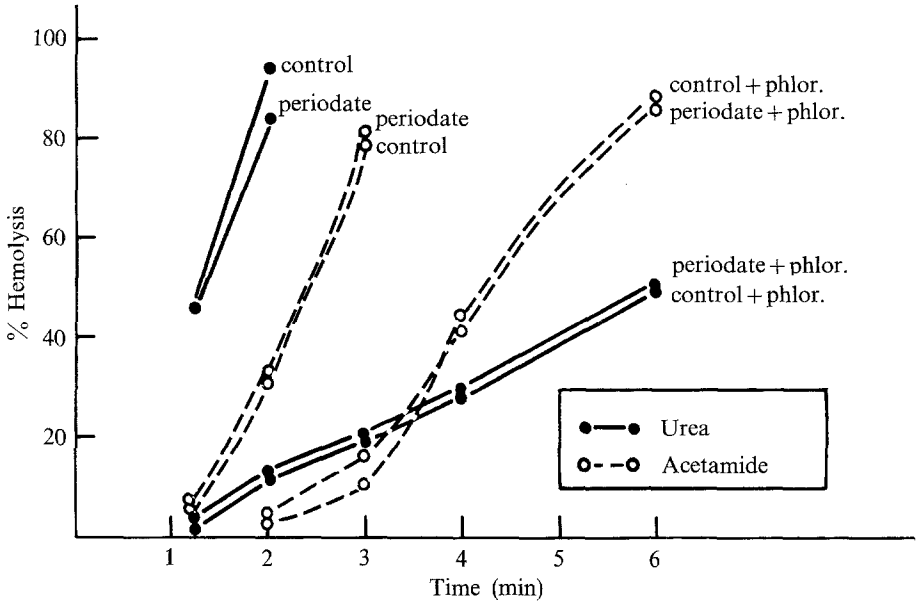


Fig. 1. Osmotic hemolysis curves of control and periodate oxidized erythrocytes in 2 M urea and 2 M acetamide solutions in the absence and presence of $6 \times 10^{-4}$ M phloretin ($n=4$)

and/or reduction of the sialoglycoprotein has no observable effect on urea and acetamide permeability.

Treatment with the proteolytic enzymes pronase and trypsin did not affect urea and acetamide permeability (Table 1). In addition, the phloretin effect was not altered suggesting that its action is not directly associated with surface proteins hydrolyzed by these enzymes. Both trypsin and pronase released tritium into the supernatant during proteolysis and determination of radioactivity in membranes revealed that trypsin released 50% and pronase 75% of the total radioactivity. In Fig. 2*B* and *C* the gel patterns after trypsin and pronase treatment show the loss of the glycoprotein bands with corresponding loss of radioactivity. In addition, in agreement with others (Bender, Garan & Berg, 1971; Triplett & Carraway, 1972; Cabantchik & Rothstein, 1974), trypsin did not cause a major change in protein pattern and pronase led to loss of proteins in band III.

Fig. 3 shows the osmotic hemolysis curves for erythrocytes treated with tannic acid. In one set of experiments, erythrocytes were pretreated with $10^{-4}$ M tannic acid and then washed free of the excess. In a second set of experiments normal cells were placed into amide solutions containing $10^{-4}$ M tannic acid. Normal erythrocytes displayed a decrease in permeability when suspended in a tannic acid containing solution. That this effect was not

