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CARBAMYLLATION OF ERYTHROCYTE MEMBRANE COMPONENTS: 
AN IN VITRO AND IN VIVO STUDY

by

Daniel Joseph Trepanier

A DISSERTATION
Submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
1995
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ABSTRACT

CARBAMYLLATION OF ERYTHROCYTE MEMBRANE COMPONENTS:
AN IN VITRO AND IN VIVO STUDY

by

DANIEL JOSEPH TREPANIER

Cyanate exists in equilibrium with urea and in its reactive form, isocyanic acid, forms a stable adduct with protein amino groups (carbamylation). Both carbamylated hemoglobin and carbamylated plasma proteins are elevated in patients with chronic renal failure (uremia) and are believed to be responsible for some of the pathophysiological consequences of uremia. The present study focused upon an in vitro / in vivo investigation of the carbamylation of erythrocyte membrane cytoskeletal and phospholipid components.

The time course binding pattern of $[^{14}C]$ cyanate to the individual cytoskeletal membrane proteins demonstrated that spectrin and ankyrin were the most extensively carbamylated proteins, incorporating $18.3 \pm 1.6$ and $10.7 \pm 3$ mol cyanate / mol protein, respectively, following a 10-h incubation. The degree of carbamylation was directly correlated with protein molecular weight, indicating the nonspecific nature of the binding. For in vivo studies, erythrocyte ghosts were digested with proteinase K and the released peptides colorimetrically assayed for carbamylation using diacetyl monoxime. Erythrocytes from uremics were found to have a greater level of carbamylation relative to nonuremics ($47.09 \pm 7.90$ and $25.89 \pm 6.92$ nmol homocitrulline / mg proteolyzed protein released, respectively.)
In addition, measurements of membrane fluidity, using the fluorescence polarization probe — fluorescein lithocholic acid, provided evidence of membrane alterations as a result of *in vitro* carbamylation.

Solvent extraction of erythrocyte ghost membranes incubated with \(^{14}\text{C}\) cyanate followed by thin-layer chromatography and autoradiography clearly indicated the specific modification *in vitro* of the amino containing headgroups of both phosphatidylethanolamine and phosphatidylserine by cyanate. The cyanate incorporated following a 15-h incubation was 15.75 ± 0.09 and 13.34 ± 0.81 mol %, respectively. Separation of carbamylated phosphatidylethanolamine from native phosphatidylethanolamine by thin-layer chromatography was achieved in a solvent system of chloroform/methanol/ammonia (65/35/5, v/v/v).

Treatment of erythrocyte lipid / Triton X-100 micelles, constructed from solvent extracts of ghost membranes from normal individuals, with phospholipase D released a chromogenic species which reacted with diacetyl. The results provide preliminary evidence for the presence of carbamylated phospholipid in the native erythrocyte membrane. The level of carbamylated phosphatidylethanolamine in the native erythrocyte membrane was estimated to be 2.85 ± 0.65 mol %.

It is suggested that the increased level of membrane carbamylation in uremics may contribute to the decreased erythrocyte survival time observed in these individuals.
DEDICATION

To My Family,
for their continued love and support.

To Deirdre,
for her love, courage, and tolerance.
ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor, Dr. Roger J. Thibert for providing me with the appropriate blend of research guidance and independence, and for unwaveringly being my "biggest fan".

I would also like to acknowledge the critical contributions made to this dissertation by Drs. K.E. Taylor, K. Adeli, A.H. Warner, T.F. Draisey and T.M. Annesley.

The continued help and support of Dean Lois K. Smedick and the entire staff at the Faculty of Graduate Studies and Research is greatly appreciated.

I would also like to acknowledge the gracious assistance of Mr. M. Goodwin and the clinical laboratory staff at the Salvation Army Grace Hospital, Hotel-Dieu Hospital, and Bioscience Laboratories. This research project could not have been completed without their support.

I would like to extend my best wishes to all the students, staff and faculty of the Department of Chemistry and Biochemistry. I thank you for your help, stimulating discussions and, most of all your friendships.
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Reaction of Phospholipid Headgroups and Phospholipid Micelles with Diacetyl
LIST OF ABBREVIATIONS

aa: acetic acid

Carb-PE: carbamylated phosphatidylethanolamine

Carb-PS: carbamylated phosphatidylserine

Cer: ceramide

Chol: cholesterol

DPM: disintegrations per minute

EDTA: ethylenediaminetetraacetic acid

FLA: fluorescein lithocholic acid

HPLC: high-performance liquid chromatography

HSA: human serum albumin

kDa: kilodaltons

K_d: dissociation constant

Meth: methanol

PA phosphatidic acid

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PK: proteinase K

PLD: phospholipase D

PMSF: phenylmethylsulfonyl fluoride
PS: phosphatidylserine
rpm: revolutions per minute
SD: standard deviation
SDS: sodium dodecyl sulfate
SM: sphingomyelin
Std: standard
TLC: thin-layer chromatography
Tris: tris-(hydroxymethyl)-aminomethane
CHAPTER 1

INTRODUCTION

1.1 Overview

The general purpose behind this work was to investigate the in vitro binding of isocyanate (carbamylation) to the erythrocyte membrane and to establish whether the level of binding is increased in individuals with chronic renal failure (uremia). What follows is a review of information pertinent to this investigation which is succeeded by a more specific discussion concerning the purpose(s) of the present research.

1.2 The Erythrocyte Cytoskeleton and Lipid Bilayer

Mature erythrocytes of mammals consist only of cytoplasm (containing all enzymes of glycolysis and the hexose monophosphate shunt, providing ATP and NADH/NADPH, respectively) and a cell membrane — they lack a nucleus, mitochondria, and all other organelles (Nakao, 1990), and no new membrane synthesis or even significant repair occurs (Devlin, 1992) during its 120-day lifespan (Ralston, 1990). The shape of the erythrocyte in isotonic solution is a smooth biconcave disk. Essentially all the cytoplasmic contents of the cell can be released by osmotic hemolysis (Dodge et al., 1963) to give erythrocyte ghosts which are pure plasma membranes. During the life span of the erythrocyte, it endures more than 500,000 turbulent passages through the circulation and travels a distance of approximately 300 miles (Ralston, 1990), much of it through capillaries with diameters much less than that of the erythrocyte (8 μm). The
membrane of the erythrocyte must be deformable, not only to traverse the capillaries but also to maintain a low viscosity of blood for efficient circulation. It has been said that "if red cells were rigid, blood would have the consistency of brick or concrete" (Dintenfass, 1971). The shape, mechanical stability and deformability of the erythrocyte membrane come from a direct association of plasma membrane spanning proteins (integral proteins) and an underlying fibrous network of proteins (peripheral proteins) referred to as the cytoskeleton (Stryer, 1988).

All of the erythrocyte membrane proteins are solubilized in sodium dodecyl sulfate (SDS) solution and most of the membrane proteins can be separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as monomer peptides. The protein bands are termed band 1 (α spectrin, 240 kDa), 2 (β spectrin, 220 kDa), 2.1 (ankyrin, 180 kDa), 3 (anion channel, 100 kDa), 4.1 (78 kDa), 4.2, 4.5, 4.9, 5 (actin, 43 kDa), 6, 7 and hemoglobin, according to their order of separation by SDS-PAGE (Fairbanks et al., 1971). In addition, some protein bands appear as glycoproteins containing a large amount of carbohydrate, after periodic acid-Schiff reagent (PAS) staining instead of Coomassie Brilliant Blue stain for protein (Fairbanks et al., 1971). When erythrocyte membranes are incubated in low ionic strength solutions [containing ethylenediaminetetraacetic acid (EDTA)], spectrin and actin complexed to band 4.1 are extracted and the remaining membrane endovesiculates to form vesicles that are 80-90% inside out (Bennet and Branton, 1977). Treatment of the membrane with dilute acid or base releases up to 50% of
the membrane protein (Steck, 1974).

Lipids of the erythrocyte membrane (Figure 1), like all other biological membranes, are arranged in a bilayer, with polar headgroups facing the aqueous phase of the plasma membrane and the cytoplasm while the hydrophobic acyl chains are oriented toward the interior of the bilayer. Phospholipids and unesterified cholesterol are present in approximately equimolar amounts, and account for nearly 70% and 23% by weight of the total lipid fraction, respectively. The major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) accounting for 33, 30, 13, 24 mol%, respectively, of the total membrane phospholipid (Gennis, 1989a). Both PC and SM contain choline as a polar headgroup, whereas PE and PS contain a primary amino group in their headgroup and, as such, are referred to as aminophospholipids.

1.2.1 Cytoskeleton Interactions

1.2.1.1 Protein-Protein Interactions

Since SDS-PAGE provides stable components in the ratio 1 mol spectrin dimer (αβ): 2-3 mol actin: 1 mol band 4.1: 1 mol ankyrin, many researchers have proposed very similar assembly models (Lux, 1979; Cohen 1983) (Figure 1) supported by high-resolution electron microscopy (Tsukita et al., 1980). In vitro binding experiments have shown that these components bind to each other with a $K_d$ of $10^{-7} - 10^{-9}$ M as shown in Table 1. The specificity of binding is very high and the activity of binding is easily lost after denaturation or chemical modification
FIGURE 1

The Erythrocyte Cytoskeleton and Plasma Membrane

Legend

Panel A: Schematic diagram of the proposed mode of binding of the erythrocyte membrane skeleton to the plasma membrane. Spectrin is linked to the anion channel (band3) by ankyrin, and to glycophorin by protein 4.1, which also binds an actin filament. The figure was reproduced from Stryer (1988) without permission. Panel B: Chemical structures for the major lipid components of the erythrocyte plasma membrane.
FIGURE 1

A

Anion channel
Ankyrin
Glycophorin
Actin
Protein 4.1
Spectrin

B

MAJOR LIPID COMPONENTS

- Phosphatidic acid (PA)
- Phosphatidyl choline (PC) 25%
- Phosphatidyl ethanolamine (PE) 22%
- Phosphatidyl serine (PS) 10%

- Sphingomyelin (SM) 18%

- Cholesterol (CHOL) 25%
**TABLE 1**

**Dissociation Constants for Components of the Erythrocyte Cytoskeleton**

<table>
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<th>Interaction</th>
<th>$K_u$ (M)</th>
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<tr>
<td>Spectrin dimer—dimer</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>Spectrin—band 4.1</td>
<td>$= 10^{-7}$</td>
</tr>
<tr>
<td>Spectrin—ankyrin</td>
<td>$5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Band 3—ankyrin</td>
<td>$5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Band 4.1—glycophorin</td>
<td>$1 \times 10^{-7}$</td>
</tr>
<tr>
<td>Spectrin—PS</td>
<td>$7 \times 10^{-7}$</td>
</tr>
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</table>

PS, phosphatidylserine. The table was adapted from Nakao (1990).
(Nakao, 1990).

Spectrin is the largest, both in size and in quantity, of the main components of the erythrocyte cytoskeleton. It consists of two subunits, α (240 kDa) and β (220 kDa). The subunits are rod-shaped molecules about 100 nm long, and are arranged side-by-side and antiparallel to form heterodimers. They have many 108-amino-acid repeating units and are homologous (Speicher and Marchesi, 1984; Speicher, 1986). The spectrin heterodimers are arranged in head-to-head fashion to form tetramers (Tyler 1980) with a length of 200 nm. Spectrin is bound to the membrane by linkages to ankyrin and band 4.1 at different sites (Figure 1). Ankyrin forms a cross-link between the β chain of spectrin (Calvert et al., 1980) and the amino-terminal cytosolic domain of the anion channel (band 3) (Bennett and Stenbuck, 1980). Band 4.1, which consists of two nearly identical chains, promotes the binding of actin filaments to the carboxy-terminal portion of both spectrin chains (Correas et al., 1986). Band 4.1 also links this spectrin-actin complex to the cytosolic face of glycophorin (Anderson and Lovrien, 1984). Several spectrin tetramers insert at each protein 4.1 junction complex to form a continuous meshwork underlying the plasma membrane (Stryer, 1988).

1.2.1.2 Protein-Lipid Interactions

The distribution of lipids in the erythrocyte membrane has been clearly demonstrated to be highly asymmetric (Verkleij et al., 1973), so that 75-80% of the phospholipids containing choline (i.e., PC and SM) are found in the outer monolayer, while the inner monolayer retains most of the aminophospholipid
(virtually all PS and 80% of the PE). Cholesterol is detectable on both sides of the bilayer, because it can rapidly move from one side to the other. Since these phospholipids do not adopt an asymmetric distribution in constructed liposomes (Scott et al., 1984), it is clear that mechanisms exist for the control and maintenance of aminophospholipid asymmetry. As currently understood, this state is maintained through two mechanisms. First, Haest (1982) proposed that cytoskeletal proteins contribute to the maintenance of the aminophospholipids inside orientation by selective interactions that do not occur with the cholinephospholipids. Secondly, Seigneuret and Devaux (1984) were the first to report on the existence of an ATP-dependent protein transporter (flippase) process that specifically moves aminophospholipids from the outer to the inner leaflet of the cell membrane. Experiments using several model membrane systems provide evidence in support of direct membrane cytoskeleton-PS interactions. Studies with liposomes and monolayer lipid films have demonstrated that spectrin (Maksymiw et al., 1987) and band 4.1 (Shiffer et al., 1988) specifically interact with PS. Evidence for a direct association between the cytoskeleton and the lipid bilayer in vivo comes from observations that dysfunctional mutant spectrin (Franck et al., 1985) or quantitative deficiencies of either spectrin (Williamson et al., 1982) or protein 4.1 (Mohandas et al., 1985) have an altered phospholipid asymmetry.

1.3 Erythrocyte Membrane Fluidity

For an ordinary liquid such as water or oil, the term “fluidity” is defined as the inverse of viscosity, which is essentially a measure of the frictional resistance
encountered when adjacent "layers" of fluid are moving with different velocities (Gennis, 1989b). In a biological membrane fluidity does not have a precise definition but is usually taken to mean the relative motional freedom of membrane constituents, particularly the fatty acyl chains of phospholipids (Harris and Simon, 1987). Fluidity is most commonly measured by observing the motion of spin probes or fluorescent probes incorporated into the bilayer (Section 1.3.1). It is the accepted view that membrane functions are critically dependent on the maintenance of optimal membrane fluidity (Gennis, 1989b). It is generally assumed that the function of transmembrane proteins requires a conformational change and/or lateral motion, which depends in part upon the interactions of both annular (lipid boundary immediately adjacent to the protein) and bulk lipid with the protein subunits (Gennis, 1989b). Pathological and in vitro induced changes in membrane fluidity have been correlated with changes in many membrane functions, including enzyme activity, ligand-receptor interactions and bilayer transport (Spector and Yorek, 1985; McElhaney, 1982; Santini et al., 1992).

Alterations (increases or decreases) in membrane fluidity have been directly correlated with the following:

1) Increases in membrane cholesterol decrease membrane fluidity (Ferretti et al., 1988). Due to its rigid planar shape cholesterol orients perpendicular to the membrane normal with the OH group in the vicinity of the fatty acyl carbonyls (i.e., just below the polar head group) (Franks, 1976) and restricts the angular motion of the fatty acyl chains;
2) An increased degree of saturation of fatty acyl chains decreases membrane fluidity (Cooper, 1977). Phospholipids containing fully saturated acyl chains can pack together tightly. The cis-double bond, however, is inflexible and introduces a bend in the chain. This disturbs or loosens the packing of the acyl chains thereby increasing their freedom of rotation;

3) Lipid peroxidation leads to a decreased fluidity (Jain, 1988). Lipid peroxidation results in the accumulation of malondialdehyde (an end product) which is capable of crosslinking various lipid components;

4) Nonenzymatic glycosylation of erythrocyte membrane proteins leads to a decreased membrane fluidity. Nonenzymatic protein glycosylation involves an initially reversible Schiff base adduct between a deproteinated lysine side-chain and the glucose carbonyl group which slowly rearranges, in an irreversible Amadori rearrangement, to a stable ketoamine (Schleicher and Wieland, 1989). Miller et al. (1980) established that diabetics had a much increased degree of nonenzymatic glycosylation of erythrocyte membrane proteins than control individuals. Watala (1988) subsequently demonstrated a direct reciprocal relationship between erythrocyte membrane fluidity and in vitro glycated membrane protein \((r = 0.91)\). A chemical pathway leading from membrane protein glycosylation (or presumably any covalent modification) to an alteration in membrane fluidity has, however, never been proposed. More recently it has been demonstrated that incubation of erythrocytes in hyperglycemic solution causes a loss of phospholipid asymmetry (Wilson et al., 1993). Preliminary evidence indicated that this reflected an
increased passive phospholipid flip-flop caused by a secondary effect of hyperglycemia such as nonenzymatic protein glycosylation. One might propose, therefore, that nonenzymatic glycosylation of the peripheral membrane protein alters its electrostatic interaction with aminophospholipid headgroups (Section 1.2.1.2) leading to a concomitantly altered phospholipid asymmetry and membrane fluidity. There is, in fact, considerable and growing interest in the details of how peripheral proteins such as spectrin (Maksymiw et al., 1987) and myelin basic protein (Surewicz et al., 1987) bind to and influence the bilayer.

1.3.1 Measurement of Membrane Fluidity: Fluorescence Polarization
(Lakowicz, 1983; Stubbs, 1983)

The techniques used to study membrane fluidity include invasive probing using fluorescence polarization (fluorescence probe), electron spin resonance (spin probe), and the noninvasive technique of $^2$H-NMR. The method of fluorescence polarization was employed in our studies.

The basic principle of measuring fluorescence polarization involves the partitioning of a polar fluorophore into the lipid bilayer which is excited with vertically polarized light ($z$-polarized in an $xyz$ coordinate system) and the steady-state emission measured. In general, this gives information about the degree of rotation of the probe and, hence, the fluidity of the membrane.

