Biochimica et Biophysica Acta, 469 (1977) 301—310

BBA 77791

EVALUATION OF A NUCLEAR MAGNETIC RESONANCE TECHNIQUE FOR THE STUDY OF WATER EXCHANGE THROUGH ERYTHROCYTE MEMBRANES IN NORMAL AND PATHOLOGICAL SUBJECTS

VASILE V. MORARIU [a] and GHEORGHE BENGA [b]

[a] *Institute for Isotopic and Molecular Technology, 3400 Cluj-Napoca, P.B.Box 243 and* [b] *Department of Biochemistry, Faculty of Medicine, Medical and Pharmaceutical Institute, 3400 Cluj-Napoca (Romania)*

(Received January 10th, 1977)
(Revised manuscript received May 9th, 1977)

Summary

A critical evaluation has been performed of a NMR technique for the investigation of the water exchange time through erythrocyte membranes, based on doping with a paramagnetic salt. The conclusions are the following:

1. The preparation of the samples for the NMR measurement plays an essential role in obtaining precise and reproducible values of the water exchange time through erythrocyte membranes. A method for optimum mixing of the whole blood with the doping solution is described. The values of the relaxation times of erythrocyte water protons obtained by using this method were reproducible to an accuracy ranging between 2%—6%.

2. The theoretical basis of the method has been evaluated; the equations that should be employed for the calculation of the water exchange time are discussed.

3. The water exchange time through normal erythrocyte membranes is 6.0 ± 0.5 ms. This value is not influenced by the presence of the doping paramagnetic solution; determinations by atomic absorbtion spectroscopy showed no significant penetration of the Mn^{2+} into red blood cells.

4. The temperature dependence of the water exchange time through the erythrocyte membranes is characterised by values of the apparent activation energy ranging between 6 and 8 kcal/mol. These values are similar to the activation energies of the molecular rotational motion or of the diffusion of water in mono- or submonolayers of adsorbed water.

5. It has been found that the exchange time of water through erythrocyte membranes increased by 13—55% in patients with certain diseases such as Gaucher's disease, essential hyperlipemia, obstructive jaundice, chronic hepatitis, nephrotic syndrome.

6. Our findings suggest that the NMR doping method is potentially useful for

further studies of the physiology and pathology of erythrocyte water permeability.

Introduction

The physiology and pathology of erythrocyte water permeability has not been widely investigated because of the experimental difficulties involved. The method of Viera et al. [1] is based on radioactive tracers, requires large quantities of blood (about 100 ml) and involves a number of chemical procedures. In recent years there have been improvements in the tracer method [2]. However, it is considered that such measurements are experimentally impractical [3] and therefore of limited use for clinical applications.

A simple and rapid NMR method, using standard, commercially available equipment, has been proposed by Conlon and Outhred [4]. This method, requiring 1 ml or less of blood is, in principle, useful for extensive studies in normal and pathological conditions.

The principle of this method consists of doping the plasma with a paramagnetic solution ($MnCl_2$) and subsequent measurement of the spin-spin relaxation time (T_2) of the erythrocyte water proton. This relaxation time is correlated with the water exchange time through the membrane, and this in turn is inversely related to the water permeability of the erythrocyte membrane. To our knowledge no studies of the water permeability of erythrocytes in pathological conditions have been reported.

The aim of this work was to make a critical evaluation of the NMR method of Conlon and Outhred [4], studying the theoretical basis and the experimental factors which may influence the measurement of the exchange time, such as the preparation of samples and the effect of temperature. Examples are given to illustrate the clinical application of the method to the measurement of the water exchange time in patients with several diseases where alterations of the lipid composition of the erythrocyte membranes occurred.

