AD-A045 450 WAYNE STATE UNIV DETROIT MICH DEPT OF BIOLOGY F/G 6/3
CONTRIBUTIONS OF MEMBRANE COMPONENTS TO INTRACELLULAR WATER ORD--ETC(U)
SEP 77 P D MORSE N00014-76-C-1167
UNCLASSIFIED NL

1 OF 1
AD A045450

END
DATE FILMED
11-77
DDC

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

(12) (11)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Annual Report No. 1
2. GOVT ACCESSION NO.:
3. RECIPIENT'S CATALOG NUMBER:

4. TITLE (and Subtitle): (6) Contributions of Membrane Components to Intracellular Water Order: The Current Status.

5. TYPE OF REPORT & PERIOD COVERED: Annual Report Oct. 1, 1976-Sept. 30, 1977

6. PERFORMING ORG. REPORT NUMBER:

7. AUTHOR(s): (10) Philip D. Morse, II

8. CONTRACT OR GRANT NUMBER(s): (15) N00014-76-C-1167

9. PERFORMING ORGANIZATION NAME AND ADDRESS:
Wayne State University
Department of Biology
Detroit, Michigan 48202

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NR 207-009

11. CONTROLLING OFFICE NAME AND ADDRESS:
Office of Naval Research
Biophysics Program, 800 North Quincy Street
Arlington, Virginia 22217

12. REPORT DATE: (11) 27 Sep[illegible]77

13. NUMBER OF PAGES: ~~29~~ (12) 31p

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office):
Office of Naval Research Branch Office
536 South Clark Street
Chicago, Illinois 60605

15. SECURITY CLASS. (of this report): Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

DISTRIBUTION STATEMENT A
Approved for public release; Distribution Unlimited

16. DISTRIBUTION STATEMENT (of this Report)

Distribution of this document is unlimited.

DDC RECEIVED OCT 20 1977 B

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

(9) Rept. no. 1 (Annual) 1 Oct 76-30 Sep 77

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Electron spin resonance, spin labels, membranes, red blood cells, red blood cell ghosts, artificial lipid vesicles, chaotropic agents, intracellular water order, membrane proteins, membrane lipids, chloroplasts, gel electrophoresis

are described

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report describes the progress made in this laboratory during the previous year (October 1, 1976 to September 30, 1977). ~~I describe~~ the methods for measuring intracellular viscosity by electron spin resonance and show results obtained for red blood cells, red blood cell ghosts, chloroplasts, and artificial lipid vesicles. The conclusions from these studies are that membrane proteins may be strong contributors to water order within cells and organelles.

(OVER)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE S/N 0102-014-6601

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

AD A045450

AD No. DDC FILE COPY

400719

Block 20. (continued)

Detailed descriptions are provided for the methods I have developed for use in this project. These include perparing a sample for measuring its internal viscosity with the spin label TEMPAMINE (2,2,6,6-tetramethyl piperidine-N-oxyl-4-amine), spin labeling cell and organelle membranes, and preparation of hemoglobin-free resealed red blood cell ghosts.

The conclusions derived from this past year of wark are provided along with my ideas on directions this research could take in the future.

OFFICE OF NAVAL RESEARCH
BIOLOGICAL & MEDICAL SCIENCES DIVISION
MEDICAL AND DENTAL SCIENCES PROGRAM, CODE 444
DISTRIBUTION LIST FOR TECHNICAL, ANNUAL AND FINAL REPORTS

Number of Copies

(12) Administrator, Defense Documentation Center
Cameron Station
Alexandria, Virginia 22314

(6) Director, Naval Research Laboratory
Attention: Technical Information Division
Code 2627
Washington, D. C. 20375

(6) Office of Naval Research
Attention: Code 102IP (ONRL DOC)
800 N. Quincy Street
Arlington, Virginia 22217

(3) Office of Naval Research
Medical and Dental Sciences
Code 444
Arlington, Virginia 22217

(1) Commanding Officer
Naval Medical Research and Development Command
National Naval Medical Center
Bethesda, Maryland 20014

(1) Chief, Bureau of Medicine and Surgery
Department of the Navy
Washington, D. C. 20375

(2) Technical Reference Library
Naval Medical Research Institute
National Naval Medical Center
Bethesda, Maryland 20014

(1) Office of Naval Research Branch Office
495 Summer Street
Boston, Massachusetts 02210

Enclosure (3)

(1) Office of Naval Research Branch Office
536 South Clark Street
Chicago, Illinois 60605

(1) Office of Naval Research Branch Office
1030 East Green Street
Pasadena, California 91101

(1) Office of Naval Research
Contract Administrator for Southeastern Area
2110 G Street, N.W.
Washington, D. C. 20037

(1) Commanding Officer
Naval Medical Research Unit No. 2
Box 14
APO San Francisco 96263

(1) Commanding Officer
Naval Medical Research Unit No. 3
FPO New York 09527

(1) Officer in Charge
Submarine Medical Research Laboratory
Naval Submarine Base, New London
Groton, Connecticut 06342

(1) Scientific Library
Naval Medical Field Research Laboratory
Camp Lejeune, North Carolina 28542

(1) Scientific Library
Naval Aerospace Medical Research Institute
Naval Aerospace Medical Center
Pensacola, Florida 32512

(1) Commanding Officer
Naval Air Development Center
Attn: Aerospace Medical Research Department
Warminster, Pennsylvania 18974

(1) Scientific Library
Naval Biomedical Research Laboratory
Naval Supply Center
Oakland, California 94625

(1) Commander, Army Research Office
P. O. Box 12211
Research Triangle Park
North Carolina 27709

(1) Director, Life Sciences Division
Air Force Office of Scientific Research
1400 Wilson Boulevard
Arlington, Virginia 22209

(1) Commanding General
Army Medical Research and Development Command
Forrestal Building
Washington, D. C. 20314

(1) Department of the Army
U. S. Army Science and
Technology Center - Far East
APO San Francisco 96328

(1) Assistant Chief for Technology
Office of Naval Research, Code 200
800 N. Quincy Street
Arlington, Virginia 22217

(1) Office of Naval Research
Resident Representative
University of Michigan
139 Cooley Building
2355 Bonisteel Blvd.
Ann Arbor, Michigan 48109

(1) Dr. Gilbert Ling
Pennsylvania Hospital
Department of Molecular Biology
8th and Spruce Streets
Philadelphia, Pennsylvania 19107

(1) Dr. Freeman W. Cope
Biochemistry Laboratory
U.S. Naval Air Development Center
Department of the Navy
Warminister, Pennsylvania 18974

(1) Dr. Carlton F. Hazlewood, Professor
Department of Pediatrics
Baylor College of Medicine
1200 Moursund
Houston, Texas 77030

OFFICE OF NAVAL RESEARCH

Contract N00014-76-C-1167

Task No. NR 207-009

"Contributions of Membrane Components to Intracellular Water Order: The Current Status"

by

Philip D. Morse, II

Wayne State University
Department of Biology
Detroit, Michigan 48202

27 September 1977

CONTRIBUTIONS OF MEMBRANE COMPONENTS TO INTRACELLULAR WATER ORDER: THE CURRENT STATUS

ORIGINAL OBJECTIVES

The original objectives stated in my initial proposal are:

1) How far does ordered water extend into the cell?

2) What are the contributions of the lipid and protein constituents of the membrane to water order?

3) Does the water inside a cell represent a different state of water compared to bulk water?

It is the purpose of this report to outline the progress made on these questions during the last year (October 1, 1976 to September 30, 1977).