For steady-state measurements (continuous illumination) emission intensity ($I$) is measured parallel ($II$) and perpendicular ($\perp$) to the polarized exciting light. Historically, the data has, in most cases, been reported as polarization ($P$), where:
\[ P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \]  

[eq.1]

However, from a mathematical and theoretical viewpoint, the preferable expression is in terms of anisotropy (A), where:

\[ A = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \]  

[eq.2]

Both P and A are, therefore, related to the ratio \( I_\parallel / I_\perp \). The maximum and minimum values of A are 0.4 and 0, respectively.

The degree of loss of the original plane of polarized light, subsequent to absorption by the fluorophore is dependent upon the fluorescent lifetime of the fluorophore (\( T_f \), typically less than 10 nsec), and the rotational time of the fluorophore (\( T_c \), in nsec). These dependencies have been mathematically correlated by Perrin (1926) into the following relationship:

\[ A = A_0 \times (1 + T_f / T_c). \]  

[eq.3]

where, \( A_0 \) (limiting anisotropy) refers to the anisotropy observed in the absence of rotational depolarization, i.e., anisotropy observed when the probe is immobile during its fluorescent lifetime. Since the maximum value of A is 0.4, then any value of \( A_0 \) less than 0.4 is due to an intrinsic angle between the absorption and emission dipoles which serves to depolarize the original plane of polarization even in the absence of molecular rotation.

The rotational rate of a fluorophore is dependent on, the temperature (T), the viscosity (N), and the volume of the rotating fluorophore (V) as follows:
\[ N = \frac{T_0 \cdot RT}{V} \]  

[eq.4]

From a comparison of equations 3 and 4, one can obtain a measure of the viscosity of a solution by a simple measure of \( A \) (assuming \( A_0 \) and \( T_f \) are known).

Although many probes of membrane fluidity exist, by far the most common probe is diphenylhexatriene (DPH). This is so because DPH has a high extinction coefficient but does not fluoresce in aqueous solution (Shinitzky and Barenholz, 1974) and distributes throughout the hydrophobic core of the lipid bilayers (i.e., both the inner and outer leaflets) (Harris and Simon, 1987). It is important to realize that since each probe has a characteristic molecular structure and polarity, they would not all be expected to be found in the same location(s) within the bilayer labyrinth. Some probes may be adjacent to protein molecules, or trapped by protein aggregation, whereas others may be in different lipid domains with different physical states [e.g., the DPH analogue, trimethylammonium-DPH, contains a charged group and is anchored at the surface to the membrane (Prendergast et al., 1981)]. An excellent study of membrane fluidity probes and their varied locations within a membrane has been reported (Ben-Yashar and Barenholz, 1991). In any case, a particular fluorescent probe only relays information concerning the fluidity of its immediate microenvironment, which in most cases is undefined, and is not sufficient to characterize the physical state of the entire membrane. Relative changes in the polarization of one probe in a particular membrane as a result of disease or experimental manipulation, however, have great clinical utility since they are highly suggestive of a gross alteration in
membrane structure.

1.4 Urea-Isocyanate Equilibrium in Aqueous Solutions.

It has long been known that urea exists in equilibrium with ammonium cyanate. Knowledge of the interrelation between these two species began with the historic synthesis of urea from ammonium cyanate by Wöhler (1828). Wöhler’s synthesis is now known to result from the dissociation of ammonium cyanate into ammonia and isocyanic acid, followed by addition (Conant and Blatt, 1959):

\[
\text{NH}_4\text{NCO} \rightleftharpoons \text{NH}_3 + \text{HN} = \text{C} = \text{O} \rightleftharpoons \text{H}_2\text{NCONH}_2
\]

ammonium cyanate

ammonia isocyanic acid

urea

In fact, in an aqueous solution of urea the following reactions occur (Hagel et al., 1971):

\[
\text{H}_2\text{NCONH}_2 \rightleftharpoons \text{NH}_3 + \text{HNCO}
\]

\[
\text{HNCO} + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{CO}_2
\]

Taking into account the equilibrium constant of the urea-isocyanic acid equilibrium and the rate of hydrolysis of isocyanic acid into ammonia and CO\(_2\), the formation of ammonia and isocyanic acid in an aqueous solution of pure 8 M urea at a given pH, ionic strength, and temperature was calculated by Hagel et al. (1971). From these results, as well as those of Dirnhuber and Schütz (1948) it would appear that at equilibrium an 8 M urea solution at pH > 6 would be 0.02 M with respect to isocyanic acid.

Isocyanic acid is very reactive. Besides the above mentioned reactions,
isocyanic acid reacts with alcohols to form urethanes (alkyl carbamates) (Conant and Blatt, 1959):

\[ \text{HN} = \text{C} = \text{O} + \text{C}_2\text{H}_5\text{OH} \rightarrow \text{H}_2\text{NCOOC}_2\text{H}_5 \]

urethane

Isocyanic acid also reacts with primary and secondary amines to form substituted ureas (Conant and Blatt, 1959):

\[ \text{HN} = \text{C} = \text{O} + \text{H}_2\text{NR} \rightarrow \text{H}_2\text{NCONHR} \]

\[ \text{HN} = \text{C} = \text{O} + \text{R}_2\text{NH} \rightarrow \text{H}_2\text{NCONR}_2 \]

1.4.1 Reactions of Isocyanate with Amino Acids and Proteins

The carbamylation of the \( \alpha \)-amino groups of amino acids by isocyanic acid has been known for some time. For example, in 1947 Nye and Mitchell synthesized carbamyl aspartic acid in good yield by allowing equimolar amounts of the monopotassium salt of the amino acid and KNCO to stand in aqueous solution at room temperature. In 1949 Schütz stated that cyanate would react readily with the \( \varepsilon \)-amino and sulfhydryl groups of amino acids, although he gave no data, and suggested that the same reaction might also occur with proteins. It was not until 1960 that direct evidence was provided for the reaction of isocyanic acid with protein. During the course of a study of the influences of denaturing agents on the reaction of iodoacetate with ribonuclease, Stark et al. (1960) found that when control solutions, which contained no iodoacetate, were kept in 8 M urea at 40° C and then dialyzed to remove urea, ion-exchange chromatography
generated a large unexpected peak in a position just ahead of that normally ascribed to ribonuclease A. From the position of the peak, it was hypothesized that, in the urea solution, a chemical reaction had taken place which had made the protein less basic. Upon amino acid analysis of an acid hydrolyzate of a sample of ribonuclease that had been incubated with urea, the lysine content was found to be less than that of ribonuclease A and, in addition, a new peak appeared on the ion exchange column just in front of and partly overlapping the position of valine. The amount of this new amino acid increased and the amount of lysine concomitantly decreased as the time during which ribonuclease A was exposed to urea was increased. In addition a homocitrulline (ε-carbamyl lysine) standard eluted at the same position as the new peak. It was concluded that a chemical reaction had taken place between isocyanic acid and the ε-amino groups of lysine residues in ribonuclease A.

Further studies have shown that cyanate reacts with amino, sulfhydryl, phenolic, carboxyl, and imidazole groups of proteins, to form carbamyl derivatives.

1.4.1.1 Reaction of Cyanate with Protein Amino Groups

The reaction of cyanate with amino groups (RNH₂, where R represents

![Diagram of reaction between cyanate and amino group]
protein) involves nucleophilic addition of the unprotonated amino nitrogen to neutral isocyanic acid (Stark, 1964), the reactive form of cyanate. The reaction rate depends on the pKa of the amino group (typically 6-7 for the α-amino group and 9-10 for the ε-amino group of lysine residues), the pKa of the isocyanic acid (approximately 4) (Smyth, 1967), and the pH of the medium. Consequently, the concentration of isocyanic acid decreases by a factor of 10 for each rise in pH whereas the concentration of the uncharged form of the amino group increases correspondingly. Since the rate of reaction is proportional to the product of the concentrations of the reactive species at each pH value, the rate is, therefore, essentially constant over the pH range of 5 to 8. As a result of their lower pKa values at physiological pH the α-amino groups of proteins are expected to be carbamylated 50 to 100 times faster than the ε-amino group of lysine residues (Stark, 1965a).

1.4.1.2 Reaction of Cyanate with Sulphydryl Groups

Sulphydryl groups (cysteine) react with cyanate (Stark, 1964) more rapidly

\[
\begin{align*}
\text{H--O--H} & \quad \text{H--O}^- \\
\quad \text{H} & \quad \text{R}--\text{S}^\prime \\
\text{N} & \quad \text{R}--\text{S}--\text{C} \quad \text{NH}_2 \\
\text{C} & \quad \text{Carbamylated Protein Cysteine Group}
\end{align*}
\]

\[
\begin{align*}
\text{Protein Cysteine Group} & \quad \text{k}_1 \\
\text{k}_1 & \quad \text{k}_1
\end{align*}
\]
than do amino groups, but the reaction is readily reversible. The value of \( k_1 \) is constant between pH 6 and 8, but \( k_4 \) depends on the OH\(^-\) concentration. Therefore, the position of the equilibrium constant is dependent on pH. At pH 5 and lower carbamylmercaptans are relatively stable. Between pH 6 and 8 (at 25° C) their half-life varies from 11 to 6 minutes.

### 1.4.1.3 Reaction of Cyanate with Protein Phenolic Hydroxyl Groups

The phenolic hydroxyl group of tyrosine reacts readily with cyanate (Smyth, 1967) in a reversible reaction similar to the one that occurs with sulphydryl groups. The mechanism involves isocyanic acid and the negatively charged phenolic anion:

\[
\begin{align*}
\text{H–O} & \xlink \text{H} & \xlink \text{H} \\
\text{H} & \xlink \text{N} & \xlink \text{C} & \xlink \text{O} & \xlink \text{O} & \xlink \text{NH}_2 \\
\text{R} & \xlink \text{O}^{-} & \xlink \text{O}^{-} & \xlink \text{O}^{-} & \xlink \text{O}^{-} & \xlink \text{O}^{-} \\
\text{Protein Tyrosine Group} & \xlink \text{Carbamylated Protein Tyrosine Group}
\end{align*}
\]

The rate of formation of O-carbamyltyrosine is independent of pH over the range 5 to 9. The carbamyl derivative is stable in acidic medium but decomposes rapidly in neutral and alkaline solutions.
1.4.1.4 Reaction of Cyanate with Protein Carboxyl Groups

Cyanate reacts with carboxyl groups (aspartate and glutamate) to form mixed anhydrides of carbamic acid (Stark, 1965a):

\[
\begin{align*}
\text{H} & \quad \text{O} \quad \text{H} \\
\text{O} & \quad \text{C} \quad \text{O} \\
\text{R} & \quad \text{C} \quad \text{O} \\
\text{N} & \quad \text{C} \quad \text{O} \\
\end{align*}
\]

Protein Carboxyl Group

\[
\begin{align*}
\text{H} & \quad \text{O}^{-} \\
\text{O} & \quad \text{C} \quad \text{O} \\
\text{R} & \quad \text{C} \quad \text{O} \\
\text{NH}_2 & \quad \text{C} \quad \text{O} \\
\end{align*}
\]

Carbamylated Protein Carboxyl Group

The rates of both the forward and the reverse reactions vary with pH. An increase in 1 pH unit decreases 100-fold the equilibrium concentration of carbamyl-carboxylate. Therefore its formation, which proceeds readily at pH 5, can be avoided entirely at pH 7.

1.4.1.5 Reaction of Cyanate with Protein Imidazole Groups

The imidazole group of histidine reacts reversibly with cyanate (Stark,
1965b) to form carbamylimidazole. At a pH greater than 7 carbamylimidazole is very unstable.

From the above reactions of cyanate with protein functional groups it is evident that, while many reactions are possible, at physiological pH the only significant reactions are those of isocyanic acid with α- and ε-amino groups to yield α-carbamyl amino acids and homocitrulline (carbamyl lysine), respectively.

1.5 Measurement Methods for Carbamylated Proteins

Methods have been developed for the analysis of both α- and ε-amino group carbamylation.

1.5.1 α-Amino Group Carbamylation Analysis (Stark and Smyth, 1963)

1) This method involves treatment of protein with hot 6 M HCL for 1 h to generate a mixture of hydrolyzed amino acids, peptides and the terminal α-carbamylated amino acid of the protein. Under acidic conditions α-carbamylated amino acids are known to spontaneously cyclized to form hydantoins. The hydantoin, because of its neutral character is separated from the other charged amino acids by ion-exchange chromatography. The hydantoin is then hydrolyzed for 20 h in 0.2 M NaOH or 96 h in 6 M HCL at 100° C. Under these conditions the original amino acid is regenerated along with the liberation of ammonia and CO₂. The amino acid is quantitated by amino acid analysis using either ion-exchange chromatography of reversed-phase HPLC. Because the exact amino acid can be identified, this method has also be used to determine the NH₂-terminal residue in proteins.
2) The method of Kwan et al. (1990) involves treatment of the protein in a mixture of 6 M HCL and 8 M acetic acid for 2 h at 100° C. The resulting hydantoins are isolated from the amino acid mixture by solvent extraction in ethyl acetate and analyzed by octadecyl silica-bonded reversed-phase HPLC (Apex II) with UV detection or by gas chromatography (Manning et al., 1973).

1.5.2 ε - Amino Group Carboxamidation (Homocitrulline) Analysis

1) The method of Stark et al. (1960) involves hydrolysis of the protein in 6 N HCL at 110° C for 22 h. Homocitrulline is fairly stable to acid hydrolysis. When heated at 110 C for 22 h in 6 N HCL only 24% of the homocitrulline decomposes and appears as lysine whereas the terminal hydantoin carbamyl group has been converted to ammonia and CO₂. Homocitrulline is then measured by amino acid analysis. (NOTE: S-carbamyl cysteine is not stable to acid hydrolysis; the products formed are predominantly ammonia and cysteine (Stark et al., 1960).

2) The recent method of Koshiishi and Imanari (1989) involves nitrosylation of carbamyl lysine residues by nitrite in acidic solution followed by degradation of nitrosourea to cyanate ion in alkali solution which is detected by a cyanate ion analyzer.

3) The carbamido-diacetyl reaction of Fearon (1939) has been the basis for a number of colorimetric methods used to determine urea and carbamyl derivatives. The identity of the final chromogen formed has not been confirmed. Venjamin and Vakirtzi-Lemonais (1970) have proposed the formation of a glycoluril, while others have proposed the generation of a diazone (Kaplan, 1989).
The reaction is thought to proceed as follows (Venjamin and Vakirtzi-Lemonais, 1970):

The diacetyl monoxime does not directly react with urea but is first hydrolyzed to form diacetyl and hydroxylamine. The unstable diacetyl condenses with urea and substituted ureas in an acidic environment to form either a 1,3-diazole derivative, or a glycoluril, which absorb in the 525-550 nm region. Many chemical modifications of the assay have been made to enhance or stabilize the absorption of the chromogen, either directly (such as thiosemicarbazide (Coulombe
and Favreau, 1963)) or indirectly, by elimination of the hydroxylamine by compounds such as potassium persulfate (Ormsby, 1942). The method of Wybenga et al. (1971) which uses thiosemicarbazide and cadmium ion, produces a stable chromogen (i.e., less than 5% decrease in absorbance per hour) and is utilized throughout this dissertation as a means of measuring carbamylation (Section 2.2.3.3).

4) The most recent means of detecting protein carbamylation has resulted from the production of polyclonal antibodies specific for homocitrulline (Kraus et al., 1991; Kraus et al., 1994). An enzyme-linked immunosorbent assay (ELISA) was developed and shown to be capable of detecting a number of carbamylated proteins. At present the antibodies are not commercially available and the technique has only qualitative use. The technique, however, has great potential utility in both research and clinical settings.

1.6 Carbamylated Proteins in Vitro: Sickle Cell Anemia

The list of proteins that have been carbamylated in vitro is extensive and is the subject of a review (Carreras et al., 1976). The effects of carbamylation range from: decreased activities of enzymes such as phosphorylases a and b (Huang and Madsen, 1966) and pyruvate kinase (De Furia et al., 1972); to loss of antibody sites on anti-ρ-azobenzene arsonate (Chen et al., 1962); to loss of biological activity of a number of hormones such as insulin and thyroid-stimulating hormone (Cole, 1961); and altered binding affinities of CO₂, O₂, and DPG in hemoglobin (Carreras et al., 1976). Carbamylation can, therefore, be considered
an amino group specific reaction rather than a protein specific reaction.

1.6.1 Carbamylated Hemoglobin

The most extensively studied *in vitro* carbamylation is that for hemoglobin (Uvelli *et al.*, 1980). This is directly due to the finding that the carbamylation of the NH₂-terminal valine residue of hemoglobin S prevents the gelling of deoxygenated hemoglobin and the sickling of the erythrocytes (Cerami and Manning, 1971) and, as such, lengthens the survival of the erythrocytes (Gillette *et al.*, 1971). Not surprisingly, therefore, cyanate was used throughout the 1970's as an anti-sickling agent (Harkness and Roth, 1975).

1.6.2 Erythrocyte Carbamylation

Much, if not all, of the interest in carbamylated proteins came about as a result of cyanate treatment for sickle cell anemia. It was of interest, at the time, to establish the potential effects of cyanate on the various constituents of blood in order to establish a dose which maximized its anti-sickling effect while minimizing any adverse effects on other proteins. Besides the *in vitro* effects mentioned above, investigations were undertaken to establish the effect of *in vitro* incubation of cyanate with the erythrocyte. De Furia *et al.* (1972) showed that incubation of erythrocytes with 10 mM potassium cyanate at physiological pH and temperature for 1 h did not significantly decrease erythrocyte metabolism and function. Only the pyruvate kinase activity of the carbamylated cells was decreased (approximately 25%). With higher concentrations of cyanate (25-50 mM), Carreras-Barnes *et al.* (1972) found extensive incorporation of [¹⁴C] cyanate into the membrane of
erythrocytes. Further studies have shown the increased autohemolysis (Diederich et al., 1971) and altered membrane surface charge (Durocher et al., 1973) of in vitro carbamylated erythrocytes. Since the integrity of the erythrocyte membrane is a major determinant of the erythrocyte lifetime, these alterations were proposed to shorten cell life (Lane and Burka, 1976). In fact, Lane and Burka demonstrated that rabbit erythrocytes which were carbamylated in vitro with [14C] cyanate and injected back into circulation have a decreased survival time relative to control erythrocytes. The decreased survival time (16-63%) was directly correlated to the concentration of cyanate to which the erythrocytes were exposed. It was further suggested by Lane and Burka (1976) that the advantageous effect of carbamylation in decreasing sickling may be counterbalanced by deleterious effects on the cell membrane which decrease life span since "in no study was it shown that treated cells had been restored to a fully normal life span". By the end of the 1970's cyanate treatment for sickle cell anemia was abandoned due to the appearance of peripheral neuropathies (Peterson et al., 1974), subcapsular cataracts, and weight loss in subjects maintained on long-term oral cyanate therapy (Harkness and Roth, 1975). Much of the research, therefore, concerning protein carbamylation was concomitantly dropped.