Theory

The NMR doping method of measuring the water exchange time in erythrocytes will be outlined in this section. The spin-spin relaxation time of water inside the isolated erythrocytes (T_{2a}) is about 140 ms and is much longer than the time required for water to exchange through the membrane (the water exchange time, T_{ae}), which is about 10 ms. If the relaxation time in plasma (T_{2b}) is made much shorter than the exchange time (by adding a paramagnetic ion, such as Mn^{2+}), the observed relaxation time of the erythrocytes (T'_{2a}) would be dominated by the exchange process through the membrane.

The spin-spin relaxation time was evaluated from a logarithmic plot of the nuclear spin-echo as a function of the time interval 2τ where τ is the time interval between the radiofrequency pulses [5]. When the system is characterised by a single relaxation time, the plot is a straight line and the relaxation time is the reciprocal of the slope. For a system characterised by two relaxation times as for the blood doped with Mn^{2+}, the plot consists of two lines, as

shown in Fig. 1. The longer relaxation time, T'_{2a}, corresponds to the segment a, and the intercept with the vertical axis A, is proportional to the concentration of the water protons in phase a, i.e. in the red cells. The intercept of the segment (a + b) is (A + B), which is proportional to the concentration of the water protons in the whole sample. Subtraction of segment a from (a + b) gives the segment b whose $(\text{slope})^{-1}$ is the relaxation time T_{2b} of the doped plasma.

The observed relaxation time T'_{2a} of the erythrocytes will be shortened compared to T_{2a} because of the proton exchange between erythrocytes and plasma. The T'_{2a} value is given by the Eqn. 1 [6]:

$$T'^{-1}_{2a} = 1/2\{T^{-1}_{2a} + T^{-1}_{2b} + \tau_a^{-1} + \tau_b^{-1} - [(T^{-1}_{2a} - T^{-1}_{2b} + \tau_a^{-1} + \tau_b^{-1})^2 + 4/\tau_a\tau_b]^{1/2}\} \quad (1)$$

where τ_a and τ_b are the life times of the protons in the two phases. The rates of transfer or exchange between phases are, by definition, $1/\tau_a$ and $1/\tau_b$ and they are reciprocally related to the population of the phases according to the relationship: $P_a/\tau_a = P_b/\tau_b$.

Eqn. 1 is not convenient for practical purposes of τ_a calculation. However, simple approximations can be considered when particular conditions are fulfilled. A first approximation can be made when $T_{2a} >> T_{2b}$. This approximation is valid in the case of our present experiments as the fraction of water molecules in plasma is comparable to that in erythrocytes and typical values for T_{2a} and T_{2b} are 140 ms, [4] and 0.93 ms respectively (for the blood doped with a 40 mM $MnCl_2$ solution). Then Eqn. 1 becomes:

$$T'^{-1}_{2a} = T^{-1}_{2a}(\tau_a P_b + P_a T_{2b})/(\tau_a P_b + T_{2b}) + P_b/(P_b\tau_a + T_{2b}) \quad (2)$$

This is the same asEqn. 16 of ref. 7, rewritten in a suitable form such as to

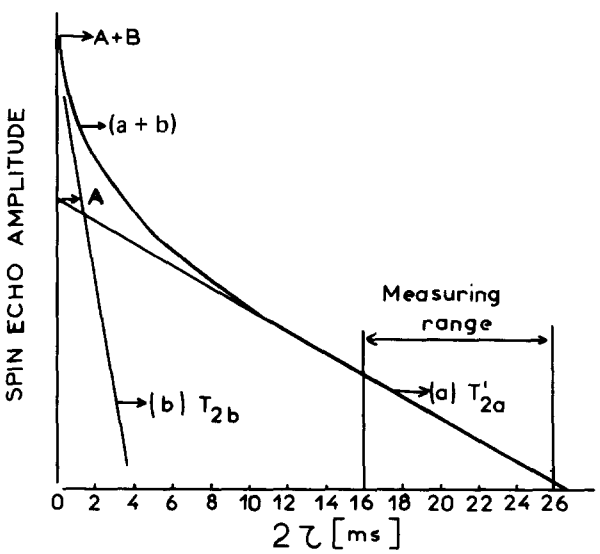

Fig. 1. The decay in time of the water proton spin-echo in a blood sample doped with $MnCl_2$. Component b results from rapid decay of spin-echo in plasma. Component a results from slower decay of spin-echo in red blood cells. The T'_{2a} value was estimated from the decay of the spin echo-amplitude between 6 and 24 ms.