OBJECTIVES MET DURING THE REPORTING PERIOD

Description of the method

The primary method described for studying the internal viscosity, and hence the state of water, within cells is electron spin resonance (ESR). This method makes use of the fact that the lifetime of the excited state of a spin label is rather long (10^{-8} sec.). When the spin label is tumbling rapidly (10^{11} rotations / sec.) this gives rise to three narrow spectral lines which arise from the interaction of the electron with the magnetic moment of the nucleus. Because of magnetic asymmetry of the nitrogen-oxygen free radical bond, the three lines broaden at different rates as the spin label tumbles more slowly. This allows use of the equation (1):

$$\tau_c = kW_o\left\{\left[\frac{h_o}{h_{-1}}\right]^{\frac{1}{2}} - 1\right\}$$

where τ_c is the rotational correlation time, k is a constant (6.5×10^{-10}) which takes into account the contributions of microwave frequency and proton interaction to the residual linewidth, W_o is the width of the midfield line, h_o and h_{-1} are the heights of the mid- and highfield lines, respectively. It can be seen immediately that as τ_c decreases, h_o/h_{-1} decreases.

In the presence of a number of transition metals with rapid relaxation times (10^{-13} sec. for Ni^{++} and Fe^{++}), collisions between the spin label and the transition metal cause the spin label to relax more rapidly. Thus, the spectral lines broaden. In the presence of 80 mM $K_3Fe(CN)_6$, a positively charged spin label (2,2,6,6-tetramethyl piperidine-N-oxyl-4-amine) TEMPAMINE has less than 1% of its original signal remaining (Figure 1, reference 2). If, however, the spin label can occupy an environment away from the transition metal (broadening agent), its signal returns and this signal is representative of that environment. This is the basis for measuring the internal viscosity of cells by ESR. A necessary requirement for the broadening agent is that it remains outside the cell. This requirement is met by nickel chloride in some cases and with potassium ferricyanide in others.

It should be pointed out that τ_c is used in a relative way. None of the spin labels used in this study are perfectly spherically symmetrical and therefore their tumbling is not completely isotropic. Absolute values of τ_c are not really obtainable. However, τ_c is a useful parameter to compare the tumbling rates of the same spin label is two different environment. Thus, for the remainder of this report, changes in spin label motion will be referenced to spin label motion in bulk water.

Use of potassium ferricyanide and TEMPAMINE

In the original proposal, a series of membrane systems were to be studied ranging from simple lipid vesicles prepared by artificial means, through sarcoplasmic reticular vesicles, which contain a single enzyme, the Ca^{++}-Mg^{++}-dependent ATPase, to the very complex, but well understood red blood cell membrane.

Because the proposal culminated in a study of a very complex system, it was first necessary to develop an adequate procedure to study the internal

viscosity of the red blood cell by electron spin resonance. Previous methods to determine the internal viscosity of cells or organelles (3) are not applicable to the red blood cell because they require toxic amounts of nickel chloride to remove the spin label signal from outside the cell. For this reason, I developed a method to determine internal viscosity of the red blood cell using a different spin broadening agent, potassium ferricyanide, and the spin label previously described, TEMPAMINE. The charge attraction between TEMPAMINE and the potassium ferricyanide allows isotonic concentrations of potassium ferricyanide to be used, and potassium ferricyanide is not permeable to the red blood cell, even over several hours. For these reasons, I have been using ferricyanide and TEMPAMINE as the spin label-spin broadening system of choice. Details of the procedure are in reference [illegible] closed).

Inter[illegible]viscosity of the red blood cell

I intially found that the internal viscosity of the red blood cell was about four times greater than that of water (2). I investigated this further by studying the internal viscosity of the red blood cell as a function of pH, ionic strength, and temperature. This data, shown in Figures 1A, 2A, and 3A, respectively (closed circles), show that a) as ionic strength increases, internal viscosity increases; b) as temperature increases, internal viscosity decreases up to 40°C at which point it increases sharply, and c) that red blood cell internal viscosity shows a maximum at pH 8. All these results with the exception of the viscosity rise above 40°C, could be attributed to the hemoglobin inside the red cell.

To further test this idea, I developed a method to remove all the hemoglobin from the red cell, leaving an intact, ion impermeable "ghost" (see METHODS). Using this preparation, I repeated the above studies (Figures 1A, 2A, and 3A, open circles). I found that the ghosts behaved similarly to the intact red blood cells, but that the internal viscosity

was about a factor of 2-3 times less than the intact cells. This data indicates that, in contrast to my original idea, hemoglobin cannot be uniquely responsible for the internal viscosity changes observed, since hemoglobin is less than 1% of the remaining protein. Therefore, some of my initial results may be due to contributions from membrane components.

To test the contribution of membrane lipid on the internal viscosity of the red blood cell, I used two spin label probes, 7N14 (2-hexyl-2-heptyl-5,5-dimethyl oxazolidine-N-oxyl) which probes the interior of the membrane hydrocarbon region, and 2N14 (2-dodceyl,2,5,5-trimethyl oxazolidine-N-oxyl) which probes the polar groups of the membrane. Again, the motion of the spin labels in the membrane were studied as a function of pH, ionic strength, and temperature (Figures 1B, 2B, and 3B, respectively). The results show that the lipid portion of the membrane does not respond to changes in pH or ionic strength and changes only as expected for temperature. A sharp increase in membrane fluidity at temperatures above 40^{o}C was not observed. Therefore, I must conclude that the membrane proteins are responsible in some part for the changes in internal viscosity of the red blood cell and the red cell ghost. In the intact red cell, however, hemoglobin provides the overriding influence.

Prelininary electron micrographs show that the "ghost" membrane is shaped essentially as a flacid sac and that there is a definate matrix of osmium-staining material with the lumen of the sac. A good candidate for this material is the protein spectrin which is found on the inner surface of the red blood cell membrane. Experiments are underway to use specific antibodies against spectrin to determine if this is indeed the case.

In summary, these sets of experiments have demonstrated that 1) the spin label method can be used to determine the internal viscosity of the red blood cell, 2) hemoglobin exters the major influence on the internal viscosity of the intact red blood cell, 3) membrane proteins also influence

the internal viscosity of the red blood cell, and 4) that this influence may be recognizable in the intact red cell.

An interesting side-light is the possibility that this method may be used clinically to determine the state of a patient by measuring the internal viscosity of the red cells. For example, a patient suffering from dehydration, which would increase the osmolarity of the blood, would show increased viscosity of the red cells. This may be an important finding from a medical aspect and should be pursued (see FUTURE DIRECTIONS): however, it is presently outside the scope of this proposal.

Internal viscosity of chloroplasts

The spinach chloroplast presents an excellent contrast to the red cell. The chloroplast is shaped like a small, thin sac with a miminal amount of trapped internal volume. This is in distinct contrast to the red cell which has considerable trapped internal volume. In addition, the chloroplast is an energy transducing membrane which is capable of synthesizing ATP. The distribution of enzymes within the chloroplast membrane is an unresolved question. Some proteins may be found in the internal aqueous region. I choose to study the chloroplast internal viscosity to contrast this membrane system with that of the red blood cell.

I found that 80 mM $K_3Fe(CN)_6$ does not affect the chloroplast membranes and that electron transport and ATP synthesis were not greatly influenced by ferricyanide at these concentrations. However, ferricyanide does cross the chloroplast membrane slowly. Extensive studies on model systems assured me that this does not have an effect on the measured internal viscosity of the chloroplasts. In contrast to the red blood cell, the chloroplast has an internal viscosity of about 15 to 17 times greater than water. It also accumulates TEMPAMINE so that the internal concentrations are about 3.5 times that of the external concentrations. Again, in contrast to red blood cells, the internal viscosity is not drastically affected by temperature

(Figure 4). The effect of pH and ionic strength were not studied.

In summary, the chloroplast behaves in a much different fashion than the red blood cell-red cell ghost. This may be due to the totally different nature and function of the chloroplast membrane. One must also consider that the trapped aqueous interior of the chloroplast is many times smaller than that of the red cell so that proteins attached to the membrane may influence all the water trapped by the chloroplast.