1.7 Carbamylated Protein and Uremia

Urea is the major nitrogen-containing metabolic end-product of protein metabolism in man, comprising over 75% of the nonprotein nitrogen eventually excreted (Rock et al., 1986). The biosynthesis of urea from amino nitrogen-derived
ammonia is carried out exclusively by hepatic enzymes of the urea cycle, and over 90% of urea is excreted by the kidney. Urea is neither actively reabsorbed nor secreted by the tubules but is filtered freely by the glomerulus (Rock et al., 1986).

As a consequence of chronic kidney failure (uremia) the plasma levels of urea are greatly increased (30-50 mM) above normal (3-7 mM) (Rock et al., 1986). Because of the equilibrium between urea and isocyanic acid (Section 1.4), uremic individuals would be expected to have an increased level of isocyanic acid. Harding and Rixon (1980) speculated that up to 1 mM cyanate might be present in the blood of uremics. Actual analysis for cyanate in the plasma of normal and uremic individuals, however, clearly shows that cyanate is not detectable (detection limit, < 1 μM) (Koshiishi and Imanari, 1990). The generally accepted view is that since isocyanic acid is a very reactive species, that its free ion concentration is kept low by reactions with plasma proteins (Koshiishii and Imanari, 1990; Kraus et al., 1994).

It has been noted by Kwan and Carr (1992) that the toxic manifestations of cyanate therapy in sickle cell anemia (Section 1.6.2) are "not dissimilar to some of the symptomatology seen in uremia, thus raising the possibility of protein carbamylation having a role in the mechanism of uremic toxicity". What follows is an overview of the carbamylation of hemoglobin, plasma proteins, and polymorphonuclear neutrophils in uremia. A discussion of erythrocyte membrane carbamylation in uremia is included in the statement of research purpose (Section 1.8).
1.7.1 Hemoglobin Carbamylation

Flückiger et al. (1981) were the first to demonstrate the presence of carbamylated protein in uremia. They showed elevated levels of carbamylated hemoglobin which correlated well with the time-average urea concentrations in hemodialysis patients. More recently, others have shown (Kwan et al., 1992) that the level of carbamylated hemoglobin (by HPLC analysis of carbamyl valine) is approximately 5-fold higher than normal individuals and that the levels are independent of age and hemodialysis procedure.

1.7.2 Plasma Protein Carbamylation

Because of the nonspecific nature of carbamylation it is not surprising, therefore, that the levels of plasma protein carbamylation have also been shown to be qualitatively increased in uremics (Erill et al., 1980; Oimoni et al., 1981). In fact, the observed decreased plasma protein binding of some acidic drugs in uremics has been proposed to result from the increased levels of carbamylated plasma proteins (Erill et al., 1980). Koshiishi and Imanari (1989) have recently devised a quantitative method based upon the degradation of carbamyl lysine groups (homocitrulline) in plasma protein to cyanate ion by nitrite (Section 1.5.2). The method was, however, only applied to the plasma of normal and renal failure induced rats.

1.7.3 Polymorphonuclear Neutrophil Carbamylation

The binding of polyclonal antibodies developed against homocitrulline (Section 1.5.2) to polymorphonuclear neutrophils (PMN) (i.e., leukocytes) from
normal and uremic individuals was visually demonstrated using a fluorescein isothiocyanate-labelled secondary antibody and fluorescence microscopy (Kraus et al., 1994). In PMN's from normal individuals a strong linear fluorescence surrounds the nucleus. Since the outer nuclear membrane is continuous with the endoplasmic reticulum, this suggests that the endoplasmic reticulum is a carbamylated cellular organelle. In PMN's from uremics there is binding of the antibody to cell surface proteins as well as homogeneously throughout the cytoplasm.

1.8 Research Purpose: Carbamylation of Erythrocyte Membrane Components

Besides the demonstration by Carreras-Barnes et al. (1972) that [14C] cyanate was extensively incorporated into the erythrocyte membrane under physiological conditions, and that this in vitro carbamylation results in a decreased rabbit erythrocyte lifetime (Lane and Burka, 1976) and an altered membrane surface charge (Durocher et al., 1973), there has been no documentation in the literature concerning carbamylation of the erythrocyte membrane. The intrinsic levels of membrane protein carbamylation and whether these levels are increased in uremics is not known.

Since, like hemoglobin, the membrane proteins of erythrocytes are not turned-over during the 120-day lifespan of the erythrocyte, it would not be surprising to find a significant level of carbamylation among these proteins. In fact, Schleicher et al. (1981) have shown that, in diabetics, the levels of erythrocyte membrane protein glycosylation (i.e., covalent modification of ε-amino group of
lysine residues by glucose in a manner analogous to carbamylation) and glycosylated hemoglobin are essentially equivalent (i.e., = 2.5 nmol lysine-bound glucose / mg protein), despite the fact that the plasma concentration of hemoglobin (160-180 mg/mL) is approximately 70-fold higher than the concentration of erythrocyte membrane protein (2-3 mg/mL).

The hematological consequences of uremia on the erythrocyte include a decreased survival time (Eschbach et al., 1977), a decreased (Ca$^{2+}$ + Mg$^{2+}$)-ATPase pump activity (Shalev, 1991), a decreased deformability (Kikuchi et al., 1982), and a decreased membrane fluidity (Komidori et al., 1985). Since, as mentioned, the integrity of the erythrocyte membrane is a major determinant of the erythrocyte life span (Lane and Burka, 1976), it is conceivable, therefore, that should uremics have an increased level of carbamylated membrane protein this may critically alter key binding domains within the cytoskeletal matrix and, at least partially, contribute to the observed decreased erythrocyte lifetime in uremics. In this regard, it is interesting to note that a decreased erythrocyte survival time is also observed in diabetics (Peterson et al., 1977) and is believed to be, at least partially, a consequence of the increased levels of glycosylation of the membrane proteins.

Because the reaction between cyanate and protein is amino group specific rather than protein specific, it is also considered a possibility that carbamylation may extend beyond the level of protein to other non-protein primary amino group containing compounds. One such potential target is the erythrocyte membrane
lipid. The erythrocyte membrane contains nearly equivalent amounts (w/w) of lipid and protein and, together, phosphatidylserine (PS) and phosphatidylethanolamine (PE) make up approximately 35 mole% of the total erythrocyte membrane phospholipid (Gennis, 1989a). Both phospholipids contain a potentially accessible primary amino group in their polar headgroup, and are relatively easy to label with exogenous NH$_2$-specific chemical reagents such as trinitrobenzene sulfonic acid (TNBS) (Gennis, 1989c).

The purpose of the research, therefore, was twofold:

1. To establish the degree of membrane protein carbamylation in erythrocytes from both nonuremics and uremics, and to characterize the \textit{in vitro} binding of isocyanic acid to the individual proteins of the cytoskeletal matrix, and;

2. to study the potential \textit{in vitro} binding of cyanate to the erythrocyte membrane aminophospholipids (PS and PE), and to investigate whether their carbamyl derivatives can be detected in the native erythrocyte membrane.
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Biochemicals

Whole blood (in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant) was provided by the hematology departments at the Hotel Dieu Hospital (Windsor, ON) and the Salvation Army-Grace Hospital (Windsor, ON).

Distilled deionized water was prepared using a Barnstead FI-Stream distiller (Sybron Corp. Dubuque, IA) and a Milli-Q water purification system (Millipore Corp. Milford, MA).

The following materials were purchased from BDH Chemicals (Toronto, ON): Sodium phosphate (mono and diphosphates); sodium chloride; tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCL); chloroform (OmniSolv®); methanol (OmniSolv®); glacial acetic acid; perchloric acid; hydrochloric acid; diacetyl monoxime; manganese chloride; ethanolamine; mercury; phenol reagent (Folin and Ciocalteu reagent); sulfuric and orthophosphoric acids.

The following materials were purchased from the Sigma Chemical Company (St.Louis, MO): phenylmethysulfonylfluoride (PMSF); choline chloride; L-serine; urea; ethylenediaminetetraacetic acid (EDTA); ascorbic acid; calcium chloride; Triton X-100; hydrogen peroxide (30%); Sigma-Fluor™ scintillation fluid; protein standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); phospholipase D (PLD) from Streptomyces Chromofuscus; lipids (Egg yolk
L-α-phosphatidylethanolamine (Type III); L-α-phosphatidylcholine (Type III-E); sphingomyelin; bovine brain L-α-phosphatidy1-serine; and anhydrous cholesterol); ammonium molybdate tetrahydrate and bovine serum albumin (BSA).

Sodium cyanate was from Aldrich Chemical Company (Milwaukee, WI). Potassium [14C] cyanate (1 mCi) was purchased from Amersham International (Buckinghamshire, UK). Sodium azide, potassium (mono and diphosphates), sodium carbonate, copper (II) sulfate pentahydrate, and sodium potassium tartrate were obtained from Fisher Scientific Co. (Toronto, ON). Ultrapure electrophoresis reagents (acrylamide, ammonium persulfate, Bromophenol Blue, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide) and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Richmond, CA). Sodium dodecyl sulphate (SDS) was obtained from United States Biochemical Corporation (Cleveland, OH). Homocitrulline was from ICN Biomedical (Aurora, OH). Cadmium sulfate was from Mallinckrodt Chemical Works (St.Louis, MO). ENHANCE™ was purchased from Dupont Canada (Toronto, ON). Proteinase K was obtained from Boehringer Mannheim (Laval, QUE, Canada). Cadmium chloride was obtained from Anachemia Chemicals (Montreal, QUE, Canada). Thiosemicarbazide was from Eastman Organic Chemicals (Rochester, NY). Fluorescein lithocholic acid (FLA, supplied in methanolic solution) and TDx dilution buffer (0.1M sodium phosphate, 1 mg/mL sodium azide, 0.1 mg/mL bovine gamma-globulin, pH 7.4) were obtained from Abbott Laboratories (Abbott Park, IL). Ammonium hydroxide was obtained from McArthur Chemical Co (Montreal, QUE). L-lysine was from Fluka Chemika-
Biochemika (New York, NY). Kodak GBX developer and replenisher, and fixer were obtained from Picker International Canada (Brampton, ON). Hydroxyethylurea was purchased from TCI America (Portland, OR).

2.1.2 Supplies

Thin-layer chromatography (TLC) plates (glass-backed Silica Gel 60, 0.25 mm) were purchased from BDH Chemicals (Toronto, ON).

Kodak X-Omat AR5 film was obtained from Picker International Canada (Brampton, ON).

2.2 Methods

2.2.1 General Methods

2.2.1.1 Protein Quantitation

All protein determinations were conducted using a modified Lowry procedure (Marcel and Haas, 1978). Bovine serum albumin (BSA) was used as the standard and samples were read at 660 nm.

2.2.1.2 Phospholipid Quantitation

Phospholipid was quantitated as phosphorus (Higgins, 1987) using ascorbic acid as the reducing agent (Ames, 1966). Potassium monophosphate was used as the standard and samples were read at 826 nm.

2.2.1.3 Absorption Spectrophotometry

All absorption measurements were performed on a Shimadzu UV-160 absorption spectrophotometer (Kyoto, Japan) and represent the average of at least duplicate determinations.
2.2.1.4 Liquid Scintillation Counting

All liquid scintillation counting was performed on an LS 6500 Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA). Unless otherwise indicated, polyethylene scintillation vials and 10 mL of scintillation cocktail were used.

2.2.2 Erythrocyte Membrane Protein Carbamylation: In Vitro Studies

2.2.2.1 Preparation of Erythrocyte Ghosts

Unsealed erythrocyte ghosts were prepared by the method of hypotonic hemolysis (Dodge et al., 1963) in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and stored in 10 mM sodium phosphate, pH 7.4. Long storage periods (> 24 h) included 0.2 mg/mL sodium azide as an antibacterial.

2.2.2.2 Carbamylation of Ghost Membrane Protein with [*14C]* Cyanate and Quantitation of Bound Cyanate

Fresh (< 12 h) whole blood from nonuremic individuals was obtained in tubes containing EDTA as anticoagulant. Ghost cells (2 mg/mL membrane protein) were prepared and incubated in 10 mM phosphate buffer, pH 7.4, containing 25 mM sodium cyanate and 0.5 mM potassium [*14C]* cyanate (specific activity = 52 mCi/mmol) at 37 °C. One volume of reaction mixture was removed at various time intervals (1-10 h) and added to an equivalent volume of 2 M lysine in water to terminate any further reaction between cyanate and erythrocyte membrane protein. (This represents an approximately 67-fold molar excess of lysine to cyanate). The samples were then stored at 5 °C until all time points were collected. All samples were then centrifuged (12,000 x g for 30 min at 5 °C, Sorvall Instruments RC2-B
and SS-34 fixed angle rotor) and washed to remove unbound cyanate. Total cyanate (nmol) incorporation into the erythrocyte ghosts (DPM / mg ghost protein) was measured by liquid scintillation counting in 10 mL Sigma-Fluor™, with a counting efficiency of 96%, and quantitated as follows:

\[
\text{DPM / mg ghost protein} \times \left( \frac{\text{cyanate}}{[^{14}\text{C}] \text{cyanate}} \right) = \left( \frac{52 \text{ Ci mole}^{-1}}{(2.22 \times 10^{12} \text{ DPM Ci}^{-1})} \right)
\]

where \((\text{cyanate} / [^{14}\text{C}] \text{cyanate})\) is the ratio of unlabelled to labelled cyanate in the reaction mixture. The final concentration of \([^{14}\text{C}] \text{cyanate}\) was calculated as \([ (\text{stock} \ [^{14}\text{C}] \text{cyanate}) \ (\text{volume of stock} \ [^{14}\text{C}] \text{cyanate}) / \text{total assay volume} \]). Stock \([^{14}\text{C}] \text{cyanate}\) was prepared by the addition of 1.89 mL water to 1 mCi of crystalline potassium \([^{14}\text{C}] \text{cyanate}\) and the DPM of an aliquot determined and converted to stock \([^{14}\text{C}] \text{cyanate}\) according to \([ (\text{DPM of aliquot}) / (52 \text{ Ci mole}^{-1}) ] \ (2.22 \times 10^{12} \text{ DPM Ci}^{-1}) \).

2.2.2.3 SDS-Polyacrylamide Gel Electrophoresis and Fluorography of \(^{14}\text{C}-\text{Carbamylated Ghost Membrane Protein}

Separation of the major erythrocyte membrane proteins was performed by SDS-PAGE essentially as described by Laemmli (1970). Gels were composed of 5% polyacrylamide (w/v) stacking and either 8% or 15% polyacrylamide (w/v) resolving gels. Electrophoresis was performed at 180 V for 60 min using the Mini-PROTEAN II cell (Bio-Rad Laboratories, Richmond, CA) or at 66 V for 16 h using a standard apparatus. The gels were stained with Coomassie Brilliant Blue R-250,
1 g/L in methanol-acetic acid-water (40/10/50, v/v/v), and destained with methanol-acetic acid-water (40/10/50, v/v/v). Stained SDS-PAGE gels were fluorographed by incubating in ENHANCE™ (Dupont Canada, Toronto, ON). The gels were dried and exposed to Kodak X-Omat AR5 film at -80°C for 12 days.

2.2.2.4 Quantitation of Cyanate Binding in Ghost Membrane Proteins

The electrophoretograph of the SDS-PAGE separated ¹⁴C-carbamylated ghost membrane proteins was scanned by an imaging densitometer (Bio-Rad Laboratories, model GS-670). The individual proteins were quantitated (µg) by use of the volume analysis program (Molecular Analyst, Bio-Rad Laboratories) as a percentage of the loading concentration (typically 20-30 µg of membrane protein) and converted to nmol protein using the known molecular weights (Gennis, 1989c): spectrin (230 kDa); ankyrin (174k Da); anion channel (95 kDa); band 4.1 (80 kDa); band 4.2 (70 kDa); actin (43 kDa); and band 7 (29 kDa). [¹⁴C] Cyanate incorporation (nmol) into the individual membrane protein was determined by scalpel excision of the protein band followed by solubilization in 30% hydrogen peroxide (Miller et al., 1980) for 24 h at 37 °C and subsequent ¹⁴C liquid scintillation counting in 10 mL Sigma-Fluor™ with a counting efficiency of 95%. Quantitation of cyanate bound (nmol) / mg protein was calculated as above (Section 2.2.2.2).

2.2.3 Erythrocyte Membrane Protein Carbamylation: In Vivo Studies

2.2.3.1 Proteinase K Treatment of Erythrocyte Ghosts

Fresh (< 12 h) whole blood from nonuremic individuals was obtained in tubes
containing EDTA as anticoagulant. Erythrocyte ghosts (3.5 mL containing 3.5 mg of membrane protein) in 10 mM sodium phosphate, pH 7.4 were brought to 37 °C and proteinase K (PK) (typically 10-80 µL of a 8 mg/mL stock) added. After incubation (1-2 h) the sample was centrifuged (48,000 x g for 1 h at 10 °C, Beckman JL-HS and SA-20 fixed angle rotor) and the supernatant lyophilized (Labconco Freeze-Dryer, Kansas City, MO) to 1 mL. Aliquots of 100 µL were removed prior to and subsequent to incubation with PK and monitored for proteolysis, using SDS-PAGE and protein quantitation.