include τ_a. Eqn. 2 can be further simplified if $T_{2b}/\tau_b < 0.3$ [7] to the form shown in Eqn. 3:

$$T'^{-1}_{2a} = T_{2a} + P_b/(P_b \tau_a + T_{2b}) \quad (3)$$

If $T_{2b}/\tau_b \ll 1$, we further obtain:

$$T'^{-1}_{2a} = \tau_a^{-1} + T_{2a}^{-1} \quad (4)$$

The life time τ_a of the protons in the erythrocytes, which is the quantity of interest to us, was called by Conlon and Outhred [4] the water exchange time, T_{ae}. We can easily calculate the value of T_{ae} from Eqns. 3 and 4. The value of P_b was estimated on the basis of the average hematocrit and hemoglobin content of the adult blood and it was found to be equal to 0.734. The average value of T'_{2a} at 37°C is 7.0 ms; T_{2a} = 140 ms and T_{2b} = 0.93 ms. The values of T_{ae} calculated using Eqns. 3 and 4 are 6.0 ms and 7.35 ms, respectively. Therefore Eqn. 4 leads to a positive error of 12%. On the other hand Eqn. 3 is a very good approximation of Eqn. 2, the difference being not greater than 1%. We conclude therefore that Eqn. 3 is best suited for the calculation of the water exchange time through erythrocyte membranes in our conditions of doping. The same equation may be employed for other doping solutions of lower manganese concentration. At higher concentrations, Eqn. 4 may also become applicable. Conlon and Outhred [4] have shown that the measured exchange time, T_{ae}, tends to the real exchange time, T_e, through the membrane at low packed cell volume (below 40%) and at sufficiently high concentration in plasma, such as to have T_{2b} smaller than 2 ms. This ensures that there is no influence from the back flux of water.

The exchange time T_e is related to the water permeability of erythrocytes (P) by the equation:

$$P = V/(A T_e) \quad (5)$$

where V is the cell colume and A is the surface area of the erythrocyte.

Experimental

Human blood was obtained by venipuncture, in heparinised tubes and used within 4 h. The sample was made by mixing 1 ml of blood and 0.5 ml of 20 or 40 mM $MnCl_2$ solution in 0.15 M NaCl. Analytical grade chemicals were used throughout.

The NMR measurements have been performed with a Bruker-Physik SXP spectrometer, at 90 MHz. The 90° pulse had a width of 5.5 μs. The spin-spin relaxation time has been measured by the 90° 2 τ 180° method [5], where τ is the time interval between the radiofrequency pulses. Phase sensitive detection has been used and the field was stabilised with the BSN 15 external field stabiliser. The temperature was controlled with the variable temperature unit of the spectrometer to ±0.5°C. The measurements were made within several minutes after the preparation of the sample.

The relation time T'_{2a} was measured from the spin-echo attenuation in the region $16 < 2\tau < 26$ ms, which ocrresponds to the slowest decaying fraction. The logarithmic plot of the nuclear spin-echo as a function of the time interval 2 is linear in this region. (Fig. 2)