Internal viscosity of artificial lipid vesicles

The above studies suggest that the membrane proteins have an overriding influence on the state of water within the cell and close to the membrane. However, the role of lipid on the state of intracellular water cannot be completely dismissed. Preliminary results using artificial lipid vesicles of about 250 Å diameter made from dipalmitoyl phosphatidyl choline (liquid crystal-crystal transition temperature: 41°C) show that spin label motion within these vesicles is reduced by a factor of 25-35 over that of bulk water at temperatures below 41°C. However, above this temperature, spin label motion becomes much more rapid (viscosity decreases). This suggests that the lipids themselves may have an influence on cell water. The apparent contradiction between this data and that obtained from the red blood cells can be resolved when one considers that red blood cell membranes have a molar ratio of cholesterol to phospholipid of 1:1. The role of cholesterol in this case seems to be that of "smoothing" out the phase transition of the phospholipids. Current concepts of the membrane suggest that this is necessary to keep the membrane fluid so that membrane proteins are able to move around within the plane of the membrane; certainly membrane enzymes in artificial lipid systems do show an enormous decrease in activity when the lipids go through a phase transition to the crystalline state. However, another role may be to minimize the influence lipids have on water immediately adjacent to the membrane polar region. At the moment, this is speculation.

Effect of chaotropic agents on membrane structure

In addition to the above spin label studies, I have been studying the influence of chaotropic agents (agents which alter water structure) on the red blood cell membrane and its enzymes. This study has been undertaken in three parts. Part 1 is the study of the enzyme acetylcholine esterase, a membrane bound enzyme which is found on the outer half of the red blood cell membrane. Part 2 is a study of the enzyme glyceraldehyde-3-phosphate dehydrogenase which is bound by salt linkages to the inner half of the red cell membrane. Part 3 is a gel electrophoresis study of the distribution of membrane proteins. In all these studies, the effects of chaotropic agents has been studied. Preliminary data in these experiments has been promising.

Acetylcholine esterase is associated with the lipids of the red cell membrane and requires them for activity. Therefore, one can ask the following question: Do chaotropic agents have any effect on the packing density of lipids within the membrane and how would this influence the enzyme? I answered this question by subjecting the ghost membranes to varying concentrations of different salts and measuring the acetylcholine esterase activity (5). The results show that ions which decrease water structure, for example KSCN, inhibit the enzyme more rapidly (at lower salt concentrations) than do neutral salts such as NaCl. More interesting is that LiCl, a water structure maker, causes an increase in the activity of the enzyme. Using a one molar concentration of salt as a reference, KSCN would reduce activity by a factor of 2-4 compared to NaCl, while LiCl would activate the enzyme by a factor of 2-4. The use of NaCl as a reference eliminates salt effects from this consideration. Attempts to show that these agents alter membrane lipid packing, as measured by electron spin resonance, have been unsuccessful. I must therefore conclude that chaotropic agents affect the enzyme directly and that changes in water structure have only a direct effect on protein conformation, not lipid packing.

The second study using glyceraldehyde-3-phosphate dehydrogenase, was less convincing. All salts studied, including LiCl, NaCl, and KSCN, inhibited the enzyme at low concentrations (1 molar or less). Because this enzyme is attached to the membrane only through salt linkages, probably the salts are removing the enzyme from the membrane, and no information about water order immediately adjacent to the membrane can be obtained from these experiments.

The third study made use of gel electrophoresis and proteolysis to study the redistribution of membrane proteins within the membrane as a function of water order. In these studies, trypsin was used with varying concentrations of the salts. The rationale behind this experimental method is that trypsin will cleave off any portion of the membrane proteins which are outside the membrane. Thus, if chaotropic agents cause changes in the degree to which these proteins are accessable to trypsin, one could conclude that the proteins either have risen out of the membrane to some extent, or that the lipids are less tightly packed and trypsin can have access to more of the membrane proteins. Because ESR data is contrary to the former conclusion, the latter is the more probable.

Preliminary experiments have shown that the method is feasable, that is, trypsin will act on membrane proteins over a considerable range of salt concentrations. However, I have not yet obtained good gel electrophoresis patterns of the proteins because the remaining salt causes interference during separation of the proteins on the gel. Further effort in this direction will be directed toward elimination of this problem.

Membrane spin labels

The synthesis and purification of the various membrane spin labels has been discussed in the Status Report (March 28, 1977) and will not be discussed here. At present all spin labels required to carry out the research in the original proposal are available in highly purified form.

METHODS

The following is a detailed description of the methods developed by me and employed during the reporting period.

Measurement of internal viscosity of cell and organelle systems using ESR

An appropriate amount of red blood cells (100 µl of cells are required per ESR run) are washed 3 times in 150 mM NaCl-5 mM phosphate buffer, pH 8 (PBS). Each wash consists of a 3-fold dilution of the cells with PBS followed by centrifugation at 2000 g for 5 minutes at 0°C and aspiration of the supernatant. The cells are then suspended in the solution of interest (i.e., pH, ionic strength) containing 80 mM $K_3Fe(CN)_6$, and washed once again as above. 99 µl of the cell suspension is placed in a 10 X 75 mm test tube and 1 µl of 100 mM TEMPAMINE in water is added. The solution is mixed briefly, drawn into a 75 µl capillary tube, and the ESR spectrum measured. Typical spectrometer settings for these measurements are: 40 gauss sweep, 0.5 gauss modulation. Other settings are as required.

If the solution of cells or organelles contains less than about 5% trapped volume (5 µl in the total 100 µl sample), the remaining ferricyanide-broadened signal will begin to interfere with the ESR signal which arises from the internal aqueous space of the cells. In this case, a sample containing no organelles or cells (only 80 mM ferricyanide, 1 mM TEMPAMINE) is made up and is run directly after the sample containing the cells of interest. The difference between these two signals is the actual spectrum which contains the information about the internal viscosity of the cells. This method can be performed by hand, but it is much easier to do with a signal averager, where one spectrum is subtracted from another. Extreme stability of the spectrometer is required for meaningful measurements by this method.

Preparation of hemoglobin-free red blood cell ghosts

25 ml. of blood is washed as above, except that extreme care is taken

to remove the buffy coat (white cells). 1 ml. of washed blood is suspended in 34 ml. of 5 mM phosphate buffer, pH 8 (5P8) and centrifuged at 30,000 g for 15 minutes in the cold. The hemolyzed cells are at the bottom of the tube and a red button of unlysed cells and residual white cells is aspirated along with the supernatant. This procedure is repeated 3-4 times until the supernatant is colorless and the cells are white. Gel electrophoresis has shown that in ghosts prepared in this manner, hemoglobin contributes less than 1% to the remaining protein (primarily membrane protein).

The cells are resealed by incubation in PBS at 37°C for 1 hour. At this time, the cells appear to be sealed as determined by assaying for glyceraldehyde-3-phosphate dehydrogenase, an enzyme attached to the inner half of the membrane. Inaccessability of this enzyme is indicative of "resealing". However, when the ghosts are assayed by ESR as described previously, only a ferricyanide-broadened TEMPAMINE signal is obtained which indicates that the cells are not yet sealed to ferricyanide. Incubation at 4°C overnight then seals the cells to ferricyanide. Thus, "resealed" ghosts are only resealed to ferricyanide, which has a molecular weight of about 200. Experiments are currently underway to determine leakage of sodium and potassium from these cells by atomic absorption.

Labeling of organelle or cell membranes with membrane spin labels

An appropriate amount of 20 mM spin label in ethanol is added to the bottom of a 10 x 75 mm test tube and the ethanol removed by vacuum. "An appropriate amount" means that a ratio of one spin label per 50 membrane phospholipids is maintained to prevent perturbation of the membrane by the spin labels. Once the spin label solution is dry, 100 µl of sample is placed in the tube and the entire solution vortexed 10 times (5 sec. on-5 sec. off) to exchange the spin label from the wall of the test tube into the membrane of the cell under study. After this mixing process, 75 µl of the membrane suspension are removed in a capillary tube, and the ESR

spectrum taken.

CONCLUSIONS

The conclusions derived from research sponsored during the previous year are:

1) Electron spin resonance is a reliable, reproducible, and rapid way to determine the internal viscosity of cells and organelles.