2.2.3.2 Measurement of Membrane Protein Proteolysis and Release by Proteinase K.

Erythrocyte ghosts (1.7 mL containing 0.85 mg membrane protein) were incubated in the absence and presence of 1 or 10 µL of a 5 mg/mL stock solution of PK in water. At various time intervals, a 500-µL aliquot of the samples was removed and immediately centrifuged (10,000 x g at 5 °C for 15 min, Sorvall Instruments, Microspin 24S, Wilmington, DE) to sediment the ghost cell membrane. An aliquot (100 µL) of the clear supernatant was removed and assayed for protein content. For each time point the percentage protein released from the membrane (corrected for protein content due to PK addition) was calculated as \[ 1 - \left( \frac{A - B}{A} \right) \times 100 \], where A is the total protein concentration at time zero, and B is the concentration of protein in the supernatant. The Lowry method of protein quantitation (Marcel and Haas, 1978) is essentially a measure of the concentration of tyrosine and phenylalanine residues and as such is not effected by proteolysis.
2.2.3.3 Reaction of Diacetyl Monoxime with Native and Proteinase-Treated Ghosts

The method of Wybenga et al. (1971) for the determination of urea and substituted ureas (carbamyl derivatives) was modified and employed for measurement of carbamylated erythrocyte membrane protein. Briefly, 500 μL of the lyophylized and PK proteolyzed membrane protein (1 mL containing approximately 1 mg protein) was added to 2.5 mL of urea-nitrogen reagent (0.83 M sulfuric acid, 1.13 M orthophosphoric acid, 0.55 mM thiosemicarbazide and 2.6 mM cadmium sulfate) and 500 μL of 3% diacetyl monoxime in water. As controls, erythrocyte ghosts (1 mg/mL) in 10 mM sodium phosphate, pH 7.4, were incubated in the absence of PK for 1 h at 37 °C. The sample was centrifuged (48,000 x g for 1 h) and the supernatant removed and lyophylized to 1 mL, while the ghost pellet was diluted up to 1 mL with 10 mM phosphate, pH 7.4. An aliquot (500 μL) of both samples was added to the diacetyl monoxime reaction mixture, as above, and all samples were incubated for 30 min at 100 °C in a dry heater block, cooled for 5 min in a beaker of cold water, and the absorption spectra recorded (400-600 nm). The chromogen(s) formed absorb maximally at 530 nm. A homocitrulline (carbamyl lysine) standard curve (0-30 nmol) was constructed using serial dilutions of a stock solution (200 μM) in 10 mM sodium phosphate, pH 7.4.

2.2.3.4 Time Course and Stability of the Homocitrulline-Diacetyl Reaction Complex

A series of homocitrulline (20 nmol) and control samples (buffer alone, 10
mM sodium phosphate, pH 7.4.) were reacted with diacetyl at 100°C, as above, for 10-60 min. At various time intervals (from 10 to 60 min) a control and homocitrulline sample were removed from the heat, cooled for 5 min and the absorbances (530 nm) recorded (the absorbance of the control sample was subtracted from the absorbance of the sample containing homocitrulline). All samples were again read (530 nm) 60 min post reaction to determine the photostability of the reaction complex.

2.2.3.5 Quantitation of Membrane Protein Carbamylation in Uremics and Nonuremics

Erythrocytes (< 3 days) from 6 uremics on a hemodialysis program, and 6 nonuremic individuals (random samples from individuals not on dialysis) were obtained in tubes containing EDTA as anticoagulant. For each sample, unsealed erythrocyte ghosts were prepared and suspended in 3.5 mL of 10 mM phosphate buffer, pH 7.4, PK (80 μL of a 8 mg/mL stock) was added, and the sample incubated at 37 °C for 1 h. The samples were then centrifuged (48,000 x g for 1 h) and the supernatant removed (2.5 - 3 mL) and lyophylized to a final volume of 1 mL. Each sample was assayed for protein content and reactivity with diacetyl, as above. Proteolyzed membrane protein released was determined and corrected for the measurable presence of PK as follows:

\[ \text{mg/mL proteolyzed protein released} = D - \frac{D}{C} \left( B - \frac{C}{A} \right) \]

where:

A = Concentration of ghost protein (mg/mL) measured prior to addition of PK;
B = Concentration of protein (mg/mL) measured subsequent to 1-h incubation of the ghosts with PK (in all cases B is simply the sum of A + PK (mg/mL));

C = Concentration of protein (mg/mL) measured in the supernatant subsequent to 1-h incubation with PK and centrifugation;

D = Concentration of protein (mg/mL) measured in the lyophylizate.

The percentage of proteolyzed protein released from the membrane as a result of PK treatment was calculated from: \[ \left( 1 - \frac{(B - C)}{A} \right) \times 100. \]

The reactivity of 500 μL of each lyophylizate with diacetyl was conducted as above. Results are quoted as homocitrulline (nmol) / mg of proteolyzed protein released.

2.2.4 Erythrocyte Membrane Fluidity: In Vitro Studies

2.2.4.1 Erythrocyte Ghost Preparation

Erythrocyte ghosts were prepared by the method of hypotonic hemolysis (Dodge et al., 1963) and resealed in phosphate buffered saline (PBS) (0.152 M NaCl in 10 mM phosphate buffer, pH = 7.4) for 40 min at 37°C (Steck and Kant, 1974).

2.2.4.2 Carbamylation of Ghost Membrane Protein

In vitro carbamylation of erythrocyte membrane ghosts was performed by incubation of ghost suspensions (1.5 mL containing 5 mg membrane protein) with 3.5 mL of 10 mM potassium phosphate buffer, pH 7.4, containing increasing final sodium cyanate concentrations (0, 10, 20, 40, and 60 mM) at 37°C. Following a 4-
h incubation, the suspensions were washed by centrifugation (30 min at 4000 x g) four times with PBS to remove any unreacted cyanate, stored overnight at 5°C, and then made up to a membrane concentration of 1 mg/mL.

### 2.2.4.3 Measurement of Membrane Fluidity

Determination of membrane fluidity was performed on an Abbott TDx Analyzer™ (Abbott Laboratories, Abbott Park, IL) by incorporation of the lipophilic bile acid derivative, fluorescein lithocholic acid (FLA) into the ghost membranes with subsequent measurement of steady-state fluorescence polarization. The Abbott TDx Analyzer™ is a filter fluorometer (excitation wavelength 485 ± 8 nm, emission wavelength 525-550 nm) in which polarization of the excitation beam is controlled electronically with a liquid crystal (Popelka et al., 1981).

The membrane probe (FLA), was chosen because its excitation maximum (494 nm) and emission maximum (515 nm) overlap well with the spectral characteristics of the Abbott TDx Analyzer. A methanolic solution of FLA (supplied by Abbott Laboratories) was diluted with TDx dilution buffer (0.1 M sodium phosphate, 1 mg/mL sodium azide, 0.1 mg/mL bovine gamma-globulin, pH 7.4) such that the addition of 50 μL of this stock solution to 1.5 mL of TDx dilution buffer yielded an intensity of ≈ 2000 units (signal gain = 5) when assayed by the TDx photo-check procedure.

Erythrocyte membrane fluidity was measured as follows: An aliquot (100 μL) of carbamylated ghosts (1 mg/mL suspension in PBS), prepared as above, was added to 1.4 mL TDx dilution buffer. Background fluorescence intensity (I_blank) and
polarization ($P_{\text{blank}}$) readings were obtained using the photo-check procedure. The membrane probe, FLA, was then added to the samples and incubated in the TDx analyzer for 10 min. The photo-check procedure was again initiated to obtain the final fluorescence intensity ($I_{\text{total}}$) and polarization ($P_{\text{total}}$) readings. Net polarization ($P_{\text{net}}$) was calculated as follows (Tait et al., 1986):

Step 1: 
$$I_{\text{net}} = I_{\text{total}} - I_{\text{blank}}$$

Step 2: Polarization readings are converted to anisotropy ($r$).
$$r = 2P / (3 - P)$$

Step 3:
$$r_{\text{net}} = \frac{[r_{\text{total}}I_{\text{total}} - r_{\text{blank}}I_{\text{blank}}]}{I_{\text{net}}}$$

Step 4:
$$P_{\text{net}} = 3 r_{\text{net}} / 2 + r_{\text{net}}$$

Since fluorescence polarization is inversely proportional to fluidity, membrane fluidity results are quoted as 1/polarization ($P^{-1}$).

2.2.4.4 Polarization of Fluorescein Lithocholic Acid as a Function of Ghost Cell Concentration

Control or carbamylated ghost cells (50-200 µL of a 1 mg/mL suspension) were made up to a final volume of 1.4 mL with TDx dilution buffer and the background polarization recorded. Stock FLA solution (50 µL) was added to each ghost suspension and the total polarization value recorded.

2.2.5 Erythrocyte Membrane Fluidity: In Vivo Studies

2.2.5.1 Patients

Uremic patients consisted of patients on hemodialysis, 2 females and 5
males, mean age 54.3 ± 20 (20-80).

Mean pre-dialysis values of serum urea for the patients was 26.1 ± 4.5 mM (16.4-32.9). All patients were nondiabetics.

A control group consisted of 6 individuals with normal levels of serum urea and glucose.

Serum levels of glucose and urea were determined on a clinical chemistry analyzer (Kodak Ektachem 700XR).

2.2.5.2 Measurement of Membrane Fluidity

Erythrocyte ghosts were prepared from fresh whole blood. All samples were made up to 1 mg/mL using PBS, stored at 5°C, and used for subsequent membrane fluidity analysis within 24 h.

Membrane fluidity was measured as in the in vitro studies (Section 2.2.4.3), with the following exceptions: in all cases, 200 μL of erythrocyte ghost preparation (1 mg/mL) were added to 1.3 mL of TDx dilution buffer; 25 μL of a stock solution of FLA was added such that the probe fluorescent intensity was = 2000 units (signal gain = 5).

2.2.6 Erythrocyte Membrane Lipid Carbamylation: In Vitro Studies

2.2.6.1 ¹⁴C-Carbamylation of Erythrocyte Ghosts for Lipid Studies

Erythrocyte ghosts (1.5 mg/mL membrane protein) were incubated in 10 mM sodium phosphate, 25 mM sodium cyanate, and 0.5 mM potassium [¹⁴C] cyanate, pH 7.4, for 15 h at 37°C. The sample was subsequently centrifuged (12,000 x g for 30 min at 5°C) and washed to remove unbound cyanate. The carbamylated
membranes were brought up to approximately 50% hematocrit with 10 mM sodium phosphate, pH 7.4, and generally contained 1-2 mg/mL membrane protein.

2.2.6.2 Extraction of Erythrocyte Membrane Lipids

Native and/or ^14^C-carbamylated ghost cells were centrifuged (12,000 x g for 20 min at 10°C, Sorvall Instruments RC2-B and SS-34 fixed-angle rotor) and the supernatant discarded. The lipid components of the erythrocyte membrane pellet (1-2 mL) were extracted into chloroform by the method of Folch et al. (1957) and the solvent evaporated (Brinkmann Rotavapor RE 120, Brinkmann Instruments Inc., Westbury, NY) to approximately 0.5 mL and stored in small (1.5 mL) glass vials at -20°C.

2.2.6.3 Quantitation of [^1^C] Cyanate Binding to Membrane Protein and Lipid Fractions

Aliquots (100 µL) of washed ^14^C-carbamylated ghosts (1-2 mg/mL) were removed and incorporation of cyanate (nmol) into the total cell, protein [resolved by SDS-PAGE and excised from the gel as previously described (Section 2.2.2.4)] and lipid (by solvent extraction, as above) fractions was measured by liquid scintillation counting in 10 mL Sigma-Fluor™, with a counting efficiency of 96%, and quantitated as follows:

\[
\text{DPM / fraction} \times \left( \frac{\text{cyanate} / \left[ ^{14} \text{C} \right] \text{cyanate}}{52 \text{ Ci mole}^{-1} \times 2.22 \times 10^{12} \text{ DPM Ci}^{-1}} \right)
\]

where (cyanate / [^14^C] cyanate) is the ratio of unlabelled to labelled cyanate in the reaction mixture. The final concentration of [^14^C] cyanate was calculated as [ (mM stock [^14^C] cyanate) (volume of stock [^14^C] cyanate) / total assay volume ]. Stock
[\textsuperscript{14}C] cyanate was prepared by the addition of 1.89 mL water to 1 mCi of crystalline potassium [\textsuperscript{14}C] cyanate and the DPM of an aliquot determined and converted to mM stock [\textsuperscript{14}C] cyanate according to \(( \text{DPM of aliquot} / (52 \text{ Ci mole}^{-1}) \times (2.22 \times 10^{12} \text{ DPM Ci}^{-1}) \) \}. Greater than 85\% of the total cell counts were recovered in the combined lipid and protein fractions.

\textbf{2.2.6.4 Preparation and \textsuperscript{14}C-Carbamylation of Phosphatidylethanolamine / Phosphatidylcholine Liposomes}

Phosphatidylethanolamine (PE) / phosphatidylcholine (PC) liposomes were prepared by adding 5 mg of egg yolk PC (50 \mu L of a 100 mg/mL stock solution of egg yolk PC in chloroform) and 4 mg of egg yolk PE (400 \mu L of a 10 mg/mL stock solution of PE in chloroform) to 2 mL chloroform. The phospholipid mixture was rotary evaporated to dryness and 2 mL of buffer A (5 mM sodium phosphate, 100 mM sodium chloride, pH 8.0) added. The suspension was sonicated (Branson 1200, Branson Cleaning Equipment Company, Shelton, CT) for 10 min at 10\(^{\circ}\)C and 1 mL of buffer B (5 mM sodium phosphate, 100 mM sodium cyanate, pH 8.0) and 200 \mu L of stock [\textsuperscript{14}C] cyanate in water (prepared as above) were added. The liposomes were incubated at 30\(^{\circ}\)C for 16 h and then exhaustively dialyzed against buffer A to remove unbound cyanate. The washed liposomes were then solvent extracted into chloroform as above.

\textbf{2.2.6.5 Thin-Layer Chromatography, Autoradiography and Imaging Densitometry}

Thin-layer chromatography (TLC) of solvent extracts of native and [\textsuperscript{14}C] cyanate treated erythrocyte membrane lipid, and egg yolk PE/PC liposomes was
performed. Briefly, TLC plates (20 x 20 cm) were spotted with sample (typically 5-10 μL) using a 5-μL Hamilton syringe and developed in a solvent system (appropriate to the lipid being investigated) to within 0.5 cm from the top of the plate. The TLC plate was air-dried and stained for either phospholipid or total lipid visualization. Phospholipids were visualized by spraying the plates with a phospholipid specific stain according to the procedure of Vaskovsky and Kostevsky (1968). Total lipids were visualized by dipping the plate in a manganese chloride-sulfuric acid derivatizing reagent followed by heat activation at 110°C for 40 min (Conte and Bishop, 1988). Stained plates were scanned by imaging densitometry and, in some cases, the phospholipids were quantitated by use of the volume analysis program (Molecular Analyst, Bio-Rad Laboratories). Chemical quantitation of the individual phospholipids, and cyanate incorporation was conducted by phosphorus analysis (Section 2.2.1.2) and scintillation counting (Section 2.2.6.3), respectively, of spots scraped from the TLC plate. Autoradiography was performed on the stained plates by exposure to Kodak X-Omat AR5 film at -80°C for 3 days.

2.2.6.6 Time Course of Erythrocyte Phosphatidylethanolamine Carbamylation

Fresh (≤ 12 h) whole blood from nonuremic individuals was obtained in tubes containing EDTA as anticoagulant. Ghost cells (2 mg/mL membrane protein) were prepared and incubated with 25 mM sodium cyanate in 10 mM phosphate buffer, pH 7.4, at 37 °C. One volume of reaction mixture was removed at various time intervals (1-10 h) and added to an equivalent volume of 2 M lysine in water to terminate any further reaction between cyanate and erythrocyte membrane lipid.
The samples were then stored at 5 °C until all time points were collected. All samples were then centrifuged (12,000 x g for 30 min at 5 °C, Sorvall Instruments RC2-B and SS-34 fixed-angle rotor) and washed to remove unbound cyanate. All samples were made up to an equivalent concentration (1.5 mg/mL membrane protein) and the lipids extracted as described (Section 2.2.6.2), applied to TLC plates, and resolved in chloroform/methanol/ammonia (65/35/5, v/v/v).

2.2.7 Erythrocyte Membrane Lipid Carbamylation: In Vivo Studies

2.2.7.1 Preparation of Erythrocyte Membrane Lipid/Triton X-100 Micelles and Treatment with Phospholipase D

Erythrocyte ghost membrane pellets (1-2 mL containing 2-3 mg protein) in 10 mM sodium phosphate, pH 7.4, were added to 25-30 mL of chloroform / methanol (2:1, v/v) and the lipids extracted, as above. The chloroform solvent was removed by rotary evaporation and the lipids left to vacuum dry for 15 min. Subsequently, 500 μL of buffer C (50 mM Tris-HCL, 10 mM CaCl₂, 1% Triton X-100, pH 8.0) were added and the suspension sonicated for 10 min at 10°C (Holbrook et al., 1991). The resulting phospholipid / Triton X-100 micelle suspension was incubated with 80 μL of freshly prepared phospholipase D (PLD, 1 mg/mL) in water for 2 h at 37°C.

2.2.7.2 Preparation of Egg Yolk Phospholipid / Triton X-100 Micelles, Cholesterol / Triton X-100 Micelles, Phospholipid Headgroup Alcohols, and Treatment with Phospholipase D

All phospholipid and cholesterol / Triton X-100 micelles were prepared by rotary evaporation of commercially purified egg yolk phospholipid (in chloroform
suspension) to dryness followed by sonication (10 min) in 1.0 mL of buffer C. Phosphatidylcholine (PC) / Triton X-100 micelles were prepared using 17 µL of a 100 mg/mL PC stock in chloroform to give a final concentration of 2.3 µmol PC / 1 mL buffer C. Phosphatidylethanolamine (PE) / Triton X-100 micelles were prepared using 137 µL of a 10 mg/mL PE stock in chloroform to give a final concentration of 1.8 µmol PE / 1 mL buffer C. Phosphatidylserine (PS) / Triton X-100 micelles were prepared using 68 µL of a 10 mg/mL PS stock in chloroform to give a final concentration of 0.91 µmol PS / 1 mL buffer C. Sphingomyelin (SM) / Triton X-100 micelles were prepared using 27 µL of a 50 mg/mL SM stock in chloroform to give a final concentration of 1.8 µmol SM / 1 mL buffer C. Cholesterol / Triton X-100 micelles were prepared using 132 µL of a 10 mg/mL cholesterol stock in chloroform to give a final concentration of 2.6 µmol cholesterol / 1 mL buffer C. Triton X-100 micelles containing more than one phospholipid were prepared in an identical manner using the same concentrations of each phospholipid indicated above.