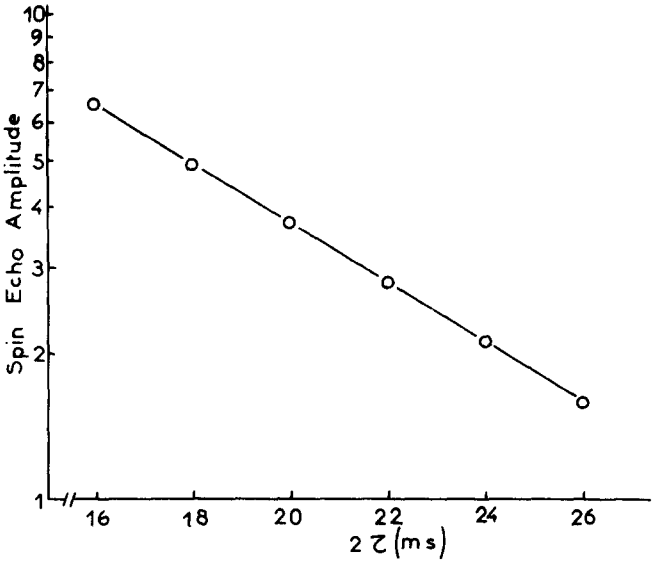

Fig. 2. Spin-echo attenuation versus time (2 τ) for a sample of normal blood doped with 40 mM $MnCl_2$ (0.5 ml of solution to 1 ml of whole blood), at 37°C. For details see the text. The value of T'_{2a} is 7.1 ms and T_{ae} = 6.1 ms.

The Mn^{2+} content of the red blood cells was determined by atomic absorption spectroscopy using a Perkin-Elmer 300 spectrophotometer. The counting of red blood cells was performed using a Celloscope 101 (Lars Ljunberg, Stockholm).

Results and Discussion

(1) Factors influencing the measurement of the water exchange time (T_{ae})

We realized that the results depended critically on the procedure adopted for mixing the blood with the doping solution. It was therefore necessary to find a criterion for optimum mixing. Conlon and Outhred [4] have shown that the population of water protons in the red cells, P_a defined as:

$$P_a = A/(A + B) \tag{6}$$

(see notation in Fig. 1) should be equal to the packed cell volume determined by centrifugation, which, under the experimental conditions used in ref. 4, was equal to 28%. (We made a similar dilution of the blood and obtained packed cell volume values around the same figure.)

When the manganese ion penetrates inside the red cell, the value of T_{2a} decreases and therefore the slope of segment a increases, thus resulting in a larger value of P_a, the population of water protons in the red cells. Therefore, the criterion for the optimum mixing is a P_a value close to the packed cell volume (allowing for the volume occupied by solutes inside the cells and in the plasma).

Table I lists the values of apparent exchange time, T_{ae}, of water through erythrocyte membrane obtained by using different methods of determination. It can be seen from this table that our results reproduced the results of Conlon

TABLE I

VALUES OF EXCHANGE TIME (T_{ae}) OF WATER THROUGH ERYTHROCYTE MEMBRANES OBTAINED BY USING DIFFERENT METHODS OF DETERMINATION (37°C)

In types A and B of experiments the samples were prepared by "gentle mixing" of the doping solution with the blood and calculated using the Eqn. 4, according to Conlon and Outhred [4]; in type C of experiments by the procedure for optimum mixing of the doping solution with the blood and the exchange time calculated using the Eqn. 3.

Method	No. of determinations	Concn. of additive in plasma (mM)	T_{ae} (ms)	References
Tracer	5	—	8.8 ± 1.0	1
NMR (90° 2τ 180°) type of experiment:				
A	2	9.5	9.66	4
A	5	9.5	9.4 ± 0.9	this work
B	8	24.0	8.2 ± 0.3	4
B	18	19.3	8.0 ± 0.7	this work
C	38	19.3	6.0 ± 0.5	this work

and Outhred [4] using the same method of sample preparation. The concentration of Mn^{2+} should be above 19 mM, such as to have T_{2b} smaller than 2 ms and consequently no influence from the back flux of water [4].

However, we found that a "gentle mixing" of the doping solution with the blood, as recommended by Conlon and Outhred [4], was not sufficient to ensure the optimum mixing. This resulted in relatively long relaxation times T'_{2a} (see type A and B of experiments in Table I). When the mixing was optimal, a decrease of the relaxation time occurred (see type C of experiment, in Table I).