2) The spin label-spin broadening system TEMPAMINE-ferricyanide can be used to determine the internal viscosity of red blood cells ahd ghosts.

3) Viscosity data for the red blood cell and ghost show that hemoglobin is the major protein associated with internal viscosity, but that membrane proteins cause visible changes in internal viscosity in both ghosts and intact cells.

4) TEMPAMINE-ferricyanide can be used to measure the internal viscosity of chloroplasts, a metabolically active membrane system.

5) Chloroplasts have an internal viscosity 15 times greater than bulk water and this viscosity does not change significantly with temperature.

6) The viscosity of chloroplasts is probably due to membrane-bound proteins which may exert considerable influence over water in the internal aqueous space.

7) In complex membrane systems, the lipids of the membrane probably do not influence water order within the cell or organelle system to any large extent. However, in model systems, the order of water inside artificial lipid vesicles is dependent upon the state of the lipid (whether the lipid is in a liquid crystal or crystalline state).

8) Studies to determine the influence of chaotropic agents on enzymes of the red blood cell membrane have shown that the membrane-bound enzyme acetylcholine esterase is strongly influenced by the state of water. Ions which decrease water order tend to deactivate the enzyme; ions which increase water order increase its activity. Similar studies on the enzyme glyceraldehyde-

3-phosphate dehydrogenase have given results which are not so clear. Gel electrophoresis of red cell membranes attacked by trypsin in the presence of chaotropic agents seems to be a better method of determining the influence of these agents on the distribution of proteins within the plane of the membrane.

FURTHER DIRECTIONS

Artificial lipid systems

Artificial lipid systems should be studied more closely to determine if size and lipid composition are major contributors to the internal viscosity of the internal aqueous space of these vesicles. Trapping chaotropic agents within these vesicles should alter the water order within the vesicles and may be used to study possible perturbations of potassium and sodium distribution across these membranes.

Red blood cell membranes

Intact and ghosted red blood cells should be studied in the presence of various chaotropic agents. The purpose of this experiment is to determine if information about the external water "structure" can be transmitted across the red blood cell membrane. Subsequent digestion of the protein or lipid regions of the membrane can be used to determine if proteins or lipids alone contribute to this effect.

Medical application of ESR measurement of red blood cell internal viscosity

Experimental results described above have shown that the internal viscosity of the red blood cell is sensitive to osmotic pressure, pH, and temperature. These conditions are variable in patients undergoing stress of certain types. At an ONR-sponsored meeting in Houston, Texas this year, T.I. Malinin impressed upon the conference the importance of being able to determine whether patients were or were not undergoing hemorrhagic shock. NMR studies of muscle biopsies of rabbits subjected to hemorrhagic shock did not give conclusive evidence that hemorrhagic shock would be recoginzed by this method.

Similar studies performed by electron spin resonance may be a valuable addition to this study. The red blood cell is an excellent biopsy specimen because it can be obtained easily and without damage. The sensitivity of the red blood cell to environmental changes suggests that is could be used in the study of hemorrhagic shock. The efficacy of the ESR method could be easily tested using the rabbit model system described by Dr. Malinin. Further sutdies may be warrented in this direction.

REFERENCES

1. Keith, A., Bulfield, G., and Snipes, W. (1970) Biophysical J. 10, 618-629.

2. Morse, Philip D., II. (1977) Biochem. Biophys. Res. Comm. 77, 1486-1491.

3. Morse, P. D., II, Ruhlig, M., Snipes, W., and Keith, A.D. (1975) Arch. Biochem. Biophys. 168, 40-56.

4. Steck, T.L., and Kant, J.A. (1975) in Methods in Enzymology, Volume 31, S. Fleischer and L. Packer, eds., Academic Press, New York, pps 172-180.

PUBLICATIONS DURING THE REPORTING PERIOD

1) Morse, Philip D., II (1977) Biochem. Biophys. Res. Comm. 77, 1486-1491.

2) Morse, Philip D., II (1978) Chem. Phys. Lipids, accepted for publication

MANUSCRIPTS IN PROGRESS

1) Morse, Philip D., II, Buttrey, D., Pote, K., and Lusczakoski, D. "Synthesis and purfication of hydrocarbon spin labels".

2) Morse, Philip D., II, Kavieff, R., and Lusczakoski, D. "Properties of hydrocarbon spin labels in dipalmitoyl lecithin multilayers".

3) Berg, S., Lusczakoski, D., and Morse, P.D., II. "Internal viscosity of chloroplasts".

4) Simpson, D., Lusczakoski, D., Rotole, G., Palazzo, R., and Morse, Philip D., II. "A new method for preparation of sealed hemoglobin-free red cell ghosts".

5) Morse, Philip D., II, and Simpson, D. "The internal viscosity of red blood cells and hemoglobin-free resealed ghosts"

FIGURE LEGENDS

Figure 1A. Internal viscosity of red blood cells (●) and ghosts (○) as a function of pH. The method is described in the body of this report. All points were taken in triplicate. The point represents the mean of the three measurements and the error bars represent the standard deviation of the mean. If no error bars are present, the error was less than the representative size of the point.

Figure 1B. Rotational correlation time of 2N14 (▲) and 7N14 (△) in the membrane of the red blood cell as a function of pH. The method is described in the body of this report. Each point represents a single experiment.

Figure 2A. Internal viscosity of red blood cells (●) and ghosts (○) as a function of osmotic strength. Remaining data as in Figure 1A.

Figure 2B. Rotational correlation time of 2N14 (▲) and 7N14 (△) in the membrane of the red blood cell as a function of osmotic strength. Remaining data as in Figure 1B.

Figure 3A. Internal viscosity of red blood cells (●) and ghosts (○) as a function of temperature. Remaining data as in Figure 1A.

Figure 3B. Rotational correlation time of 2N14 (▲) and 7N14 (▲) in the membrane of the red blood cell as a function of temperature. Remaining data as in Figure 1B.

Figure 4. Internal viscosity of chloroplasts as a function of temperature. Each point is the average of 3 to 5 trials.