Ethanolamine (1.82 µmol/500 µL buffer C) was prepared by adding 110 µL of ethanolamine to 390 µL of buffer C. Choline (4.1 µmol/500 µL buffer C) was prepared by adding 100 µL of a 5.72 mg/mL choline stock in water to 400 µL of buffer C. Serine (0.91 µmol/500 µL buffer C) was prepared by adding 100 µL of a 0.957 mg/mL serine stock in water to 400 µL of buffer C.

All of the above solutions (500 µL) were incubated in the absence (80 µL of buffer C) and presence of phospholipase D (80 µL of a 220 U/mL stock in
water) for 2 h at 37°C.

2.2.7.3 Measurement of Phospholipase D-Catalyzed Phospholipid Hydrolysis by Thin-Layer Chromatography and Imaging Densitometry

Erythrocyte membrane lipid / Triton X-100 micelles and egg yolk phospholipid / Triton X-100 micelles, prepared as described above, were incubated in the absence (80 μL of buffer C) and presence of phospholipase D (80 μL of a 220 U/mL stock in water) at 37°C. For the hydrolysis of native and PLD-treated egg yolk phospholipid / Triton X-100 micelles, samples were incubated for 2 h and subsequently added directly to 12 mL of chloroform/methanol (2/1, v/v). The chloroform layer was rotary evaporated to less than 500 μL and 10- to 20-μL aliquots were applied to TLC plates. For the time course of erythrocyte membrane lipid / Triton X-100 hydrolysis by PLD, 100-μL aliquots were removed at various time intervals and added directly to chloroform/methanol (2/1, v/v) to stop the reaction. Samples were treated as above and 10- to 20-μL aliquots were applied to TLC plates and the phospholipids resolved using a solvent system of chloroform/methanol/acetic acid/water [90/40/12/2, v/v/v/v (Higgins, 1987)] and stained for total lipid as described in Section 2.2.6.5. The dried TLC plates were scanned by imaging densitometry and analyzed for relative phospholipid by use of the volume analysis program (Section 2.2.6.5).

2.2.7.4 Reaction of Native and Phospholipase D-Treated Lipid / Triton X-100 Micelles with Diacetyl Monoxime

The method of Wybenga et al. (1971) for the determination of urea and substituted ureas (carbamyl derivatives) was modified and employed for
measurement of carbamylated erythrocyte membrane lipid. Briefly, the native and PLD-treated phospholipid / Triton X-100 micelle suspensions (580 μL) were added directly to 2.5 mL of urea-nitrogen reagent (0.83 M sulfuric acid, 1.13 M orthophosphoric acid, 0.55 mM thiourea, and 2.6 mM cadmium sulfate) and 500 μL of 3% diacetyl monoxime in water. A solution of PLD (80 μL of a 1 mg/mL solution in water) in buffer C was used as a control. All samples were incubated for 30 min at 100°C in a dry heater block and cooled for 5 min in a beaker of cold water. To clear the sample turbidity sodium dodecyl sulfate (SDS) was added (100 μL of a 20% SDS solution in water) prior to recording the absorption spectrum (400-650 nm). Studies with homocitrulline demonstrated that the addition of SDS does not alter the final absorbance (data not shown).

2.2.7.5 Reaction of Urea, Carbamyl Ethanolamine, and Carbamyl Serine with Diacetyl

Aliquots (100 μL) of 400 μM stock solutions of urea (24.02 μg/mL), carbamyl ethanolamine (hydroxyethylurea; 41.64 μg/mL) and carbamyl serine (8.16 μg/mL) in water were added to 400 μL of buffer (50 mM Tris-HCl, 10 mM CaCl₂, 1% Triton X-100, pH 8.0). All samples were reacted with diacetyl as above, except that SDS was not added.

2.2.7.6 Quantitation of Carbamylated Phosphatidylethanolamine in Erythrocytes of Normal Individuals

Fresh (< 10 h) pooled whole blood from nonuremic individuals (urea = 4.46 ± 0.51 mM) was obtained in tubes containing EDTA as anticoagulant. Ghost cells were prepared (Section 2.2.2.1, the last two washes were in buffer containing 50
mM Tris-HCL, 1% Triton X-100, pH 8.0) and 1.25 mL aliquots (containing 2.75 mg/mL membrane protein) were added to 25 mL of chloroform/methanol (2/1, v/v). Erythrocyte lipid/Triton X-100 micelles (in 500 µL buffer C) were prepared from the lipid extracts and aliquots were analyzed for phospholipid (Section 2.2.1.2) and protein content (Section 2.2.1.1). The erythrocyte lipid/Triton X-100 micelles were then treated in the absence and presence of PLD as above, and subsequently reacted with diacetyl. The carbamyl ethanolamine released was quantitated from a standard curve (0–40 nmol) prepared using hydroxyethylurea.
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Carbamylation of Erythrocyte Membrane Protein

The reaction of cyanate with protein occurs predominantly with the terminal amino group of the protein and the ε-amino group of lysine residues (Stark et al., 1960). The reaction with lysine yields homocitrulline (carbamyl lysine) (Stark, 1967). An extensive literature exists concerning the binding of cyanate to hemoglobin (De Furia et al., 1972; Carreras-Barnes et al., 1972; Uvellli et al., 1980; Manning et al., 1973) as a result of its antisickling effect upon erythrocytes from patients with sickle cell anemia (Gillette et al., 1971). More recently, attention has been given to the measurement of carbamylated hemoglobin as a potential index of long term uremic control (Kwan, et al., 1992; Fluckiger et al., 1981; Kwan et al., 1990), in an analogous fashion to the utility of monitoring glycated hemoglobin as an index of glycemic control in diabetes (Bunn et al., 1978). Little information exists, however, concerning the binding of cyanate to the erythrocyte membrane associated proteins. The in vitro binding of [14C] cyanate to the erythrocyte membrane has been documented (Carreras-Barnes, 1972) however, a measurement of the intrinsic levels of membrane protein carbamylation in the erythrocyte, or any other cell membrane has not been reported in the literature. The goal of the present investigation was to characterize the binding of cyanate to the individual membrane proteins of the erythrocyte and to establish whether uremic individuals have an increased level of carbamylated membrane protein. It
is felt that this information is important not only from a basic biochemical standpoint but it may also provide insights into the hematological impact of uremia on the erythrocyte [e.g., decreased lifetime (Lane and Burka, 1976), decreased (Ca^{2+} + Mg^{2+})-ATPase pump activity (Shalev, 1991), and a decreased deformability (Kikuchi et al., 1982)].

3.1.1 In Vitro Studies

The incorporation of cyanate into erythrocyte ghost membranes was measured using a mixture of nonradiolabeled cyanate and $[^{14}\text{C}]$ cyanate (50:1, mM:mM) and the reaction stopped by the addition of an equal volume of 2 M lysine to the reaction mixture. Figure 2 shows that cyanate is rapidly incorporated and proceeds in an essentially linear fashion over a 10-h incubation period. The results are in agreement with Carreras-Barnes et al. (1972). Although the reaction between membrane protein and cyanate is efficiently terminated by the addition of lysine, it is to be noted that a slight but significant incorporation of cyanate is measurable at zero time (approximately 11% of the total cyanate incorporated for the 10-h sample). This is assumed to result from a combination of 1) unbound $[^{14}\text{C}]$ cyanate trapped within the erythrocyte ghost cell and as such not depleted by repeated washings used to remove extracellular cyanate and 2) a small amount of cyanate covalently bound to the membrane (Figure 3) over its incubation time with lysine prior to removal of $[^{14}\text{C}]$ cyanate (Section 2.2.2.2). Figure 2 also shows the incorporation of glucose (20 mM, pH 7.4) (Watala, 1988) into ghost membranes over the same time period. Clearly carbamylation is a more rapid
FIGURE 2

Time Course of Erythrocyte Membrane Carbamylation

Legend

The incorporation of cyanate (25 mM sodium cyanate and 0.5 mM potassium [\(^{14}\)C] cyanate) into erythrocyte ghost membranes at 37 °C, pH 7.4, was measured as described in Section 2.2.2.2. [\(^{14}\)C] Cyanate counts (DPM) were converted to nmol cyanate incorporated. The second-order polynomial regression equation is: \[ y = 20.631 + 20.175x - 0.557x^2 \ldots, \quad r = 0.999. \] Each value represents the mean of duplicate determinations.

* Glucose (20 mM, pH 7.4) incorporation into erythrocyte ghost membranes from Walala (1988).
FIGURE 3

Time Course of Erythrocyte Membrane Protein Carbamylation as Monitored by SDS-PAGE and Autoradiography

Legend

The time course of incorporation of sodium cyanate (25 mM) and potassium [\(^{14}\)C] cyanate (0.5 mM) into erythrocyte ghost membrane protein at 37°C, pH 7.4, was monitored by SDS-PAGE (Panel A) and autoradiography of the SDS-PAGE gel (Panel B) as described in Section 2.2.2.3. Lanes 2 and 8 represent ghosts incubated with 25 mM sodium chloride for zero and 10 h, respectively. Lanes 3 - 7 represent ghosts incubated with 25 mM cyanate and 0.5mM potassium [\(^{14}\)C] cyanate (0 - 10 h). Lane 1 represents protein standards: myosin 205 kDa; β-galactosidase 116 kDa; phosphorylase b 97.4 kDa; bovine serum albumin 66 kDa; egg albumin 45 kDa; and carbonic anhydrase 29 kDa.
process than glycosylation.

To elucidate the binding pattern of $^{14}$C cyanate among the various integral and cytoskeletal matrix proteins, in vitro $^{14}$C-carbamylated erythrocyte ghost membrane protein was separated by SDS-PAGE and the resulting gel visualized by autoradiography (Figure 3). The autoradiograph clearly illustrates the binding of cyanate to all the major membrane associated proteins. Incorporation of cyanate into protein at zero-time is evident and indicates that incubation of ghost cells with lysine does not entirely stop the reaction between cyanate and the membrane protein, as indicated in Figure 2. The total amount of cyanate incorporated at time zero is, however, less than 8% of the total cyanate incorporation for the 10-h sample. To quantitate the degree of cyanate binding among the individual proteins as a function of incubation time with cyanate (Figure 4), each individually resolved membrane protein was analyzed for both cyanate incorporation (by scintillation counting of the excised band from the SDS-PAGE gel), and protein quantitation in µg (by imaging densitometry of the scanned SDS-PAGE gel and use of a volume analysis program). Figure 5 demonstrates a human serum albumin (HSA) standard curve generated using quantities of protein (0-10 µg) within the range of the various erythrocyte membrane proteins and correlating these to the volume (O.D./mm²) calculated by imaging densitometry. The results clearly demonstrate the linearity ($r = 0.998$) and hence validity of protein quantitation by imaging densitometry. The results of the above analysis (Figure 4) demonstrate that all the major integral and peripheral membrane proteins are carbamylated upon
FIGURE 4

Time Course of Cyanate Binding to Erythrocyte Cytoskeletal Proteins

Legend

The quantitation of carbamylation among the major erythrocyte cytoskeletal proteins as a function of incubation time with cyanate (25 mM sodium cyanate and 0.5 mM potassium [14C] cyanate) was determined as described in Section 2.2.2.4. Each value represents the mean of duplicate determinations.
FIGURE 6

Standard Curve for Imaging Densitometry of SDS-PAGE-Resolved Human Serum Albumin

Legend

Panel A: Lanes 1-6, SDS-PAGE of: 0.2, 1.0, 1.5, 2, 4 and 10 μg of human serum albumin (HSA), respectively, performed as in Section 2.2.2.3. Panel B: Standard curve (r = 0.998) of HSA (μg) resolved by SDS-PAGE in Panel A versus volume analysis (O.D. /mm²) of HSA by imaging densitometry using the Molecular Analyst program (Bio-Rad Laboratories). The regression equation is: $y = 0.71847x + 0.3541; r = 0.998$. 
FIGURE 6

B

Volume (O.D. / mm²)

0 2 4 6 8

HSA (μg)

0 2 4 6 8 10
incubation of ghosts cells with cyanate. The most extensively carbamylated proteins are spectrin (α and β) and ankyrin. After incubation with cyanate for 10 h, spectrin and ankyrin incorporate 18.3 ± 1.6 and 10.7 ± 3 mol cyanate / mol protein, respectively, as opposed to < 5 mol cyanate / mol protein for the remaining membrane proteins (Figure 4). The increased level of carbamylation of spectrin and ankyrin relative to the other membrane protein does not indicate that these proteins have a higher affinity for cyanate. In fact, the incorporation of cyanate into the various membrane proteins is nonspecific since, as Figure 6 demonstrates, the degree of carbamylation of the individual proteins is a simple linear function of their molecular weights. The only major protein which deviates from this linearity is the anion channel, which incorporates less cyanate than would be predicted from its molecular weight. This is most likely due to the fact that the anion channel is an integral membrane protein. Much of its bulk is embedded within the membrane lipid (Gennis, 1989c) and, as such, is not as potentially available for carbamylation as are the remaining cytosolic cytoskeletal proteins.

Despite the nonspecific nature of carbamylation among the membrane proteins, it is still evident that spectrin and ankyrin are the most extensively modified proteins upon in vitro incubation with cyanate. The significance of this finding will be discussed later (CHAPTER 4).
FIGURE 6

*In Vitro* Carbamylation of Erythrocyte Membrane Protein as a Function of Protein Molecular Weight

*Legend*

The quantitation of erythrocyte membrane protein carbamylation as a function of protein molecular weight was conducted as in Section 2.2.2.4 using the published molecular weights (Gennis, 1989c): spectrin (230 kDa); ankyrin (174 kDa); anion channel (95 kDa); band 4.1 (80 kDa); actin (43 kDa) and band 7 (29 kDa). The regression equation is: $y = 0.08581x - 2.04; r = 0.989$. 
3.1.2 In Vivo Studies

Only a few methods are available for the determination of carbamylated protein. These have been outlined in (Section 1.5) and include: an HPLC method with amino acid analysis after the hydrolysis of protein in 6 N HCL at 110 °C for 22 h (Stark et al., 1960); a method based upon nitrosylation of carbamyl lysine residues by nitrite in acidic solution followed by degradation of nitrosourea to cyanate ion in alkali solution which is detected by a cyanate ion analyzer (Koshiishi and Imanari, 1989); and a colorimetric method using diacetyl monoxime as a reagent (Wybenga et al., 1971; Hunninghake and Grisolia, 1966). Because the first two methods require relatively sophisticated equipment, the diacetyl monoxime assay was chosen to investigate the intrinsic carbamylation of the erythrocyte membrane protein.

Figure 7 shows that when diacetyl is reacted with an equimolar amount (40 nmol) of urea or homocitrulline (carbamyl lysine) the absorption spectra obtained are essentially identical. Both species absorb maximally at 530 nm, however, the calculated molar absorption coefficient of homocitrulline (17,063 L mol\(^{-1}\) cm\(^{-1}\)) is slightly less than urea (18,463 L mol\(^{-1}\) cm\(^{-1}\)). A standard curve of homocitrulline reaction with diacetyl (Figure 8) demonstrates that the detection limit of the assay is approximately 2 nmol. Figure 9 demonstrates that the optimum incubation time of the sample with diacetyl is between 25 and 35 min and the chromogen is stable for at least 60 min.

When erythrocyte ghost membranes are directly incubated with diacetyl
FIGURE 7

Absorption Spectra for the Reaction of Urea and Homocitrulline with Diacetyl

Legend

The reaction of diacetyl monoxime with urea (40 nmol) and homocitrulline (40 nmol) was conducted as described in Section 2.2.3.3. In both cases the $\lambda_{\text{max}}$ is 530 nm.
FIGURE 8
Standard Curve for the Reaction of Homocitrulline with Diacetyl

Legend

The homocitrulline standard curve was prepared as described in Section 2.2.3.3. The regression equation is: $y = 0.00416x + 0.01287$; $r = 0.997$. Each value represents the mean for duplicate determination.
FIGURE 9

Time Course and Stability of the Homocitrulline-Diacetyl Reaction Complex.

Legend

The reaction of homocitrulline with diacetyl was monitored ($\lambda = 530$ nm) as a function of: reaction time with diacetyl at 100°C (-----); and reaction complex stability 60 min post-reaction (-----), as described in Section 2.2.3.4.
monoxime, however, there is no measurable colorimetric development in the region of 530 nm. This indicates that either the level of carbamylated membrane protein is below the detection limit of the diacetyl monoxime assay, or that the conditions of the assay are not conducive to chromogen formation between diacetyl and protein carbamyl residues. In this regard the following two observations are instructive:

1) The level of glycosylation of the erythrocyte membrane protein in normal individuals has been measured to be 5-28 nmol / mg membrane protein [depending on the method used (Watala, 1988; Bryszewska and Szosland, 1988; Schleicher et al., 1981)]. Considering that glucose reacts more slowly with protein amino groups than cyanate, one would predict that the level of carbamylated membrane protein in the native erythrocyte would be above the detection limit (2 nmol) of the diacetyl monoxime assay (Figure 8);

2) The spectrin of erythrocyte ghosts is known to precipitate and aggregate at pH values less than 5.5 (Baumann et al., 1994). It is felt, therefore, that the lack of chromogen formation is most likely due to precipitation of the cytoskeletal protein within the erythrocyte ghost, as a result of the high acidity and temperature conditions of the assay. To circumvent this problem proteolytic digestion of the erythrocyte ghosts with proteinase K (PK) prior to incubation with the diacetyl monoxime reagent was investigated. It was speculated that this treatment would both release the proteins from the membrane and increase their acid solubility.