Table 1. Osmotic hemolysis

| Experiment (n)[a] | Time[b] | % Hemolysis | | | |
|---|---|---|---|---|---|
| | | Urea | Urea + phlor. | Acet. | Acet. + phlor. |
| Control (4) | 1.25 | 46 ± 5[c] | 1 ± 1 | 3 ± 1 | |
| | 2.00 | 95 ± 5 | 12 ± 3 | 33 ± 7 | 3 ± 1 |
| | 4.00 | | 28 ± 2 | | 45 ± 6 |
| Sodium periodate (4) | 1.25 | 44 ± 7 | 3 ± 1 | 5 ± 1 | |
| | 2.00 | 84 ± 3 | 13 ± 3 | 32 ± 7 | 4 ± 1 |
| | 4.00 | | 30 ± 3 | | 42 ± 5 |
| Sodium periodate + sodium borohydride (4) | 1.25 | 47 ± 7 | 1 ± 1 | 3 ± 1 | |
| | 2.00 | 93 ± 7 | 14 ± 4 | 33 ± 9 | 4 ± 1 |
| | 4.00 | | 31 ± 2 | | 48 ± 6 |
| Control (3) | 1.25 | 50 ± 3 | 3 ± 1 | 3 ± 1 | |
| | 2.00 | 103 ± 4 | 8 ± 1 | 26 ± 2 | 2 ± 1 |
| | 4.00 | | 25 ± 2 | | 42 ± 3 |
| Trypsin (3) | 1.25 | 50 ± 3 | 3 ± 1 | 3 ± 1 | |
| | 2.00 | 97 ± 2 | 9 ± 1 | 28 ± 3 | 3 ± 1 |
| | 4.00 | | 25 ± 5 | | 41 ± 3 |
| Control (3) | 1.25 | 43 ± 5 | 0 ± 0 | 2 ± 1 | |
| | 2.00 | 98 ± 10 | 9 ± 3 | 50 ± 11 | 6 ± 1 |
| | 4.00 | | 37 ± 11 | | 50 ± 2 |
| Pronase (3) | 1.25 | 51 ± 8 | 2 ± 1 | 5 ± 2 | 4 ± 2 |
| | 2.00 | 96 ± 4 | 10 ± 1 | 51 ± 4 | 57 ± 3 |
| | 4.00 | | 35 ± 10 | | |
| Control (3) | 1.25 | 56 ± 10 | 1 ± 1 | 3 ± 1 | |
| | 2.00 | 100 ± 4 | 7 ± 1 | 29 ± 5 | 5 ± 1 |
| | 4.00 | | 28 ± 2 | | 47 ± 8 |
| Tannic acid (pretreat) (3) | 1.25 | 49 ± 3 | 2 ± 1 | 4 ± 1 | |
| | 2.00 | 103 ± 5 | 9 ± 2 | 28 ± 1 | 3 ± 1 |
| | 4.00 | | 30 ± 2 | | 47 ± 11 |
| Tannic acid (direct) (3) | 4.00 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Control (3) | 1.25 | 46 ± 6 | 1 ± 1 | 1 ± 1 | |
| | 2.00 | 95 ± 10 | 8 ± 3 | 54 ± 9 | 5 ± 1 |
| | 4.00 | | 39 ± 11 | | 50 ± 2 |
| Glutaraldehyde (3) | 1.25 | 52 ± 5 | 1 ± 1 | 4 ± 2 | |
| | 2.00 | 101 ± 2 | 11 ± 1 | 49 ± 6 | 4 ± 1 |
| | 4.00 | | 41 ± 11 | | 56 ± 2 |

[a] Numbers in parentheses indicate number of experiments.
[b] In minutes.
[c] Mean ± SEM.

due to irreversible crosslinking of membrane protein is demonstrated by the normal permeability seen after 60 min of pretreatment with tannic acid (Fig. 3). The phloretin effect was also not altered by this pretreatment.