FIGURE 1

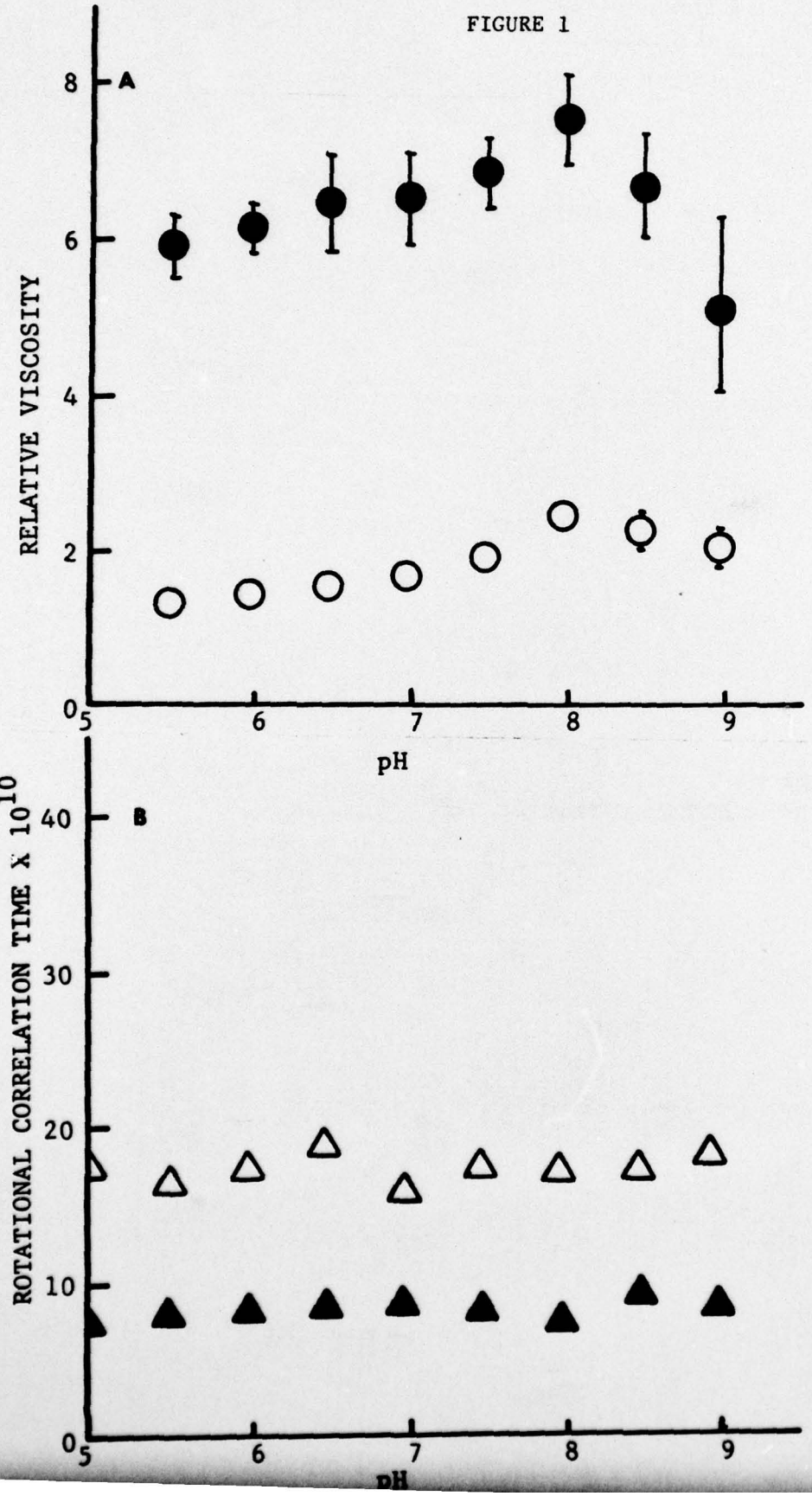

A
RELATIVE VISCOSITY
8
6
4
2
0
5
6
7
8
9
pH
B
ROTATIONAL CORRELATION TIME X 10^{10}
40
30
20
10
0
5
6
7
8
9
pH

FIGURE 2

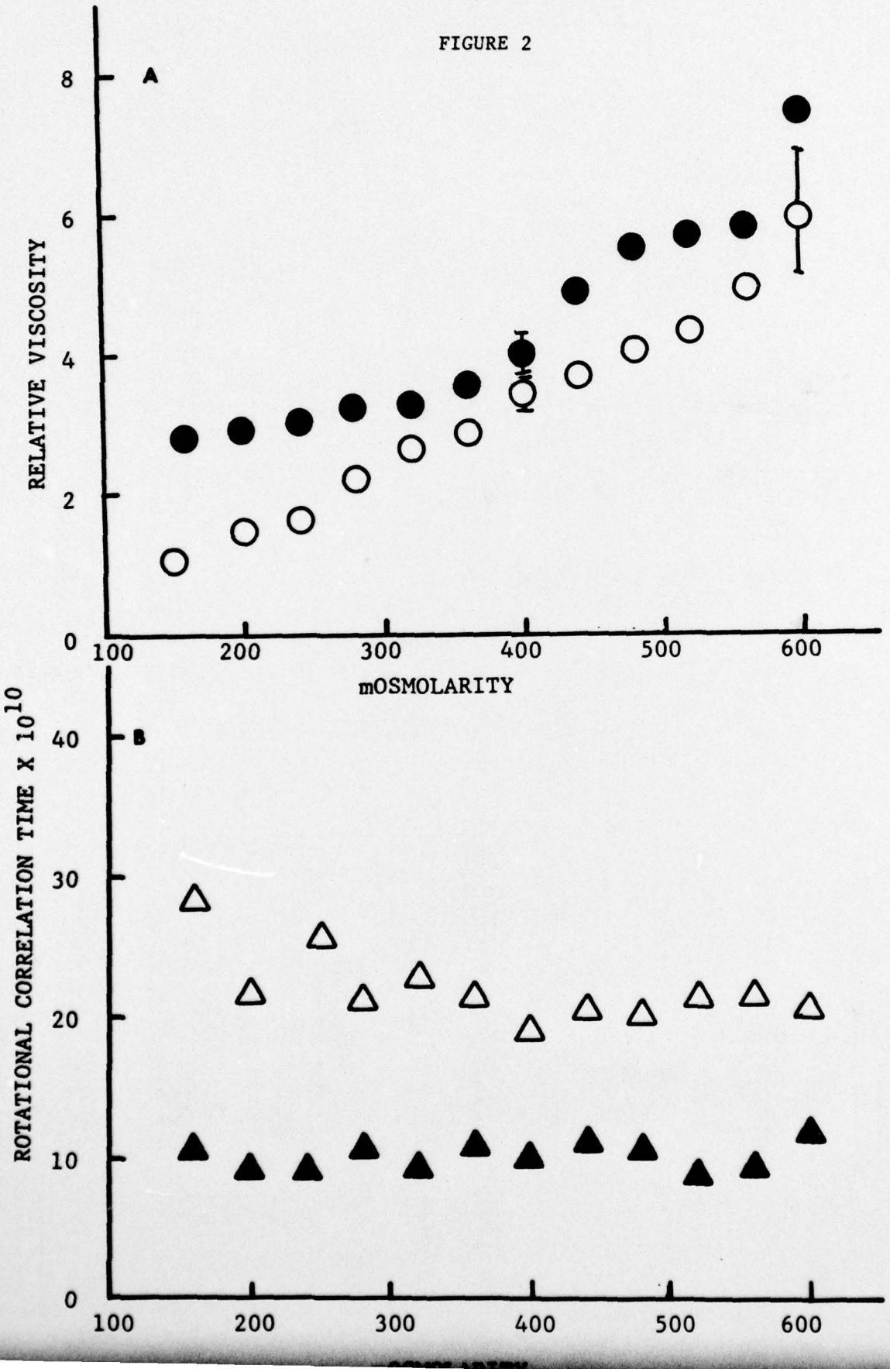
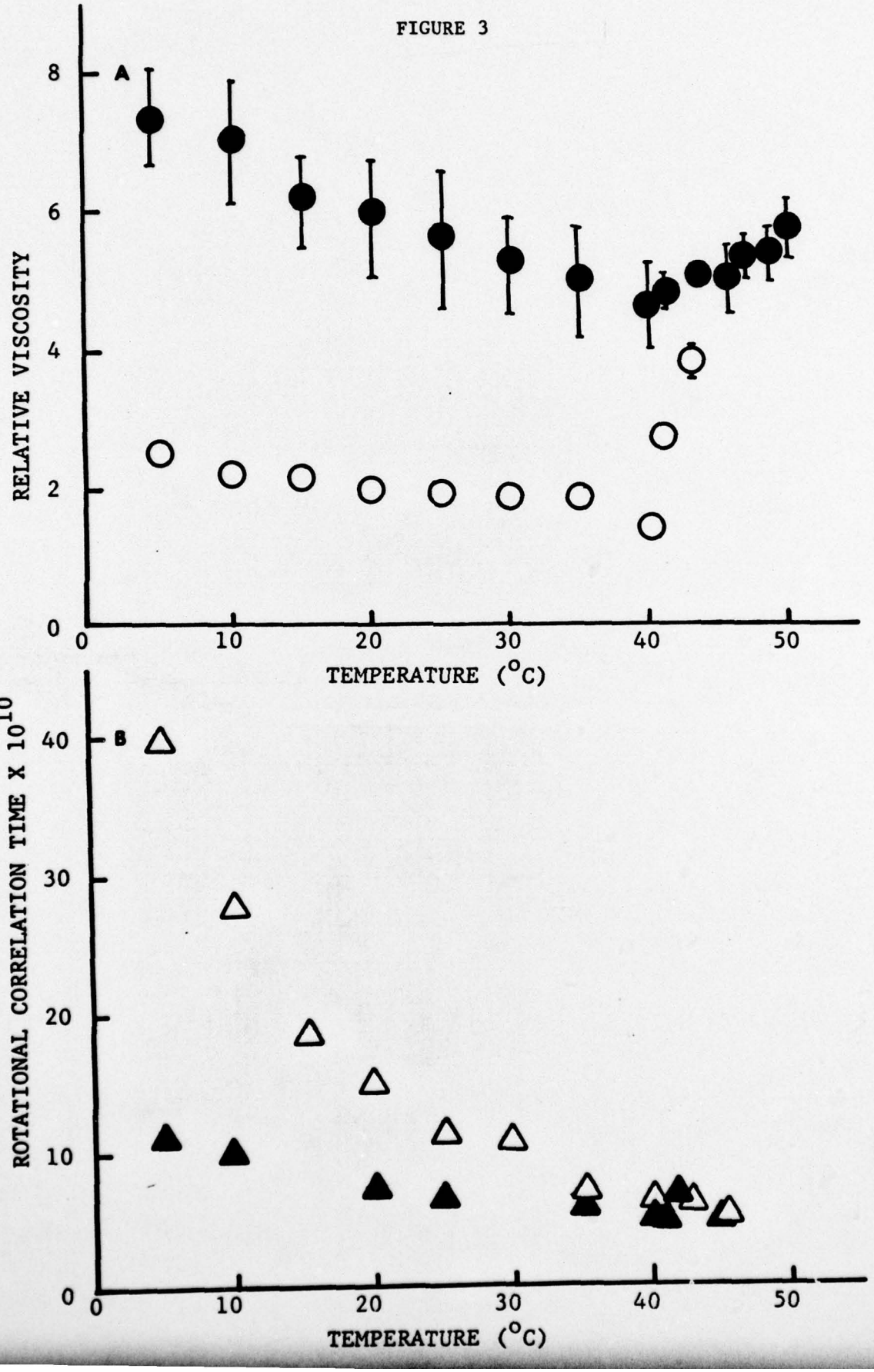