In order to achieve the optimum mixing of the doping solution with the blood we prepared the samples in two ways:

1. The doping solution was added over the blood in the NMR tube. Then the sample tube was gently rotated in an almost horizontal position about 10—15 times, at temperatures between 20 and 30°C. The sample prepared in this was gave relatively reproducible results of T'_{2a}. The standard deviation of individual measurements was found to be 6% compared to the value of 11% obtained using the tracer method [1]. However, the T'_{2a} value obtained in this way was sometimes dependent on further shakings. In this case the shaking should be gently continued until a limiting value of T'_{2a} is obtained; which value can be used for the calculation of the exchange time, by Eqn. 3.

2. The blood was pipetted on the bottom of a 150 ml Pyrex flask. The doping solution was then spread over the blood and the flask was rotated several times. The sample was then transferred to the NMR tube. This ensured the optimum mixing so that the limiting value of T'_{2a} was obtained at once. Reproducible values of T'_{2a} to an accuracy ranging between ±2% and ±8% were obtained.

Conlon and Outhred [4] reported that prolonged shaking lead to the disappearance of the two component relaxation behaviour. We have not been able to detect such an effect for normal and pathological cases. The only case when a gentle mixing resulted in the disappearance of the longer relaxation compo-

nent (T'_{2a}) was for a sample of blood for transfusion, which had been stored for about three months at 4°C. This fact suggests that the penetration of the red cell by manganese ion is significantly increased only for blood stored a long time.

We have also checked for the penetration of the Mn^{2+} into the red blood cells during brief storing of the blood in the presence of the doped plasma at room temperature. The results for four samples of blood stored for 24 h showed no significant changes, except for one sample where T'_{2a} decreased by about 10%. Therefore the penetration of Mn^{2+} into the red cells seem to be negligible. Conlon and Outhred [4] have also reported that there was very little change of T'_{2a} over a period of a few hours.

In order to check for the penetration of Mn^{2+} into the red blood cells, samples of 1 ml blood mixed with 0.5 ml of a 40 mM $MnCl_2$ solution and blanks of 1 ml blood with 0.5 ml of a 0.15 M NaCl solution were prepared. The tubes were then centrifuged for 10 min at 3000 × *g* and the sediments were washed six times with a 0.15 M NaCl solution, each time followed by centrifugation. The manganese content of the erythrocytes was measured by atomic absorption spectroscopy. No significant differences were found between samples (23.4 ± 6.0 ng $Mn/10^9$ cells) and blanks (22.2 ± 4.0 ng $Mn/10^9$ cells). The results are the mean ± standard error of the mean for five determinations. It appears therefore that the penetration of Mn^{2+} into the red blood cells is negligible.

(2) The effect of temperature on the water exchange time through the erythrocyte membranes

The temperature dependence for a sample of normal blood is shown in Fig. 3 in the form of an Arrhenius plot. The activation energy of the water exchange time through the erythrocyte membranes calculated on the basis of this plot, was found to range between 6 and 8 kcal/mol for different samples.

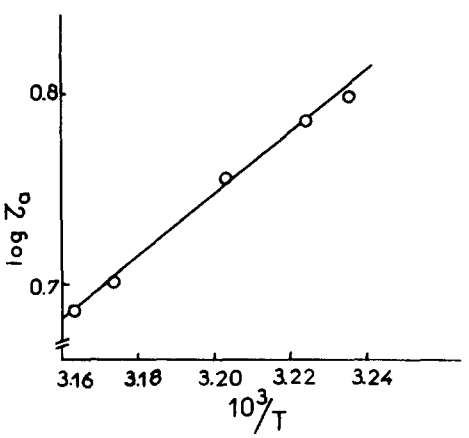

Fig. 3. The Arrhenius plot of the water exchange time through normal erythrocyte membranes. The preparation of the sample was made in optimal conditions as described in the text. Several minutes have been allowed for equilibration at each temperature. The activation energy calculated from the plot is 7.5 kcal/mol.