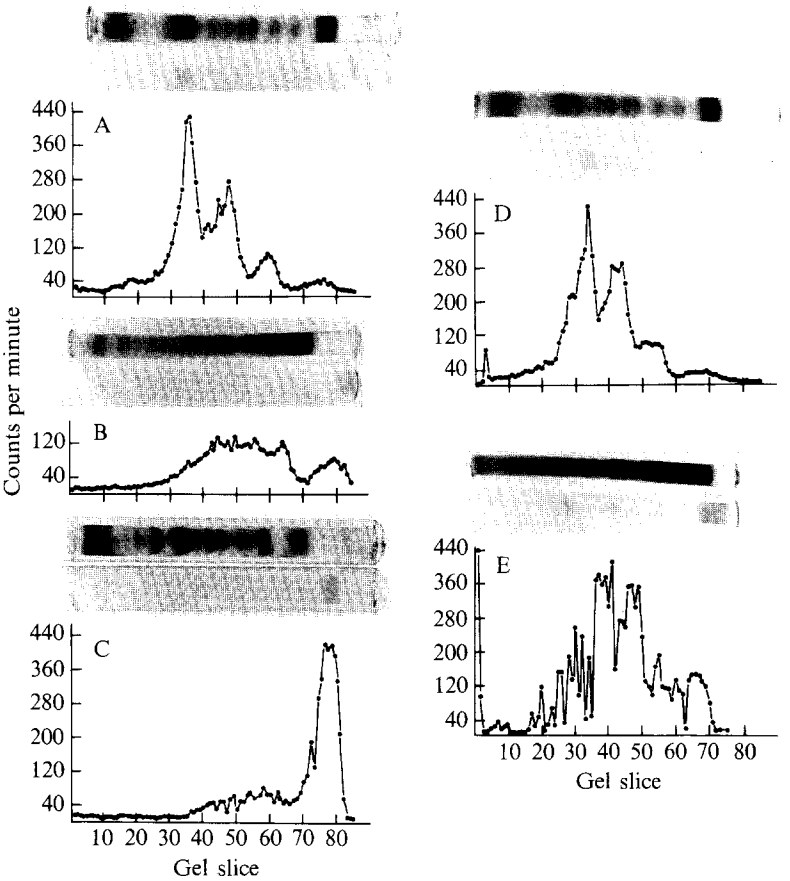


Fig. 2. Distribution of tritium label in the sialoglycoprotein of the erythrocyte membrane. The gel electrophoretic patterns in 5.6% polyacrylamide containing 1% SDS at the top are duplicate Coomassie and Schiff stained gels. (*A*) Membranes labeled with tritiated borohydride. (*B*) Labeled membranes after treatment with 0.4 mg/ml trypsin. (*C*) Labeled membranes after treatment with 0.7 mg/ml pronase. (*D*) Labeled membranes after treatment with $10^{-4}$ M tannic acid. (*E*) Labeled membranes after treatment with 0.15% glutaraldehyde

Fig. 2*D* is the gel pattern of labeled erythrocytes incubated with tannic acid $10^{-4}$ M for 60 min. The overall protein pattern appears unchanged although some radioactivity is seen in a very high molecular weight region where normally none is present. This sialoglycoprotein which has been crosslinked represents only a small amount of the membrane radioactivity and the marked decrease in amide permeability brought about by tannic acid cannot be attributed to irreversible crosslinking of surface glycoproteins.

Glutaraldehyde is a tissue fixative which leads to extensive crosslinking of proteins. Table 1 shows that 0.15% glutaraldehyde under the experi-

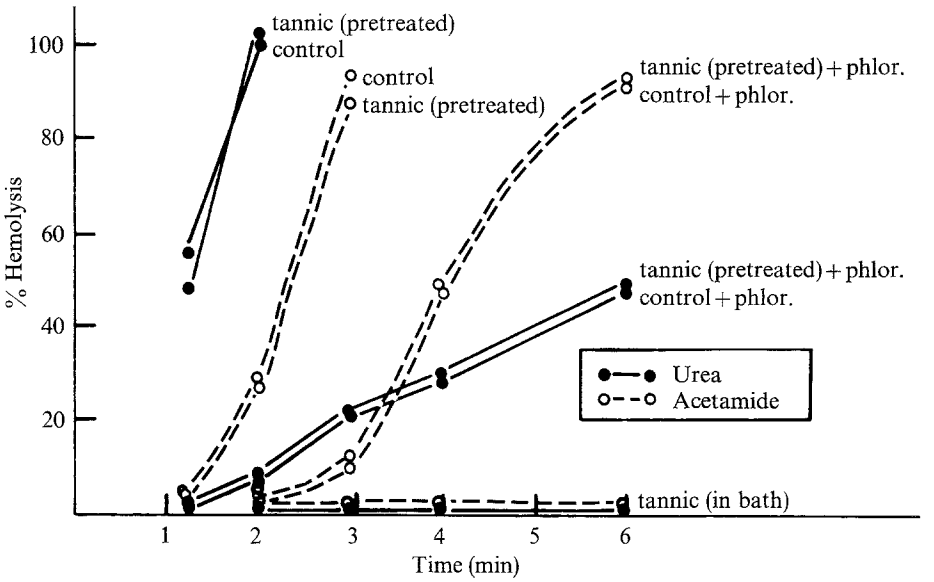


Fig. 3. Osmotic hemolysis curves of erythrocytes in the absence and presence of $10^{-4}$ M tannic acid and of erythrocytes pretreated with $10^{-4}$ M tannic acid ($n=3$)

mental conditions used had no effect on urea and acetamide-induced osmotic hemolysis either in the absence or presence of phloretin. This lack of effect is in contrast to the extensive change in the Coomassie gel pattern of the treated erythrocytes (Fig. 2*E*). The radioactivity profile suggests that crosslinking of the glycoprotein had occurred, although, as found by others (Steck, 1972; Capaldi, 1973) this was not apparent from the Schiff stain pattern. The increased amount of label associated with low molecular weight material may represent crosslinking with labeled lipid components which become modified such as glycolipids, lipid peroxides, and plasmalogens (Blumenfeld *et al.*, 1972).