Proteinase K [EC 3.4.21.14] is a nonspecific serine endopeptidase isolated
from *Tritirachium album* which typically digests proteins to small peptides and single amino acids (Boehringer Mannheim, 1995). It is used primarily in the total degradation of proteins during the isolation of DNA and RNA (Ebeling *et al*., 1974). Incubation of erythrocyte ghosts with PK as monitored by SDS-PAGE (Figure 10A, lane 4) results in the complete absence of any discernable bands corresponding to the native membrane protein (lane 3), even though both lanes contain equivalent amounts of protein. SDS-PAGE of the same samples in 15% polyacrylamide (w/v) (Figure 10B) yields only bands corresponding to PK itself (compare lane 9 with lanes 4, 5 and 6), clearly indicating the proteolysis of all membrane proteins into small fragments. The exact molecular weight range of these proteolyzed peptides was not investigated further. Figure 11 demonstrates that over the range of 0.35 - 3.5 mg % of PK / membrane protein there is a rapid release of approximately 75% of the total ghost protein from the membrane. The released peptides are easily separated from the remaining ghost cell membrane by centrifugation.

Upon incubation with diacetyl monoxime the released proteolyzed membrane peptides remain completely soluble and an easily detectable chromogen with an absorption maxima at 530 nm is observed (Figure 12). Ghost membranes or supernatant in the absence of PK, or buffer containing PK, show no detectable chromogen formation (Figure 12). It is important to note that the absorbance of ghost membranes in the absence of PK is due to sample light scattering resulting from the turbidity of the ghost cell suspension and not from any colorimetric
FIGURE 10

Proteolysis of Erythrocyte Membrane Protein by Proteinase K

Legend

The proteolysis of erythrocyte membrane protein by proteinase K (PK) was performed and monitored by SDS-PAGE as described in Section 2.2.3.1. Panel A: 8% (w/v) polyacrylamide. Panel B: 15% (w/v) polyacrylamide. Lanes 2 and 3 represent total membrane protein in absence of PK. Lane 4 represents total membrane protein after incubation with PK for 1 h at 37 °C. Lane 5 represents PK-treated ghost cell supernatant following centrifugation (48,000 x g) for 1 h. Lane 6 represents PK treated ghost cell supernatant following centrifugation and lyophilization. Lanes 7-9 represent PK (28.8 kDa) in buffer treated identically to the conditions in lanes 4-6.
FIGURE 11

Time and Concentration Dependence of Erythrocyte Membrane Protein
Proteolysis and Release by Proteinase K

Legend

The time and concentration dependence of erythrocyte membrane protein (0.5 mg/mL) proteolysis and release by proteinase K (3-30 μg/mL) was performed as described in Section 2.2.3.2. Each value represents the mean of duplicate determinations.
FIGURE 12

Absorption Spectra for the Reaction of Diacetyl with Native and Proteinase K - Treated Erythrocyte Ghosts

Legend

The reaction of diacetyl with native and proteinase K (PK)-treated erythrocyte ghosts was performed, and the chromogen was monitored (530 nm) as described in Section 2.2.3.3.
development. The results, therefore, indicate the presence of a measurable amount of carbamylated membrane protein in the native erythrocyte.

A study was next undertaken with the aim of quantitating the levels of carbamylated membrane protein in the erythrocytes of both nonuremic and uremic individuals using the above method.

Figure 13 illustrates that the reactivity of diacetyl with increasingly \(^{14}\text{C}\)-carbamylated proteolyzed membrane protein proceeds with essentially the same slope as reactivity with homocitrulline (carbamyl lysine), indicating that both species have approximately equivalent molar absorption coefficients. This validates the use of homocitrulline as a standard to quantitate the levels of carbamylated membrane protein in erythrocytes. The measurable absorbance of native proteolyzed membrane protein (i.e., unlabelled with \(^{14}\text{C}\) cyanate) clearly represents reactivity of diacetyl with intrinsically carbamylated membrane protein.

The level of carbamylated membrane protein in the erythrocytes of normal and uremic individuals was, accordingly, measured by reactivity of the PK treated ghosts with diacetyl and quantitated using homocitrulline as the standard (Figure 14). Erythrocytes from uremics were found to have a greater level of carbamylation (47.09 ± 7.80 nmol homocitrulline / mg proteolyzed protein released) relative to erythrocytes from nonuremics (25.89 ± 6.92 nmol homocitrulline / mg proteolyzed protein released). The percentage of the total proteolyzed ghost protein released from the ghost membrane of nonuremics and uremics as a result of PK treatment was 70.6 ± 3.8 and 71.07 ± 7.11 %, respectively. This indicates that the increased
FIGURE 13

Standard Curves for the Reaction of Diacetyl with $^{14}$C-Carbamylated Membrane Protein and Homocitrulline

Legend

The reaction of diacetyl with homocitrulline was performed and measured as described in Section 2.2.3.3. Erythrocyte ghost membrane protein was $^{14}$C-carbamylated and quantitated as described in Section 2.2.2.2 and subsequently treated with proteinase K (PK) to release the membrane protein for reaction with diacetyl, as described in Section 2.2.3.3.
FIGURE 14

Quantitation of Carbamylated Erythrocyte Membrane Protein in Uremics and Nonuremics

Legend

The levels of carbamylated erythrocyte membrane protein in erythrocytes of uremics (n = 6) and nonuremics (n = 6) was measured as described in Section 2.2.3.5. The values are for individuals, from which the mean (—) is calculated. A student t test analysis of the data gives a P value of 0.0005.
level of carbamylated membrane protein in uremics is not artifactually due to an increased release of protein from the erythrocytes of uremics upon PK treatment.

Taken together, the *in vitro* and *in vivo* results suggest that key protein binding domains (particularly spectrin-ankyrin associations) may be altered in the erythrocytes of uremics as a result of the increased covalent protein modifications (carbamylation) in these individuals. More direct evidence for a gross structural alteration of the erythrocyte membrane upon *in vitro* carbamylation is the subject of the next section.

### 3.2 Erythrocyte Membrane Fluidity Studies

It is the accepted view that membrane functions are critically dependent on the maintenance of optimal membrane fluidity (Gennis, 1989b). It is generally assumed that the function of transmembrane proteins requires a conformational change and/or lateral motion which depends, in part, upon the interactions of both annular (lipid boundary immediately adjacent to the protein) and bulk lipid with the protein subunits (Gennis, 1989b). Pathological and *in vitro* induced changes in membrane fluidity have been correlated with changes in many membrane functions, including enzyme activity, ligand-receptor interactions and bilayer transport (Spector and Yorek, 1985; McElhaney, 1982; Santini *et al.*, 1992). Watala (1988) has demonstrated that nonenzymatic glycosylation of erythrocyte membrane proteins leads to a decreased membrane fluidity. In fact, a direct reciprocal relationship between *in vitro* glycated membrane protein and erythrocyte membrane fluidity ($r = 0.91$) was found.
The main utility of a measurement of membrane fluidity resides not in its ability to detect small localized changes in the membrane or to derive absolute measurements of fluidity, but rather to detect gross changes in membrane structure resulting from pathological and/or in vitro induced changes in either the membrane lipid or protein. It was decided, therefore, to measure the membrane fluidity of the erythrocyte membrane as a function of in vitro membrane carbamylation as an indicator of membrane perturbation.

In this study, erythrocyte membrane fluidity was probed using the fluorescence polarization signal from the lipophilic bile acid derivative-fluorescein lithocholic acid (FLA) (Figure 15). Unlike the fluorescent molecule diphenylhexatriene (DPH), a commonly used probe of membrane fluidity [Watala et al., 1985], FLA is fluorescent in aqueous environments. Consequently, the polarization of FLA is a composite of its rotational motion outside and inside the membrane. The observed polarization value should, therefore, be dependent upon the ghost cell concentration. Figure 16 demonstrates that in the absence of ghost cell membrane the polarization of FLA is low, consistent with its high rotational rate in aqueous solution. At low ghost cell concentrations, the polarization of FLA contains a relatively large contribution of depolarizing motions from outside the membrane, and the observed polarization is consequently low. As one increases the membrane concentration, a greater fraction of the probe population will partition into the membrane, resulting in an increase in the polarization. At a ghost cell membrane concentration of approximately 0.2 mg/mL (membrane protein) the
FIGURE 15

Molecular Structure and Excitation-Emission Spectra of Fluorescein Lithocholic Acid

Legend

The emission spectrum of fluorescein lithocholic acid in 0.1 M sodium phosphate, 1 mg/mL sodium azide, 0.1 mg/mL bovine gamma-globulin, pH 7.4 (TDx buffer, Abbott Laboratories) was recorded at an excitation wavelength of 494 nm. The excitation spectrum was recorded at an emission wavelength of 514 nm.
FIGURE 16

Fluorescein ribocholeic acid

Excitation  Emission

464 nm  514 nm

Fluorescence (Arbitrary Units)

Wavelength (nm)
FIGURE 16

Polarization of Fluorescein Lithocholic Acid as a Function of Erythrocyte Ghost Membrane Concentration

Legend

The polarization of fluorescein lithocholic acid as a function of erythrocyte ghost membrane concentration was measured as described in Section 2.2.4.4. The values represent the mean of duplicate determinations.
partitioning of FLA into the membrane would appear to have reached equilibrium and, as such, the observed polarization is independent of the membrane concentration. The dependence of the polarization of FLA on the ghost cell concentration, therefore, dictates that comparative measurements of membrane fluidity using FLA can be reliably conducted using equivalent erythrocyte ghost concentrations of at least 0.2 mg/mL membrane protein. It was considered a possibility that the increasing polarization of FLA as a function of membrane concentration may, at least partially, be due to probe-induced perturbation of the membrane; however, at a constant ghost concentration, variance of the probe concentration produces no observable change in polarization over the range studied (Figure 17). Figure 18 illustrates that as the level of in vitro carbamylation of the erythrocyte membrane increases the polarization of FLA correspondingly decreases (i.e., increased fluidity). Therefore, over the range of carbamylation studied (0-60 mM incubations with cyanate for 4 h) there is an essentially linear increase in the fluidity of the erythrocyte membrane. This is consistent with the recent results of Jeevaratnam and Vaidyanathan (1992) demonstrating an increased membrane fluidity of rabbit erythrocyte membranes when incubated in vitro with methyl isocyanate.

The data is interesting on two accounts. Firstly, it provides evidence that carbamylation of the erythrocyte membrane can result in a measurable perturbation of the membrane. Secondly, the in vitro increase in membrane fluidity upon carbamylation is seemingly inconsistent with the reported decrease
FIGURE 17

Concentration Dependence of the Polarization of Fluorescein Lithocholic Acid in Erythrocyte Ghost Membranes.

Legend

Stock fluorescein lithocholic acid (FLA) in methanol was serially diluted with TDx dilution buffer (0.1 M sodium phosphate, 1 mg/mL sodium azide, 0.1 mg/mL bovine gamma-globulin, pH 7.4) such that the addition of 100 µL to 1400 µL of ghost membranes (0.085 mg /mL in TDx dilution buffer) gave the total fluorescence intensity observed on the abscissa, when measured on a TDx analyzer as described in Section 2.2.4.3. The values represent the mean of duplicate determinations.
FIGURE 18:

Erythrocyte Membrane Fluidity as a Function of *In Vitro* Carbamylation

**Legend**

The membrane fluidity of erythrocyte ghosts as a function of *in vitro* carbamylation was probed using fluorescein lithocholic acid as described in Sections 2.2.4.3 and 2.2.4.4. The second-order polynomial regression equation is:

\[ y = 0.1789 - 8.268 \times 10^{-4} x + 4.5469 \times 10^{-4} x^2 \ldots, \ r = 0.995. \]

The values represent the mean of duplicate determinations.
in fluidity of erythrocyte membranes in uremic patients on dialysis (Komidori et al., 1985), despite the fact that the levels of membrane protein carbamyla tion are increased in uremics (Figure 14). This would suggest that protein carbamyla tion is not the determinant factor leading to the decreased fluidity in these individuals. It was felt, however, that this discrepancy might be due to probe and technique differences between the present study (i.e., FLA and fluorescence polarization) and the uremic patient study [stearic acid spin labels and electron spin resonance (Komidori et al., 1985)]. However, when the membrane fluidity of erythrocytes from normal and uremic individuals was measured using FLA (Figure 19) a significantly (p < 0.05) decreased fluidity was observed in the uremic group (4.66±0.12 P⁻¹) relative to the normal group (4.96±0.20 P⁻¹), a result entirely consistent with the electron spin (ESR) study.

It is perhaps, therefore a naive view to expect that the decreased erythrocyte membrane fluidity observed in uremics can be entirely accounted for by the increased levels of carbamylated membrane protein. Clearly the impact of chronic renal failure on the erythrocyte is not limited solely to carbamyla tion. In fact, in the ESR study (Komidori et al., 1985) significant decreases of membrane phosphatidylcholine (PC) and molar ratio of phosphatidylcholine/sphingomyelin (PC/SM) were demonstrated. This was hypothesised to result from the altered lipid metabolism, common in uremics (Komidori et al., 1985), and was suggested to be involved in the altered fluidity. No correlation was, however, reported between altered phospholipid levels and decreased membrane fluidity, thereby suggesting
FIGURE 19

Membrane Fluidity of Erythrocytes from Uremics and Nonuremics.

Legend

The membrane fluidity of erythrocyte ghosts obtained from uremics \( (n = 6) \) and nonuremic controls \( (n = 6) \) was measured as described in Section 2.2.5. The values are for individuals, from which the mean \((-\)) is calculated.
FIGURE 19

Nonuremics

Uremics

Fluidity (1 / Polarization)

Patients
the possibility that other processes may be contributory. It is perhaps, then, a more inclusive hypothesis that the final observed erythrocyte membrane fluidity in uremics (or any group) is a composite of many processes, some causing increases in membrane fluidity and some causing decreases. In any case, the above data provides evidence that carbamylation of the erythrocyte membrane can result in measurable perturbations of the membrane. When this data is coupled with the findings of an increased level of carbamylated membrane protein in uremics relative to normals (Figure 14), and an extensive in vitro carbamylation of spectrin and ankyrin (Figure 4), the hypothesis that the erythrocytes of uremic individuals may have destabilized membranes, which at least partially contribute to the observed decreased erythrocyte lifetime in uremics, would appear to be strengthened.

In the next section evidence will be provided which suggests that erythrocyte membrane carbamylation may extend beyond covalent modifications of protein to direct carbamylation of membrane phospholipids.

3.3 Carbamylation of Erythrocyte Membrane Aminophospholipids

From the extensive literature concerning the binding of cyanate to protein both in vitro and in vivo (Sections 1.6 and 1.7), it is evident that under physiological conditions of pH and temperature carbamylation is amino-group specific and is not specific to any protein. It is, therefore, a possibility that carbamylation may extend beyond the level of protein to other non-protein primary amino group containing compounds of physiological importance. The
carbamylation of non-protein amino groups has not, however, been previously reported. In this regard one such potential target is the plasma membrane of the erythrocyte. The erythrocyte membrane contains nearly equivalent amounts (w/w) of lipid and protein, and together phosphatidylserine (PS) and phosphatidylethanolamine (PE) make up approximately 35 mole% of the total erythrocyte membrane phospholipid (Gennis, 1989a). Both phospholipids contain a primary amino group in their polar headgroup (Figure 1), and are potentially accessible to derivatization, since they are relatively easy to label with exogenous amino specific chemical reagents such as trinitrobenzene sulfonic acid (TNBS) (Gennis, 1989c). The following is an investigation of cyanate binding to erythrocyte membrane aminophospholipids—PS and PE.

3.3.1 In Vitro Studies

Erythrocyte ghosts were carbamylated in vitro with [14C] cyanate for 15 h and the membrane components separated into total protein and total lipid fractions (Section 2.2.6.3). Greater than 85% of the total ghost cell membrane [14C] cyanate counts were recovered. Figure 20 demonstrates extensive incorporation of cyanate into both the protein and lipid fractions. In fact, a greater degree of the total membrane cyanate incorporated is associated with the lipid components (60%) of the membrane relative to the protein components (40%). To establish that the cyanate was not just simply nonspecifically associated with the membrane lipid, thin-layer chromatography (TLC) of the extracted membrane lipid fraction was performed in a solvent system of chloroform/methanol/acetic acid/water (60/50/1/4,
FIGURE 20

Incorporation of Cyanate into Erythrocyte Membrane Protein and Lipid Fractions

Legend

\(^{14}\text{C}-\text{Carbamylated erythrocyte ghosts (1.5 mg/mL membrane protein) were prepared as described in Section 2.2.2.2. Aliquots of 100 \( \mu \text{L} \) were removed and separated into protein and lipid fractions by SDS-PAGE and solvent extraction, respectively. The amount of cyanate (nmol) incorporated into each fraction was measured as described in Section 2.2.6.3. The lipid and protein fractions of the membrane incorporated 60\% and 40\%, respectively, of the total counts (DPM) recovered. The values represent the mean of duplicate determinations.\)
v/v/v/v) followed by autoradiography of the plate stained for phospholipid (Figure 21). This solvent system has been used to completely resolve all the major classes of phospholipids (Findlay and Evans, 1987). Good TLC separation of the major erythrocyte phospholipids is clearly illustrated in Figure 21. An identical phospholipid staining pattern is observed for both native and [14C] cyanate treated ghost cells. The autoradiograph of the TLC plate, however, clearly illustrates the presence of two distinct areas of radioactivity co-migrating with phosphatidylethanolamine (PE) and phosphatidylserine (PS), respectively. All of the phospholipids separated by TLC were scraped from the plate and analyzed for both [14C] cyanate incorporation and phospholipid content (Table 2). The results confirm the covalent incorporation of cyanate into both PE (15.76 ± 0.09 mole %) and PS (13.34 ± 0.81 mole %). Under the same conditions no incorporation of [14C] cyanate into cholesterol (Chol), phosphatidylcholine (PC) or sphingomyelin (SM) was detectable (Table 2 and Figure 21). Since the degree of carbachylation of PE and PS following a 15-h incubation with cyanate are essentially equivalent (Table 2), it would appear that both phospholipids are as equally reactive towards cyanate.