A
RELATIVE VISCOSITY
0
2
4
6
8
100
200
300
400
500
600
mOSMOLARITY
B
ROTATIONAL CORRELATION TIME X 10^{10}
0
10
20
30
40
100
200
300
400
500
600

FIGURE 3


A
RELATIVE VISCOSITY
0
2
4
6
8
0
10
20
30
40
50
TEMPERATURE (°C)
B
ROTATIONAL CORRELATION TIME X 10^{10}
0
10
20
30
40
0
10
20
30
40
50
TEMPERATURE (°C)

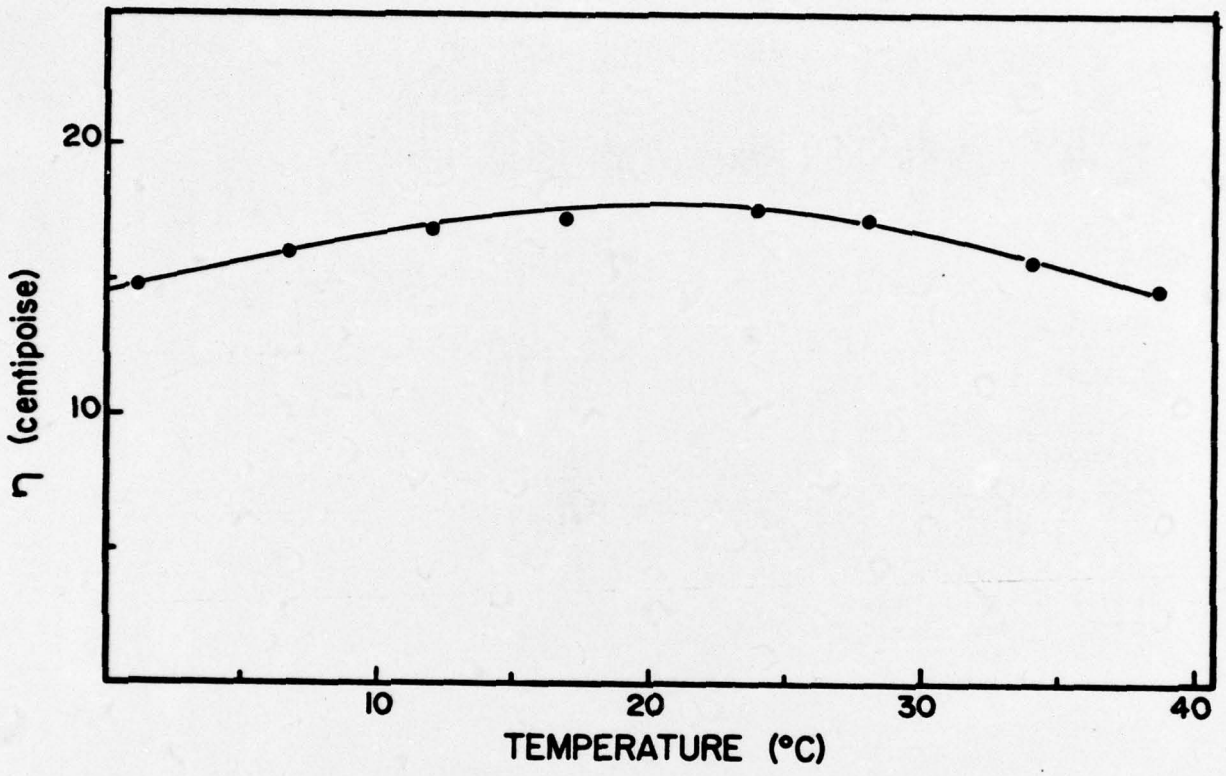


FIGURE 4

USE OF THE SPIN LABEL TEMPAMINE FOR MEASURING THE INTERNAL VISCOSITY OF RED BLOOD CELLS

Philip D. Morse, II

Department of Biology, Wayne State University
Detroit, Michigan 48202

Summary: A new spin label-spin broadening system to monitor the intracellular viscosity of red blood cells in isotonic medium at biological pH is described. The spin label 2,2,6,6-tetramethyl piperidine-N-oxyl-4-amine (TEMPAMINE) and the spin broadening agent $K_3Fe(CN)_6$ are used for this study. $K_3Fe(CN)_6$ does not penetrate red blood cells and at 80 mM completely broadens external aqueous components of the TEMPAMINE signal. Intracellular signal arising from TEMPAMINE is proportional to red cell concentration (hematocrit). TEMPAMINE samples the internal aqueous environment of red blood cells and in conjunction with $K_3Fe(CN)_6$ it appears to be very useful for measuring changes which occur in intracellular aqueous spaces.

Considerable interest has recently developed in the use of electron spin resonance spectroscopy for measuring intracellular viscosity and volume (1 - 4). The spin labels commonly used in these studies have been TEMPONE (2,2,6,6-tetramethyl piperidine-N-oxyl-4-one) and PCA (2,2,5,5-tetramethyl pyrrolidine-N-oxyl-3-carboxylic acid). Nickel chloride has been used to broaden away the spin label signals arising from the external aqueous region. However, three major difficulties have been persistently associated with these nickel/spin label systems: 1) Nickel chloride alone is about pH 3.5 and this low pH could cause membrane changes leading to artifactual data; 2) Nickel can be sucessfully brought to pH 7 by chelating it with Tris (tris-[hydroxymethyl] aminomethane) (5), but other buffers, such as phosphate, Tricine (N-tris-[hydroxymethyl] methyl glycine), and TES (N-tris-[hydroxymethyl] methyl-2-aminoethane sulfonic acid), cause nickel to precipitate and the osmotic strength of this precipitated complex is hard to

control; 3) There is substantial partitioning of TEMPONE into the hydrophobic portions of membranes which can obscure the signals arising from the internal aqueous regions of cells. PCA overcomes the third difficulty, but also requires nickel in some form as the broadening agent of choice.