These values are intermediate between the activation energy of molecular rotation in ice and that of liquid water and are similar to the activation energy of water in the adsorbed state in other systems having statistical mono- or submonolayer coverages (Table II). This suggests that the mobility of the water molecules is changed as a result of their interaction with the erythrocyte membrane. At the same time it is possible to conclude that water in the erythrocyte membrane behaves like water in adsorbed monolayers.

The passive permeability of biological membranes has been explained in terms of aqueous pathways dispersed throughout a matrix of hydrophobic lipids. Some authors have called these pathways "pores" [12—14]. It has been estimated that the equivalent radius of the "pores" for the human erythrocytes is about 3.5 Å [13], or 4.3—5.4 Å [1]. As the diameter of the water molecule is about 3 Å, these "pores" may contain between one and three statistical monolayers of water in adsorbed state. However, the existance of "pores" in membranes has been questioned by some authors [15—17], on the basis of the results obtained on model phospholipids membranes [17,18], where there was no evidence for the existance of "pores". It has been shown that water car cross a lipid bilayer membrane set up between two saline solution even though the membrane is impervious to small ions such as Na^+ and K^+. The rate of movement of water across such protein-free membranes is similar to the rate of movement across plasma membranes [15]. Although the mechanism of water diffusion through biological membranes is not known, we believe that it is possible to accept that water crosses the lipid part of the membrane by diffusing through some aqueous pathways [17]. These pathways in the membrane probably represent dynamic structures and the term "pore" should be understood in this restricted sense. Our activation energy results suggest that the mobility of the water molecules during the passage across the membrane is similar to that of the molecules in the adsorbed state. Our values are in agreement to those obtained by Viera et al. [1] using the tracer method.

(3) Clinical applications

As the lipid bilayer of the biological membranes plays a significant role in the diffusion of water, it seems reasonable to investigate the water permeability of erythrocytes in patients with diseases which involve changes in the lipid

TABLE II

VALUES OF THE ACTIVATION ENERGY OF THE WATER MOLECULES MOTION IN DIFFERENT SYSTEMS

System	Activation energy (kcal/mol)	Molecular motion	Reference
Water	4.0	molecular rotation	8
Water	4.4	diffusion	9
Ice	13.3	molecular rotation	10
H_2O/SiO_2	7.2	molecular rotation	6
H_2O/starch	6.5	molecular rotation	8
H_2O/SiO_2	6.0	diffusion	11
H_2O/erythrocyte membrane	6.0—8.0	exchange time	this work *

* For experimental details see the text.

TABLE III

THE INCREASE OF THE WATER EXCHANGE TIME THROUGH ERYTHROCYTE MEMBRANES IN SELECTED PATHOLOGICAL SUBJECTS

The results are expressed as percent increase of the water exchange time in pathological subjects, the reference being the normal blood.

Disease	$\Delta T'_{ae}$ (%)	No. of determinations
Gaucher disease		
subject A	+42	4
subject B	+13	2
Essential hyperlipemia	+32	2
Obstructive jaundice	+56	2
Chronic hepatitis	+43	1
Nephrotic syndrome		
subject A	+44	1
subject B	+16	1

composition of the red blood cell membrane. Table III shows that in such cases the exchange time of water through the red blood cell membrane is longer than in normal subjects. The results are only illustrative and it is not the aim of the present work to discuss the specific causes which lead to a change of the water permeability for each disease. However, it should be noted that both patients with Gaucher's disease (a lysosomal storage disease) and liver diseases (chronic hepatitis, obstructive jaundice) have erythrocytes with an increased content of phospholipids and cholesterol. As the present increase in phospholipid is only half of the percent increase in cholesterol, this results in an increased cholesterol : phospholipid ratio [19]. It is possible that increased levels of cholesterol may result in a decrease in the water permeability of the erythrocyte membranes. The other values of Table III were obtained from patients with diseases associated with dyslipemia (essential hyperlipemia, nephrotic syndrome). It is possible that these cases also correspond to changes in the lipid composition of the erythrocyte membrane; it is known that the lipids of red blood cells equilibrate with the plasma lipids to varying levels, as shown by incubation in vitro [19].