It is of importance to point out that proteolysis and crosslinking occurred in the intact erythrocyte and not during subsequent membrane isolation and preparation for gel electrophoresis. This is suggested by the release of tritium during proteolysis and the resistance of erythrocytes to lysis when exposed to glutaraldehyde concentrations higher than used here.

## Discussion

Urea and acetamide are amides which appear to traverse cell membranes in several tissue types from different species by way of facilitated diffusion (Levine, Franki & Hays, 1973; Kaplan *et al.*, 1974; Wieth, Funder, Gunn &

Brahm, 1974). The involvement of a protein component in the cell membrane was suggested in this specialized transport process (Hunter *et al.*, 1965; Shuchter, Franki & Hays, 1973). The possible nature of the protein and type of protein involvement remain undefined.

Several agents including phloretin, tannic acid, and chromate have been found to inhibit the movement of amides across the human erythrocyte membrane and the toad bladder (Hunter *et al.*, 1965; Levine *et al.*, 1973; Shuchter *et al.*, 1973; Kaplan *et al.*, 1974; Wieth *et al.*, 1974). The latter two agents have been implicated in crosslinking of cell membrane components.

The erythrocyte membrane contains an assembly of proteins, some exposed on the surface and others buried within. If a protein is directly involved in amide transport it may be possible to modify it with a proteolytic enzyme; similarly, it may be possible to impede its function with a non-specific protein crosslinking agent.

To aid observation of changes in the surface sialoglycoprotein in the intact erythrocyte, a selective tritium label was incorporated into this protein by periodate oxidation and subsequent reduction with tritiated borohydride. These procedures did not alter the pattern of urea and acetamide permeability.

Extensive digestion of the intact erythrocyte surface with pronase and trypsin as confirmed by gel electrophoresis did not affect the movement of urea and acetamide. This would tend to dismiss the sialoglycopeptide segment of the major sialoglycoproteins and surface segments of other proteins as being directly related to amide transport. Similar results have recently been shown for the proposed facilitated transport pathway for anions (Cabantchik & Rothstein, 1974) and glucose (Bender *et al.*, 1971) in the human erythrocyte. In addition, these membrane components do not appear to be involved with the inhibitory effect of phloretin on amide flux.

Tannic acid has been shown to impede the transport of urea across the erythrocyte membrane (Hunter *et al.*, 1965) and the isolated toad bladder (Shuchter *et al.*, 1973). In both cases tannic acid was present at the time of the flux measurements. We confirm this inhibitory effect in the human erythrocyte, but show that it is reversible. After incubation with tannic acid, crosslinking of sialoglycoprotein may have occurred to a small extent, but the overall gel pattern appeared normal. This is consistent with the fact that tannic acid does not cross the erythrocyte membrane (Herz & Kaplan, 1968) and probably does not gain entrance into the membrane. Tannic acid does irreversibly inactivate membrane-bound acetylcholinesterase which is presumed to be a surface enzyme (Herz, 1968). Thus tannic acid can irre-

versibly affect surface proteins, but its inhibition of amide transport is reversible. It would appear that no firm conclusion can be drawn as to the mode of action of tannic acid on amide movement.

The effect of glutaraldehyde on the structure of the membrane of the intact erythrocyte results in extensive crosslinking of proteins. Similar results have been shown recently for beef erythrocyte ghosts (Capaldi, 1973) and isolated human erythrocyte membranes (Steck, 1972). Despite this dramatic alteration in membrane structure, urea and acetamide permeability are unaffected. The retention of the characteristic phloretin inhibition also indicates that the facilitated diffusion pathway is undisturbed.