Thin-layer chromatography of the in vitro carbachylated membrane aminophospholipids in chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v) does not, however, separate carbachylated PE (carb-PE) or carbachylated PS (carb-PS) from PE or PS (Figure 21). It was considered unlikely that one solvent system would accomplish separation of both species on one TLC plate, and so
FIGURE 21

Incorporation of Cyanate into Erythrocyte Membrane Lipid

Legend

The incorporation of cyanate into the lipid fraction of $^{14}$C-carbamylated erythrocyte ghost membranes was investigated by thin-layer chromatography (TLC) and autoradiography of the TLC plate as described in Section 2.2.6.5. The TLC solvent system used was chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v) and the phospholipids visualized using a phospholipid specific stain according to the procedure of Vaskovsky and Kostevsky (1968). Under these conditions cholesterol migrates just slightly behind the solvent front (Higgins, 1987) and does not stain. Chol, cholesterol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. PE, PS and PC standards (10 μg of each) were applied.
FIGURE 21

Autoradiograph

Thin-Layer Chromatograph

- Erythrocyte Membrane Lipids
- \(^{14}C\)Cyanaate
- \(-^{14}C\)Cyanaate

Standards

PS PC SM
<table>
<thead>
<tr>
<th></th>
<th>Phospholipid&lt;sup&gt;a&lt;/sup&gt; (nmol)</th>
<th>Phospholipid (% of Total)</th>
<th>Phospholipid (% of Total)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Cyanate&lt;sup&gt;*&lt;/sup&gt; (nmol)</th>
<th>Cyanate/Phospholipid (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>72.79 ± 1.7</td>
<td>28.61 ± 0.59</td>
<td>25-30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>88.15 ± 3.2</td>
<td>34.65 ± 1.36</td>
<td>25-30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PS</td>
<td>30.21 ± 2.1</td>
<td>11.88 ± 0.78</td>
<td>10-15</td>
<td>4.02 ± 0.11</td>
<td>13.34 ± 0.81</td>
</tr>
<tr>
<td>PE</td>
<td>63.3 ± 0.15</td>
<td>24.88 ± 0.01</td>
<td>25-30</td>
<td>9.97 ± 0.08</td>
<td>15.76 ± 0.09</td>
</tr>
<tr>
<td>Chol</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; Chol, cholesterol; NA, not applicable; ND, not determined.

<sup>b</sup> Erythrocyte ghosts (1.5 mg/mL membrane protein) incubated with 25 mM sodium cyanate and 0.5 mM potassium [<sup>14</sup>C] cyanate for 15 h at 37°C, pH 7.4.

<sup>c</sup> The solvent system was chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v).

<sup>d</sup> Quantitated as phosphorus (Higgins, 1987) subsequent to scraping of band from TLC plate. Values represent the average of duplicate determinations.

<sup>e</sup> Actual mol percentages (Schwartz et al., 1985).

<sup>f</sup> Quantitated by scintillation counting subsequent to scraping of band from TLC plate. Values represent average of duplicate determinations.
research efforts were focused upon separation of carb-PE from PE. To accomplish this task without the potential complexity of interference from other erythrocyte phospholipids, egg yolk PE / PC liposomes were constructed [PE / PC liposomes were used since liposomes of PE alone are known to adopt a hexagonal (H₆) phase, or inverted micelle in which the PE amino headgroups are not exposed to the solvent (Gennis, 1989d)] from commercially purified PE and PC. The liposomes were carbamylated with [¹⁴C] cyanate and a number of solvent systems investigated. Complete resolution of egg yolk carb-PE from PE was accomplished using a chloroform/methanol/ammonia (65/35/5, v/v/v) solvent system (Figure 22). Under these conditions the Rᵣ value of PE shifts from 0.61 ± 0.01 (Table 3, row 1) to 0.46 ± 0.02 (Table 3, row 2), while carb-PE remains at 0.61 ± 0.01 (Table 3, row 3). This may be partially due to the different acid / base characteristics of the solvents. Since amines and amides have pKa's in the region of 9 to 10, and -1 to 0, respectively (Roberts and Caserio, 1965) a change of solvent system from chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v) (pH, 3.2) to chloroform/methanol/ammonia (65/35/5, v/v/v) is associated with a PE amine charge change from (+1) to (0) which potentially leads to the observed shift in Rᵣ value (Figure 22). In contrast, the newly formed headgroup amide of carb-PE is uncharged in both solvent systems and its Rᵣ value is unchanged (Figure 22).

The chloroform/methanol/ammonia (65/35/5, v/v/v) solvent system was next applied to the separation of ¹⁴C-carbamylated erythrocyte membrane lipids. Comparison of native with ¹⁴C-carbamylated lipids (Figure 23) shows the
FIGURE 22
Separation of Native and $^{14}$C-Carboxylated Egg Yolk Phosphatidyl-
ethanolamine

Legend

Egg Yolk phosphatidylcholine (PC) / phosphatidylethanolamine (PE)
liposomes were prepared and $^{14}$C-carboxylated as described in Section 2.2.6.4.
The phospholipids were resolved by thin-layer chromatography (TLC) in the
indicated solvent systems, and autoradiographs of the TLC plate developed as
described in Section 2.2.6.5. For clarity of presentation, the developed
autoradiograph is shown overlaid onto the stained TLC plate. The dark spots
represent areas of radioactivity. Phospholipids were visualized using the procedure
of Vaskovsky and Kostevsky (1968). Chl, chloroform; meth, methanol; aa, acetic
acid; Std, standard.
FIGURE 22

<table>
<thead>
<tr>
<th>PE Std</th>
<th>PC / PE + [14C] Cyanate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Chl/meth/aa/water (60/50/1/4)

<table>
<thead>
<tr>
<th>PC Std</th>
<th>PC / PE + [14C] Cyanate</th>
<th>PE Std</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

Chl/meth/ammonia (65/35/5)
TABLE 3
Phospholipid Quantitation, DPM and R<sub>v</sub> Values of ¹⁴C-Carbamylated and Native Phosphatidylethanolamine resolved by Thin-Layer Chromatography in Different Solvent Systems.

<table>
<thead>
<tr>
<th>Phospholipid Band</th>
<th>R&lt;sub&gt;v&lt;/sub&gt; Value</th>
<th>[¹⁴C] Counts&lt;sup&gt;c&lt;/sup&gt; (DPM)</th>
<th>Cyanate (nmol)</th>
<th>Phospholipid&lt;sup&gt;d&lt;/sup&gt; (nmol)</th>
<th>Cyanate/Phospholipid (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Egg Yolk (PE + Carb-PE)&lt;sup&gt;a&lt;/sup&gt; (Figure 22)</td>
<td>0.61 ± 0.01</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>2) Egg Yolk (PE)&lt;sup&gt;b&lt;/sup&gt; (Figure 22)</td>
<td>0.46 ± 0.02</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>3) Egg Yolk (Carb-PE)&lt;sup&gt;b&lt;/sup&gt; (Figure 22)</td>
<td>0.61 ± 0.01</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>4) Erythrocyte (PE + Carb-PE)&lt;sup&gt;a&lt;/sup&gt; (Figure 21)</td>
<td>0.61 ± 0.01</td>
<td>31983 ± 191</td>
<td>NA</td>
<td>58.9 ± 0.93</td>
<td>NA</td>
</tr>
<tr>
<td>5) Erythrocyte (Carb-PE)&lt;sup&gt;b&lt;/sup&gt; (Figure 23)</td>
<td>0.62 ± 0.01</td>
<td>31504 ± 242</td>
<td>12.28 ± 0.14</td>
<td>16.21 ± 2.1</td>
<td>0.77 ± 0.12</td>
</tr>
<tr>
<td>6) Erythrocyte (PE)&lt;sup&gt;b&lt;/sup&gt; (Figure 23)</td>
<td>0.45 ± 0.01</td>
<td>0</td>
<td>0</td>
<td>43.41 ± 1.6&lt;sub&gt;c&lt;/sub&gt;</td>
<td>NA</td>
</tr>
</tbody>
</table>

PE, phosphatidylethanolamine; Carb-PE, carbamylated phosphatidylethanolamine; NA, not applicable; ND, not determined.

<sup>a</sup> Resolved in Chloroform/Methanol/Acetic Acid/Water (60/50/1/4, v/v/v/v).
<sup>b</sup> Resolved in Chloroform/Methanol/Ammonia (65/35/5, v/v/v).
<sup>c</sup> Bands were scraped from TLC plate and scintillation counted in 10 mL Sigma-Fluor<sup>TM</sup>.
<sup>d</sup> Quantitated as phosphorus.

All values represent the mean of duplicate determinations.
FIGURE 23

Separation of Native and $^{14}$C-Carbamylated Erythrocyte Phosphatidyl-
elanolamine

Legend

The thin-layer chromatography and autoradiography of native and $^{14}$C-
carbamylated membrane lipids resolved in chloroform/methanol/ammonia (65/35/5,
v/v/v/v) was conducted as described in Section 2.2.6.5. The $^{14}$C-carbamylation and
extraction of erythrocyte membrane lipid was performed as described in Sections
2.2.6.1 and 2.2.6.2, respectively. The phospholipids were visualized using the
procedure of Vaskovsky and Kostevsky (1968). PE, phosphatidylethanolamine; PC,
phosphatidylcholine; PS, phosphatidylserine; Carb-PE, carbamylated
phosphatidylethanolamine; Std, standard; O$^{14}$CN, [$^{14}$C] cyanate.
appearance of a new band which migrates ahead of PE. The new band can be identified as carb-PE (Table 3, row 5) from the following:

1) It incorporates identical [14C] cyanate counts (Table 3, row 5) as erythrocyte carb-PE unresolved from PE (Table 3, row 4);

2) It has the same Rf value as egg yolk carb-PE (Table 3, row 3), resolved from PE, in the same solvent system and;

3) The combined nmol of separated carb-PE and PE (Table 3, rows 5 and 6, respectively) are equivalent to unresolved (PE + carb-PE) (Table 3, row 4);

4) In addition, as expected, approximately 1 (i.e., 0.77 ± 0.12) mol cyanate is incorporated per mol PE (Table 3, row 5). The autoradiograph of Figure 23 also clearly shows that the chloroform/methanol/ammonia (65/35/5, v/v/v) solvent system does not separate carbamylated-PS from PS. No further attempts were made to separate these species.

The separation of carb-PE from PE by TLC enables one to follow the carbamylation of erythrocyte membrane PE (and potentially any subcellular membrane source of PE) in vitro without the use of [14C] cyanate and autoradiography. Figure 24A clearly demonstrates the increasing carbamylation of erythrocyte PE in vitro as a function of incubation time with cyanate. Imaging densitometry of the phospholipid separated by TLC shows that by 48 h greater than 40% of the PE is carbamylated (Figure 24B).

3.3.2 In Vivo Studies

The in vitro results indicate that, in theory, TLC of native erythrocyte
FIGURE 24

Time Course of Erythrocyte Phosphatidylethanolamine Carbamylation

Legend

Panel A shows the time course of erythrocyte phosphatidylethanolamine (PE) carbamylation monitored by thin-layer chromatography (TLC) as described in Section 2.2.6.6. Membrane lipids were resolved in chloroform/methanol/ammonia (65/35/5, v/v/v/v) and visualized by dipping the TLC plate in a manganese chloride-sulfuric acid derivatizing reagent (Conte and Bishop, 1988). Panel B shows the % PE carbamylation as a function of incubation time with cyanate, where; % PE carbamylation was calculated by imaging densitometry of the scanned TLC plate.
### FIGURE 24

<table>
<thead>
<tr>
<th></th>
<th>- Cyanate</th>
<th>+ Cyanate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>0 h</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>1 h</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>5 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 h</td>
</tr>
</tbody>
</table>

A

Chol

Carb-PE

PE

PC

PS

B

![Graph showing percent carbamylated PE over time](attachment:image.png)
membrane lipid in a solvent system of chloroform/methanol/ammonia (65/35/5, v/v/v) would allow the detection of in vivo carb-PE. This is so since any band in the absence of cyanate incubation with an Rf value equivalent to carb-PE might well be intrinsically carbamylated PE. However, due to the diffuse nature of lipid content in this region (Figure 24A), phosphate analysis alone would not necessarily be indicative of the presence of in vivo carb-PE. It was also clear from previous work (Figure 12) that the direct incubation of ghost cell membranes with diacetyl does not result in the formation of a detectable chromogen. Consequently, an alternate route to investigate whether carbamylated aminophospholipids can be detected in the native erythrocyte membrane was explored.

The general concept was to treat the erythrocyte membrane lipids with phospholipase D (PLD) (EC 3.1.4.4) and to react any released carbamylated aminophospholipid headgroups with diacetyl. PLD catalyzes cleavage of the phosphodiester bond of a phospholipid (or lysophospholipid) molecule to produce phosphatidic acid (PA) and an alcohol such as choline from PC, ethanolamine from PE, serine from PS, and inositol from phosphatidylinositol (PI) (Dennis, 1983). The relative activity of PLD (Streptomyces Chromofuscus) on PC, SM, PE and PI / Triton X-100 micelles has been measured to be 100%, 25%, 31%, and 15%, respectively (Genencor International Inc., 1991). The enzyme has a pH optimum range of 7.0 - 8.5 and is activated by Ca2+ and Triton X-100 (Genencor International Inc., 1991). The analysis concept is outlined in Figure 25. Basically, the lipid components of the membrane are isolated by solvent extraction and
FIGURE 25

Proposed Reaction Scheme for the Measurement of Carbamylated Erythrocyte Aminophospholipids.

Legend

PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; Chol, cholesterol; PA, phosphatidic acid; Carb, carbamylated.
**FIGURE 25**

**Diacetyl**

**GHOST CELL**

**SOLVENT EXTRACTION**

**SONICATE (TRITON X-100)**

**ERYTHROCYTE LIPID MICELLE**

(PE, PS, PC, SM, CHOL)

**Phospholipase D**

Ca$^{2+}$

**PA**

**Ceramide**

**Cholesterol**

$\text{HO-CH}_2\text{-CH}_2\text{-N-(CH}_3)_3$  
(Choline)

$\text{HO-CH}_2\text{-CH}_2\text{-NH}_2$  
(Ethanolamine)

$\text{HO-CH}_2\text{-CH-NH}_2$  
(Serine)

$\text{HO-CH}_2\text{-CH-NH-CO-NH}_2$  
(Carb-Serine)

$\text{HO-CH}_2\text{-CH}_2\text{-NH-CO-NH}_2$  
(Carb-Ethanolamine)

**CHROMOGEN**

**NO DETECTABLE CHROMOGEN**
sonicated in a buffer containing Triton X-100. The resulting erythrocyte membrane lipid / Triton X-100 micelles are incubated with PLD. This treatment is anticipated to result in the enzymatic hydrolysis of phospholipids to yield phosphatidic acid PA (from PC, PS and PE) and ceramide (from SM) along with all the corresponding alcohol headgroups (choline from PC and SM, ethanolamine from PE, and serine from PS). If carb-PE and carb-PS exist, their hydrolysis would be expected to liberate carbamyl serine and carbamyl ethanolamine (hydroxyethylurea). Both carbamyl serine and carbamyl ethanolamine would then be available for reaction with diacetyl.

The absorbances, relative to urea, for equivalent concentrations of ethanolamine and carbamyl serine reacted with diacetyl are shown in Figure 26. Although carbamyl ethanolamine and urea have essentially equivalent molar absorption coefficients upon reaction with diacetyl, carbamyl serine does not produce any detectable chromogen with diacetyl. This indicates that either there is no reaction of diacetyl with carbamyl serine, or that the reaction product is either nonchromogenic, or unstable under the conditions of the diacetyl monoxime assay. The significance of this observation to the present work is that the liberation of carbamyl serine from erythrocyte membrane lipid micelles treated with PLD would not be expected to produce a measureable chromogen upon reaction with diacetyl. Any chromogen, therefore, generated in the presence of PLD would be suggestive of carbamyl ethanolamine specifically.

Figure 27 demonstrates that PLD efficiently catalyzes the hydrolysis of
FIGURE 26

Relative Absorbances of Urea, Carbamyl Ethanolamine, and Carbamyl Serine
with Diacetyl

Legend

The relative absorbances of the chromogens produced when urea, carbamyl
ethanolamine, and carbamyl serine are reacted with diacetyl were measured as
described in Section 2.2.7.5.
FIGURE 27
Thin-Layer Chromatography of Native and Phospholipase D-Treated Egg Yolk
Phospholipid / Triton X-100 Micelles

Legend
Thin-layer chromatography of native and phospholipase D (PLD)-treated egg yolk phospholipid / Triton X-100 micelles was performed as described in Section 2.2.7.3. The phospholipid / Triton X-100 micelles were prepared as described in Section 2.2.7.2. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; TrX, Triton X-100; Cer, ceramide; PA, phosphatidic acid.
purified egg yolk PC/Triton X-100, PE/Triton X-100, PS/Triton X-100, PC/PS/Triton X-100, and SM/Triton X-100 micelles. The solvent system utilized [chloroform/methanol/acetic acid/water (90/40/12/2, v/v/v/v)] completely resolves phosphatidic (PA) from the native phospholipids (Higgins, 1987). Interestingly, ceramide, resulting from the hydrolysis of SM, has essentially the same R<sub>v</sub> value as native PE.

The time course treatment of erythrocyte membrane lipid/Triton X-100 micelles with PLD, as monitored by TLC, is shown in Figure 28. By visual inspection it is evident that PLD catalyses the hydrolysis of erythrocyte membrane PC, PS, SM and PE into PA. The degree of hydrolysis of PE would appear to be less than the other phospholipids (lane 4), however, the lipid present in this region is most likely due to ceramide (Figure 27). Imaging densitometry of the TLC plate in Figure 28 allows an estimate of the degree of hydrolysis of the various erythrocyte phospholipids. The results (Figure 29) show that the hydrolysis is complete within a 30-min incubation with PLD. Consistent with this observation, the levels of PA rise rapidly and are essentially unchanged after 30 min. Control values of cholesterol remain unchanged over the time period studied. The less than complete hydrolysis of the phospholipids (Figure 29) does not result from PLD instability since subsequent additions of fresh PLD do not induce further hydrolysis (data not shown).