Conclusion

The NMR method for the study of water exchange through erythrocyte membranes based on doping with a paramagnetic ion is simple, fast and economical of blood. Recently, an alternative NMR method based on a pulse field gradient technique, which does not require the paramagnetic doping, has been developed theoretically [20] and tested experimentally [2]. However, the pulse gradient technique requires a rather specialised equipment c.f. the doping method for which low cost pulse NMR spectrometers are commercially available. The methodological restraint related to sample mixing has been overcome in this paper. Therefore we consider that the NMR doping method may be useful for further studies of the physiology and pathology of erythrocyte water permeability. We have already applied this method for the investigation of human epilepsy [22].

Acknowledgements

The help of Drs. B. Böhm and I. Pop in the determination of Mn is gratefully acknowledged. The authors also thank to Drs. Cornelia L. Morariu and Ileana Benga for providing the blood samples.

References

1 Viera, F.L., Sha'afi, R. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 451–466
2 Redwood, R.W., Rall, E. and Perl, W. (1974) J. Gen. Physiol. 64, 706–715
3 Sha'afi, R.I. in Membrane Transport in Red Cells (Ellory, C.V., ed.), Academic Press Inc., London, in the press
4 Conlon, R. and Outhred, R. (1972) Biochim. Biophys. Acta 288, 354–361
5 Farrar, Th.C. and Becker, E.D. (1971) Pulse and Fourier Transform N.M.R., Academic Press, New York and London
6 Woessner, D.E. (1963) J. Chem. Phys. 39, 2783–2787
7 Hazlewood, C.F., Chang, D.C., Nichols, B.L. and Woessner, D.E. (1974) Biophys. J., 14, 583–605
8 Tait, M.J., Ablett, S. and Wood, F.W. (1972) J. Colloid Interface Sci. 41, 59–68
9 Wang, J.H. (1951) J. Am. Chem. Soc. 73, 510–514
10 Onsager, L. and Runnls, L.K. (1969) J. Chem. 50, 1089–1103
11 Morariu, V.V. and Mills, R. (1972) Z. Phys. Chem. Neue Folge 79, 1–9
12 Meryman, H.T. (1972) in Biomembranes, (Mason, L.C., ed.), Vol. 3, 341, Plenum Press, New York and London
13 Rich, G.T., Sha'afi, R., Barton, T.C. and Solomon, A.K. (1967) J. Gen. Physiol. 50, 2391–2406
14 Blum, R.M. and Forster, R.E. (1970) Biochim. Biophys. Acta 203, 410–423
15 Katznar, R. and Schiemmel, H. (1969) in Handbook of Neurochemistry (Lajtha, A. ed.), Vol. 2, pp. 11–22, Plenum Press, New York and London
16 Brodsky, W.A., Shamoo, A.E. and Schwartz, I.L. (1971) in Handbook of Neurochemistry (Lajtha, A, ed.), Vol. 5, pp. 652, Plenum Press, New York and London
17 Cass, A. and Finkelstein, A. (1967) J. Gen. Physiol. 50, 1765–1784
18 Hanai, T. and Haydon, D.A. (1966) J. Theoret. Biol. 11, 370–382
19 Cooper, R.A. (1970) Seminars in Hematology 7, 296–321
20 Chang, D.B., Cooper, R.L., Young, A.C., Martin, C.J. and Ancker-Johnson, B. (1975) J. Theoret. Biol. 50, 285–308
21 Andrasko, J. (1976) Biochim. Biophys. Acta 428, 304–311
22 Benga, Gh. and Morariu, V.V. (1977) Nature 265, 636–638