I wish to report here a unique new spin label system which is not troubled by the difficulties described above. The positively charged spin label TEMPAMINE (2,2,6,6-tetremethyl-piperidine-N-oxyl-4-amine) does not partition significantly into membranes at biological pH and its signal can be broadened by potassium ferricyanide which is very soluble in this pH range. I will show that ferricyanide does not cross the membranes of red blood cells and that the remaining TEMPAMINE signal arises from an intracellular aqueous environment.

Materials and Methods

TEMPAMINE was synthesized according to Rosantsev (5). It can also be purchased in relatively pure form from Aldrich Chemical, St. Louis. $K_3Fe(CN)_6$ (Mallinckrodt) was AR grade. Red blood cells were obtained as ACD blood from the Detroit Red Cross and used well before the expiration date. Red cells were washed 3 times in phosphate buffered saline (PBS, 0.15M NaCl, 5mM sodium phosphate buffer, pH 7.5) and used immediately. ESR spectra were measured on a Varian E 109E spectrometer with temperature control. Hemoglobin spectra were measured at visable wavelengths with a GCA-McPherson spectrophotometer or a Cary/Varian 118C. Rotational correlation time (5) was calculated by the equation:

$$\tau_c = kWo\left\{\left[\frac{h_o}{h_{-1}}\right]^{1/2} - 1\right\}$$

where W_o = midfield line width, h_o = midfield line height and h_1 = high field line height. The constant k takes into account the microwave frequency at which the sample is irradiated and the magnetic anisotropy of the nitroxide moiety. Absolute measurements of τ_c are dependent on the minimum linewidth from proton interaction with the nitroxide and on the presence of other functional groups on the label. In this paper I use τ_c to compare relative changes in spin label motion and the values obtained should not be construed as representing absolute rotational correlation times.

RESULTS

Figure 1 shows the effect of ferricyanide concentration on the signal height of 10 mM TEMPAMINE in PBS alone (no cells). Signal height is reduced to nearly zero (complete broadening) by 80mM $K_3Fe(CN)_6$. At this concentration $K_3Fe(CN)_6$ is isosmolar with RBCs. The rotational correlation time of TEMPAMINE was not altered by $K_3Fe(CN)_6$.

In order to determine if ferricyanide itself contributes to the spin label spectrum, a broad scan (1000 gauss) was taken comparing 10 mM TEMPAMINE and 80 mM $K_3Fe(CN)_6$ (Figure 2A). The ferricyanide seems to produce a downward deflection around the TEMPAMINE signal but this is clearly observed only when the ferricyanide signal is considerably amplified over the TEMPAMINE signal. In Figure 2B, a more conventional magnetic field sweep is used (40 gauss) and the ferricyanide is again compared to 10mM TEMPAMINE. The increase in signal amplification of ferricyanide over TEMPAMINE in Figure 2A is 31,250x; in Figure 2B, 1000x. Thus, ferricyanide does not measurably interfere with any remaining TEMPAMINE signal.

A spin label-broadening agent system can be used for measuring intracellular viscosity only if the broadening agent remains outside the cell or membrane system under study. This was tested with the RBC's by exposing them to 80mM ferricyanide overnight at room temperature (18°C). If ferricyanide penetrated into the cells, the intracellular hemoglobin would be rapidly converted to methemoglobin and the spectral features of hemoglobin between 500 and 600 nm would be lost. Figure 3 compares spectra of hemoglobin obtained from red blood cells before and after 24 hours of exposure at room temperature to 80mM K_3FeCN_6. A slight methemoglobin peak is observed at 630 nm after 24 hours, but this represents an extremely small portion of the total hemoglobin. Although the cells were carefully washed in PBS after ferricyanide treatment, a small amount of ferricyanide may have been present during lysis of the cells. The data in Figure 3 represents an upper limit on the penetration of ferricyanide into intact red blood cells.

It is important to know that TEMPAMINE in the presence of $K_3Fe(CN)_6$ is

in fact monitoring the aqueous intracellular region and not the membrane. Because TEMPAMINE is charged at pH 7, it would not be expected to reside to any significant degree in a hydrophobic region. The hyperfine coupling (A_n) of TEMPAMINE is sensitive to solvent polarity and has a value of 15.75 gauss in water and 14.30 gauss in hexane. When the hyperfine coupling of TEMPAMINE in red blood cells is measured, it is 15.50 gauss which indicates that TEMPAMINE is sampling an aqueous environment within the cell. In addition, the dependence of the amplitude of the TEMPAMINE signal on cell concentration (hematocrit) was studied (Figure 4). It is obvious that TEMPAMINE signals increase proportionally as hematocrit increases. A slight bend in the curve is noticed at 50% hematocrit and may be due to a change in red blood cell packing at higher packing densities. The signal observed from 10mM TEMPAMINE alone (extrapolated to 100% hematocrit) is larger than expected, but falls back to its expected value when extracellular space and membrane volume are subtracted. The τ_c values for the red blood cells at several different hematocrits were $1.84 \pm .03 \times 10^{-10}$ sec. whereas the τ_c of bulk water was 4.62×10^{-11} sec. This shows that the average red blood cell interior hinders spin label rotation by a factor of 3.98 ± 0.07 relative to bulk water.

CONCLUSIONS

It is interesting to note that the measured internal viscosity of red blood cells is about 4 x that of water alone. This is surprisingly low when the concentration of hemoglobin inside the red cell is considered (3mM or 30%). Perhaps micro-domains exist in which the spin label can tumble rapidly. It is important to realize that viscosity as measured by spin labels is indeed a microviscosity; the actual translation diffusion of spin label in water is no more than 100 Å during the life time of the excited state (10^{-8} sec., ref. 2) and this distance decreases with the square root of the viscosity. This is in contrast to NMR measurements of proton diffusion in cells which takes place

over milliseconds with corresponding diffusion path lengths for water in the range of microns.

The spin label system, TEMPAMINE-ferricyanide, described in this paper offers several advantages over other spin label systems. 1) Ferricyanide can be easily brought to pH 7 with a number of different buffer systems and is therefore more compatible with biological systems. 2) Ferricyanide effectively broadens TEMPAMINE signals at isosmotic concentrations (80mM). 3) TEMPAMINE is charged and thus does not partition to any measurable degree into the membrane. It is also important to note that the remaining TEMPAMINE signal is essentially proportional to RBC hematocrit up to 100% (calculated), so that broadening of the signal by oxygen bound to hemoglobin does not measurably reduce the spin label signal.

TEMPAMINE with ferricyanide seems to be a useful spin label system for measuring intracellular or intravesicular viscosity. Preliminary experiments have also monitored red blood cell ghost resealing by a decrease of spin label signal as ferricyanide penetrates into the remaining unsealed ghosts. This spin label system should be very useful for the study of the properties of the intracellular aqueous compartments of biological systems.

This work was supported by Office of Naval Research grant N00014-76-C-1167. The author is a recepient of a Wayne State Faculty Research Award for 1976-1977.

References

1. Keith, A.D. and Snipes, W. (1974) Science 183, 666-668.
2. Morse, P.D. II, Ruhlig, M. Snipes, W. and Keith, A.D. (1975) Arch. Biochem. Biophys. 168, 40-56.
3. Henry, S., Keith, A.D., and Snipes, W. (1975) Biophys. J. 16, 641-654.
4. Hammerstedt, R.H., Amann, R.P., Rucinsky, T., Morse, P.D. II, Lepock, J.S., Snipes, W., and Keith, A.D. (1976) Biol. Reprod. 14, 381-397.
5. Keith, A., Snipes, W., and Chapman, D. (1977) Biochemistry 16, 634-641.
6. Rozantsev, E.G. (1970) Free Nitroxide Radicals. Plenum Press, New York.
7. Keith, A., Bulfield, G., and Snipes, W. (1970) Biophys. J. 10, 618-629.