The reaction of native and PLD-hydrolyzed erythrocyte lipid/Triton X-100 micelles with diacetyl was next investigated (Figure 30). Erythrocyte lipid/Triton X-
FIGURE 28

Time Course for the Hydrolysis of Erythrocyte Lipid / Triton X-100 Micelles by
Phospholipase D

Legend

The hydrolysis of erythrocyte lipid / Triton X-100 micelles by phospholipase D (PLD) was monitored by thin-layer chromatography as described in Section 2.2.7.3. Chol, cholesterol; PA, phosphatidic acid; PE, phosphatidylethanolamine; Cer, ceramide; PS, phosphatidylserine; PC, phosphatidylcholine; LysoPL, lysophospholipid; SM, sphingomyelin; TrX, Triton X-100.
FIGURE 29

Imaging Densitometry Analysis for the Hydrolysis of Erythrocyte Membrane

Phospholipids by Phospholipase D

Legend

The time course for the hydrolysis of erythrocyte membrane phospholipids by phospholipase D was monitored by imaging densitometry as described in Section 2.2.7.3. PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; PA, phosphatidic acid.
FIGURE 30

Absorption Spectra for the Reaction of Diacetyl with Native and Phospholipase
D-Treated Erythrocyte Lipid / Triton X-100 Micelles

Legend

The absorption spectra for the reaction of diacetyl with native and phospholipase D (PLD)-treated erythrocyte lipid / Triton X-100 micelles were obtained as described in Section 2.2.7.4 Buffer, 50 mM Tris-HCL, 10 mM CaCl₂, 1% Triton X-100, pH 8.0.
100 micelles untreated with PLD are shown (Figure 30) to generate a detectable absorbance at 530 nm upon reaction with diacetyl. The absorbance does not result from residual carbamylated membrane protein since no detectable protein was found upon protein analysis (Marcel and Haas, 1978) of the lipid micelles (data not shown). Treatment of erythrocyte lipid/Triton X-100 micelles with PLD results in an approximately 4-fold increase in absorbance (Figure 30). The increased absorbance does not arise from the reaction of diacetyl with PLD since a buffer blank containing PLD does not produce any absorbance at 530 nm above that of buffer alone (Figure 30). Clearly, therefore, the treatment of erythrocyte lipid/Triton X-100 micelles with PLD results in the production of a nonprotein compound(s) which is(are) reactive with diacetyl.

The incubation of PLD with the erythrocyte membrane lipid/Triton X-100 micelles clearly produces a number of reaction products (Figure 25) not present in the native micelle, which may potentially react with diacetyl. These include: PA from the hydrolysis of PC, PS and PE; ceramide, from the hydrolysis of SM; ethanolamine, from the hydrolysis of PE; choline, from the hydrolysis of PC and SM; serine, from the hydrolysis of PS; and finally, if PE is intrinsically carbamylated, carbamyl ethanolamine. Carbamyl serine is not considered as a potential reactant since it is nonchromogenic in the presence of diacetyl (Figure 26). Consequently, a study was undertaken to ascertain, by process of elimination, whether the chromogen produced upon reaction of erythrocyte membrane lipid/Triton X-100 micelles with PLD could be the reaction product of diacetyl with
carbamyl ethanolamine.

To this end the individual headgroup alcohols (choline, ethanolamine and serine) and various phospholipid/Triton X-100 micelles (constructed from commercially purified egg yolk phospholipids), in the absence and presence of PLD treatment, were investigated for chromogenic development upon incubation with diacetyl. All species were tested at concentrations equal to or greater than their individual concentrations in the erythrocyte lipid/Triton X-100 micelle suspension. The results are recorded in Figure 31. No chromogenic development with diacetyl is observed for native or PLD hydrolyzed PC, PS or SM/Triton X-100 micelles. Likewise, ethanolamine, choline and serine in the absence or presence of PLD are nonreactive with diacetyl. Interestingly, however, native PE/Triton X-100 micelles generate a detectable chromogen ($\lambda_{max} = 530$ nm) when reacted with diacetyl, while PLD treatment of the PE/Triton X-100 micelles results in an approximately 4-fold increase in the absorbance. This is analogous to the chromogenic development of erythrocyte lipid in the absence and presence of PLD (Figure 30). The reactivity of diacetyl specifically with purified egg yolk PE is also demonstrated by the observation that PE/PC/PS/SM/cholesterol/Triton X-100 micelles (simulating the composition of erythrocyte membrane lipid/Triton X-100 micelles) treated with PLD are chromogenic, however, PC/PS/SM/cholesterol/Triton X-100 micelles (i.e., without PE) are not chromogenic (Figure 31). Since the hydrolysis of PE produces only PA and ethanolamine (Figure 25), neither of which are reactive with diacetyl (Figure 31), this suggests that purified egg yolk PE is
FIGURE 31

Reaction of Phospholipid Headgroups and Phospholipid Micelles with Diacetyl

Legend

Phospholipid headgroup alcohols (choline, ethanolamine, and serine) and phospholipid micelles were prepared and incubated in the presence and absence of phospholipase D (PLD) as outlined in Section 2.2.7.2. All samples were analyzed for reaction with diacetyl as described in Section 2.2.7.4. PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; Chol, cholesterol; PE, phosphatidylethanolamine; Buffer (50 mM Tris-HCl, 10 mM CaCl₂, 1% Triton X-100, pH 8.0).
intrinsically carbamylated and that treatment with PLD releases carbamyl ethanolamine which subsequently reacts with diacetyl. The detectable chromogenic development in the absence of PLD treatment is likely due to reaction of diacetyl with, either native carbamylated PE, or the nonenzymatically hydrolyzed headgroup of carbamylated PE (carbamyl ethanolamine) resulting from the high acidity and temperature of the assay.

The presence of carb-PE in commercially purified egg yolk PE is a possibility. Commercial preparation of PE from egg yolk involves separation of PE from other egg yolk phospholipids by TLC in a solvent system of chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v) (Sigma Chemical Company, 1995). Since the work conducted with in vitro-carbamylated-PE (Section 3.3.1) clearly demonstrates that in this solvent system carb-PE is not resolved from PE (Figure 21), therefore, any intrinsic carb-PE would not be separated from commercial preparations of purified egg yolk PE.

The results of the above study, therefore, are consistent with the hypothesis that the PE (and probably PS) of the native erythrocyte membrane is intrinsically carbamylated and that incubation of native erythrocyte membrane lipid with PLD releases both carbamyl ethanolamine and carbamyl serine. However, since carbamyl serine is nonchromogenic with diacetyl, the observed absorbance (Figure 30) is likely specifically a measure of in vivo carbamylated phosphatidyl-ethanolamine.

By measuring the concentration of phospholipid in membrane lipid/Triton X-
treatment (Table 4), the level of carbamylated PE in a pool of erythrocytes from normal individuals (urea = 4.46 ± 0.51 mM, n = 12) was calculated to be 2.85 ± 0.65%.


<table>
<thead>
<tr>
<th></th>
<th>Phospholipid(^a) (nmol)</th>
<th>PE(^b) (nmol)</th>
<th>Diacetyl Assay(^c) (Abs 530 nm)</th>
<th>Carb-PE(^d) (nmol)</th>
<th>Carb PE/PE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelle (- PLD)</td>
<td>3653 ± 711</td>
<td>1059 ± 206</td>
<td>0.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelle (+ PLD)</td>
<td>same</td>
<td>same</td>
<td>0.132 ± 0.006</td>
<td>29.06 ± 1.40</td>
<td>2.85 ± 0.65</td>
</tr>
</tbody>
</table>

PE, phosphatidylethanolamine; Carb-PE, carbamylated phosphatidylethanolamine.

\(^a\) Quantitated as phosphorus (Higgins, 1987).
\(^b\) Calculated as 29% of total phospholipid (Schwartz et al., 1985).
\(^c\) Micelles reacted with diacetyl as described in Section 2.2.7.4.
\(^d\) Calculated from a carbamyl ethanolamine standard curve.

All values represent the mean of duplicate determinations.
CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Summary

In the present study the covalent modification of the erythrocyte membrane by cyanate (carbamylation) was investigated. The research goal was to elucidate and characterize the binding of cyanate among the various components of the cytoskeletal matrix and to establish whether these levels were increased in uremics relative to nonuremics. It was felt that this basic biochemical information would be useful in establishing if and how carbamylation may be playing a role in the observed decreased lifetime of the erythrocyte in uremics (Eschbach et al., 1977).

Methods were developed which demonstrated that the levels of erythrocyte membrane protein carbamylation are increased in uremics relative to normal individuals. In vitro studies indicated that spectrin and ankyrin were the most extensively carbamylated proteins of the cytoskeletal matrix. In addition, evidence for carbamylation-induced membrane destabilization was provided from measurements of membrane fluidity as a function of membrane carbamylation.

The present investigation also demonstrated the direct and specific modification in vitro of the amino containing headgroups of both phosphatidyl ethanolamine (PE) and phosphatidylyserine (PS) by cyanate. This is the first reported demonstration of the covalent modification of phospholipid headgroups by any endogenous compound. In addition, preliminary studies indicated that carbamylated PE (carb-PE) and PS (carb-PS) are natural components of the native
erythrocyte membrane. Since the present investigation demonstrated that the level of erythrocyte membrane protein carbamylation is increased in uremics, it may be hypothesized that uremics would, likewise, have increased levels of carbamylated PE and PS.

4.2 Significance of Erythrocyte Membrane Protein Carbamylation

Taken together, the in vitro and in vivo results suggest that key protein binding domains (particularly spectrin-ankyrin associations) may be altered in the erythrocytes of uremics as a result of the increased covalent protein modifications by cyanate (carbamylation) in these individuals. These are important observations since modifications of this binding are known to have deleterious consequences for the erythrocyte. Genetic deficiencies of spectrin (Agre et al., 1982) and ankyrin (Coetzer et al., 1988) (hereditary spherocytosis), as well as a multitude of specific defects in spectrin and band 4.1 (Palek, 1990) (hereditary elliptocytosis), have been documented and shown to result in altered self association of α and β spectrin (Dhermy et al., 1982) and defective spectrin-ankyrin interactions (Zail and Coetzer, 1984). Acquired ankyrin defects occur in the major hemoglobinopathies: sickle cell anemia (Platt et al., 1985), and thalassemias (Shinar et al., 1989). In these disorders, the abnormal easily denatured hemoglobin induces a functional defect in ankyrin that interferes with its ability to bind to spectrin. In all of the above cases, there is a destabilization of the erythrocyte membrane which ultimately leads to hemolytic anemia and, consequently, to a decreased erythrocyte survival time (Palek, 1990). A decreased erythrocyte survival time is also observed in
diabetes (Peterson et al., 1977) and is believed to be, at least partially, a consequence of the increased levels of nonenzymatic glycosylation of the membrane proteins (Miller et al., 1980). This is interesting since the predominant protein glycosylation site, as with carbamylation, is the ε-amino group of lysine residues.

It is conceivable, therefore, as mentioned above, that the increased level of carbamylated membrane protein in uremics may critically alter key binding domains within the cytoskeletal matrix which, at least partially, contribute to the observed decreased erythrocyte lifetime (Eschbach et al., 1977). Comparisons of spectrin-ankyrin-anion channel binding studies from erythrocytes of uremics and nonuremics are needed to strengthen this hypothesis.

4.3 Significance of Erythrocyte Membrane Aminophospholipid Carbamylation

Since the reaction of cyanate, or any endogenous compound, with aminophospholipids has not been previously reported, the physiological significance of this observation is unknown. Although the present study has not directly shown that carb-PE and/or carb-PS are increased in uremics, it can be hypothesized, however, that if this is the case, then the carbamylation of PS and/or PE may directly contribute to membrane destabilization.

In addition to the protein-protein interactions among the cytoskeletal matrix proteins (\(k_d = 5 \times 10^{-4} \) M, see Table 1) it has been demonstrated that both spectrin and protein 4.1 bind with high affinity (\(k_d = 3.3 \times 10^{-7} \) M), and selectively to the headgroup of phosphatidylserine (PS) (Cohen et al., 1988; Maksymiw et al., 1987).
The binding is electrostatic, with the negatively charged headgroup of PS interacting with positively charged regions within the binding site of the protein (Maksymiw et al., 1987; Shiffer et al., 1988). It has been proposed that these protein-lipid interactions play a role in the maintenance of phospholipid asymmetry by their capacity to "fix" phosphatidylserine to the inner leaflet (Schroit and Zwaal, 1991) since individuals with erythrocyte protein 4.1 deficiency were found to have an altered pattern of membrane lipid distributions, with significant enrichment in the outer lipid leaflet of PS (Schwartz, 1985).

Carbamylation can be expected to alter the protein-lipid interactions either through protein modification or lipid modification or, perhaps, both. Firstly, the carbamylation of PS removes a positive charge from the headgroup and, as such, changes the physiological charge of PS from (-1) to (-2). Secondly, protein lysine carbamylation removes a positive charge (which may be directly or indirectly involved in PS binding) to generate a neutral species. Either case could induce altered protein-lipid binding affinities and potentially lead to membrane destabilization.

4.4 Significance of the Carbamylation of Erythrocyte Membrane Components

The human erythrocyte cytoskeletal matrix is responsible for maintaining the cell shape, reversible deformability and membrane structural integrity of the erythrocyte. (Shiffer et al., 1988). The present study provides evidence of potentially altered protein-protein and protein-lipid interactions within the erythrocyte cytoskeletal matrix, as a result of carbamylation. It is hypothesized that
cytoskeletal binding modifications may lead to a destabilized membrane. The resulting destabilized membrane would not be expected to be as resilient to the mechanical stress incurred in the micro-circulation as the native membrane and, as such, would lead to the premature erythrocyte destruction observed in both uremics (Eschback et al., 1977) and upon in vitro carboxymylation (Lane and Burka, 1976).

Another intriguing mechanism of premature erythrocyte destruction in uremics may possibly involve PS directly. As a result of the lipid asymmetry of the erythrocyte membrane, PS normally resides entirely on the cytoplasmic side of the membrane. It has been proposed that the exposure of PS on the outer layer of the membrane may serve as a signal for recognition by phagocytes which subsequently eliminates the altered erythrocyte from circulation (Schroit and Zwaal, 1991). This was first shown by the rapid clearance of erythrocyte containing fluorescent long-chain PS but not PC analogues from the peripheral circulation of mice. (Schroit et al., 1985). When the liver and spleens of these animals were analyzed it was revealed that the cleared fluorescent erythrocytes underwent phagocytosis and accumulated in splenic macrophages and Kupffer cells. The erythrocytes from diabetes mellitus patients also display elevated levels of adherence to mononuclear phagocytes and endothelial cells (Zachowski et al., 1985) as well as a decreased lifetime (Peterson et al., 1977). This is interesting since erythrocytes from diabetics also show an altered lipid asymmetry, with PS appearing in the plasma membrane outer layer (Wali et al., 1988 and Lupu et al.,
1988). In addition, recent studies (Wilson et al., 1993) have shown that incubation of normal erythrocytes with high concentrations of glucose duplicates this effect. It was proposed that the PS reorientation may be caused by direct alteration of protein (glycosylation) or lipid (by fatty acyl chain oxidation).

It is conceivable, therefore, that carbamylation of erythrocyte components may induce the exposure of PS on the outer layer of the membrane. Indeed the altered membrane fluidity of the erythrocyte from uremics (Komidori et al., 1985) or the in vitro induced alterations of membrane fluidity as a function of carbamylation (Figure 19) may, at least partially, reflect this process. Exposure of PS could occur through carbamylation-induced alterations in the cytoskeletal protein-PS binding site leading to exposure of not only carb-PS but also PS.

Increases in intracellular calcium have also been shown to induce PS reorientation (Eitbol et al., 1987). In this regard, it is interesting to note that erythrocytes from uremics have recently been shown to have a decreased (Ca$^{2+}$ + Mg$^{2+}$ )-ATPase activity, which leads to an approximately 3-fold increase in intracellular calcium (Shalev, 1991). It is clear that an actual measurement of the lipid asymmetry of the erythrocyte membrane in uremics is needed to confirm the proposed exposure of PS.

Carbamylation may also be involved in other pathophysiological conditions observed in uremics, such as, the development of peripheral neuropathies (Asbury et al., 1963). The condition involves both segmental demyelination and axonal degeneration in peripheral nerves (Asbury et al., 1963) and evidence of uremic
neuropathy is present in approximately half of all patients on hemodialysis programs (Asbury, 1975). The polyneuropathy clinically improves following dialysis and, more dramatically, after renal transplantation (Bolton et al., 1977). It is believed, therefore, that uremic neuropathy is secondary to retained, dialyzable toxins or metabolites normally excreted by the kidney; however, the cause remains elusive. In this regard, it is interesting to note that an unrelated study demonstrated that a number of patients with sickle-cell anemia, being treated with cyanate, developed peripheral neuropathies (Peterson et al., 1974). Microscopic examination of nerve biopsies clearly showed the presence of segmental demyelination. The neuropathy clinically improved with no specific treatment after cessation of cyanate administration (Peterson et al., 1974). It is tempting, therefore, to hypothesize that carbamylation of either the myelin basic protein (Gennis, 1989a) or the abundant amounts of Schwann cell PE and PS making up the myelin sheath (Gennis, 1989a) may be playing a role here.

Finally, since our results demonstrate the carbamylation of erythrocyte membrane PE and PS under physiological conditions, it can be hypothesized that the covalent modification of aminophospholipids in vivo with other endogenous amine reactive species, such as glucose, may occur. In addition, covalent modifications of PE and PS may also extend beyond the plasma membrane to subcellular locations such as the endoplasmic reticulum or nuclear membrane.
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Abstracts


Presentations


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