Studies of the glutaraldehyde-treated toad bladder are of interest in this regard (Eggena, 1973). If the vasopressin-stimulated bladder is treated with 1% glutaraldehyde its high urea permeability remains fixed. This has been taken to indicate that the vasopressin-sensitive urea pathway can be frozen in the "open" position by crosslinking of membrane proteins. In the human erythrocyte the pathway is "open" at all times, and glutaraldehyde has no effect.

We would conclude that the sialoglycopeptide segment of the major sialoglycoproteins and surface segments of other proteins of the erythrocyte play no crucial role in either the transport of amides or their inhibition by phloretin. It would also appear that extensive crosslinking of membrane proteins and thereby, presumably, major alterations in structure have no effect on amide permeability.

This work was supported by grants AM-03858, P01-AM16281, HL-13979, and GM-16389 from the U.S. Public Health Service and by the New York State Kidney Disease Institute. Dr. Hays is a Career Investigator, Health Research Council of the City of New York. Dr. Kaplan is supported by U.S. Public Health Service Fellowship Award 5 FO2-AM53772.

## References

Bender, W. W., Garan, H., Berg, H. C. 1971. Proteins of the human erythrocyte membrane as modified by pronase. *J. Mol. Biol.* **58**:783

Blumenfeld, O. O., Gallop, P. M., Liao, T. H. 1972. Modification and introduction of a specific radioactive label into the erythrocyte membrane sialoglycoprotein. *Biochem. Biophys. Res. Commun.* **48**:242

Cabantchik, Z. I., Rothstein, A. 1974. Membrane proteins related to anion permeability of human red blood cells. *J. Membrane Biol.* **15**:227

Capaldi, R. A. 1973. A crosslinking study of the beef erythrocyte membrane: Extensive interaction of all the proteins of the membrane except for the glycoproteins. *Biochem. Biophys. Res. Commun.* **50**:656

Dodge, J. T., Mitchell, C., Hanahan, D. J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119

Eggena, P. 1973. Inhibition of vasopressin stimulated urea transport across the toad bladder by thiourea. *J. Clin. Invest.* **52**:2963

Fairbanks, G., Steck, T. L., Wallach, B. F. H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606

Herz, F. 1968. On the effects of tannic acid on erythrocyte membrane acetylcholinesterase. *Proc. Soc. Exp. Biol., N.Y.* **127**:1240

Herz, F., Kaplan, E. 1968. Effects of tannic acid on erythrocyte enzymes. *Nature* **217**:1258

Hunter, F. R., George, J., Ospina, B. 1965. Possible carriers in erythrocytes. *J. Cell. Comp. Physiol.* **65**:299

Kaplan, M. A., Hays, L., Hays, R. M. 1974. The evolution of a facilitated diffusion pathway for amides in the erythrocyte. *Amer. J. Physiol.* **226**:1327

Levine, S., Franki, N., Hays, R. M. 1973. Effects of phloretin on water and solute movement in the toad bladder. *J. Clin. Invest.* **52**:1435

Macey, R. I., Farmer, R. E. L. 1970. Inhibition of water and solute permeability in human red cells. *Biochim. Biophys. Acta* **211**:104

Rabinowitz, L., Gunther, R. A. 1973. Urea transport in elasmobranch erythrocytes. *Amer. J. Physiol.* **224**:1109

Shuchter, S. H., Franki, N., Hays, R. M. 1973. The effect of tanning agents on the permeability of the toad bladder to water and solutes. *J. Membrane Biol.* **14**:177

Steck, T. L. 1972. Crosslinking the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* **66**:295

Tishler, P. V., Epstein, C. J. 1968. A convenient method of preparing polyacrylamide gels for liquid scintillation spectrometry. *Analyt. Biochem.* **22**:89

Triplett, R. B., Carraway, K. L. 1972. Proteolytic digestion of erythrocytes, resealed ghosts, and isolated membranes. *Biochemistry* **11**:2897

Whittam, R. 1964. The diffusion and facilitated transfer of non-electrolytes. *In:* Transport and Diffusion in R.B.C. Williams and Wilkin Company, Baltimore

Wieth, J. O., Funder, J., Gunn, R. B., Brahm, J. 1974. Passive transport pathways for chloride and urea through the red cell membrane. *In:* Comparative Biochemistry and Physiology of Transport. L. Balix, editor. North Holland Publishing Company, Amsterdam