Figure Legends

Figure 1. TEMPAMINE signal height remaining as a function of ferricyanide concentration. TEMPAMINE was dissolved to a final concentration of 10 mM in 5mM phosphate buffer, pH 7.5 and increasing concentrations of $K_3Fe(CN)_6$ were added. At 80mM $K_3Fe(CN)_6$, virtually all of the TEMPAMINE signal is abolished.

Figure 2. Overlapping ESR spectra of $K_3Fe(CN)_6$ and TEMPAMINE. Each figure is composed of two separate scans. Figure 2A shows the ESR signal 80mM $K_3Fe(CN)_6$. TEMPAMINE at 10 mM is used to show the relative scale. The gain of the $K_3Fe(CN)_6$ over that of the TEMPAMINE is 31,250. Figure 2B shows a similar comparison. The gain of the $K_3Fe(CN)_6$ scan over that of the TEMPAMINE is 1000 in this case.

Figure 3. Visible wavelength scan of hemoglobin before and after 24 hour treatment with 80mM $K_3Fe(CN)_6$ at 18°C. A slight methemoglobin peak is seen at 630 nm after $K_3Fe(CN)_6$ treatment. This indicates a very slight penetration of $K_3Fe(CN)_6$ into the aqueous interior of the red blood cell.

Figure 4. Intracellular TEMPAMINE signal as a function of red blood cell hematocrit. The amount of signal present was calculated by the area under the low field line, $A = W_1^2 h_1$.

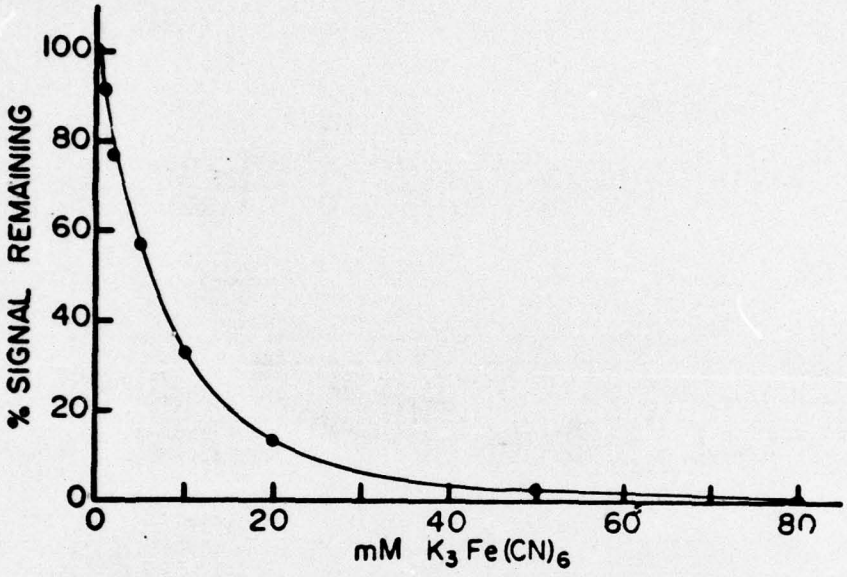


FIGURE 1

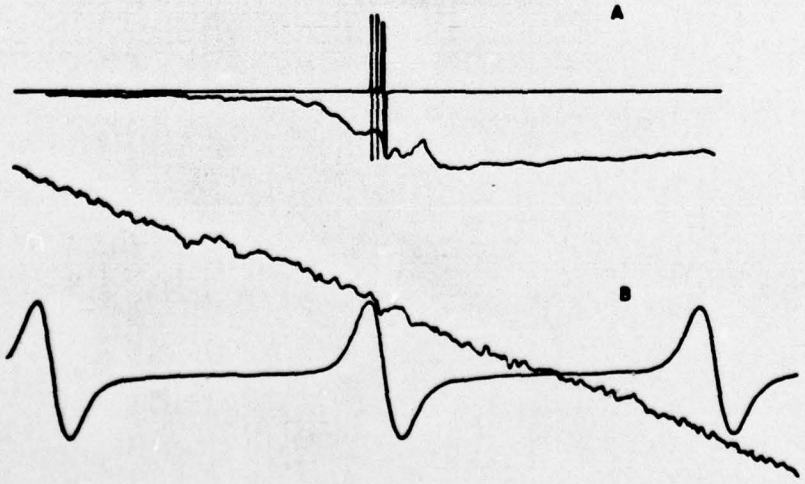


FIGURE 2

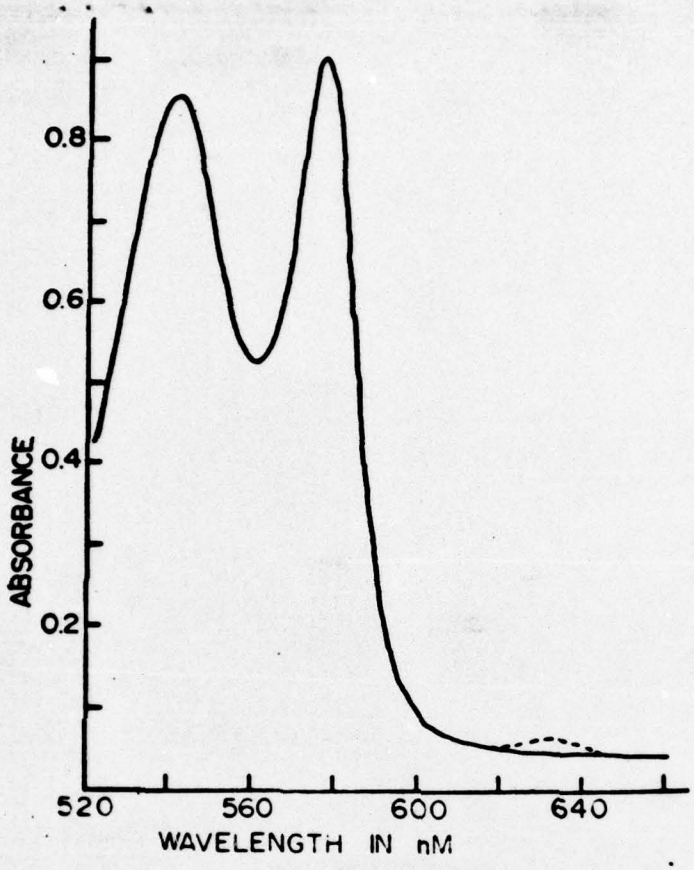


FIGURE 3

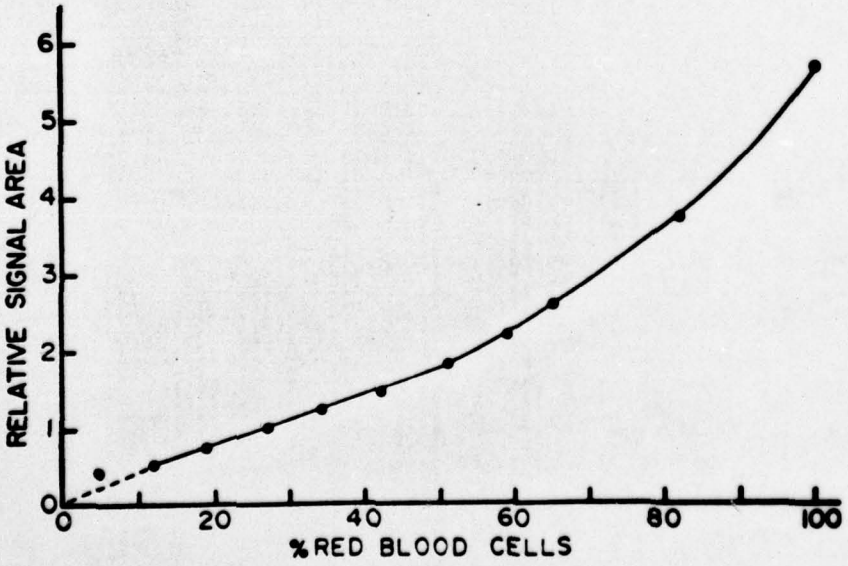


FIGURE 4