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Aan mijn ouders

STELLINGEN

1

Ofschoon in NaCl – oplossingen niet de oorspronkelijke osmotische resistentie van erythrocyten wordt bepaald, verdient deze methode voor diagnostische doeleinden toch de voorkeur.

J.V. Dacie en S.M. Lewis (1968)
Practical Haematology, p. 166
J&A. Churchill, Ltd., Londen.
Dit proefschrift (hoofdstuk 7).

2

De resultaten van de Gier et al. ondersteunen slechts tendele de hypothese dat polyhydroxy alkoholen de biologische membranen passeren als vrije, volledig gedehydrateerde molekulen.

J. de Gier, J.G. Mandersloot, J.V. Hupkes, R.N. McElhaney en W.P. van Beek (1971)
Biochim. Biophys. Acta, 233, 610

3

De verschillen in glykolytische aktiviteit van humane erythrocyten in niet–permeërende oplossingen van elektrolyten en niet-elektrolyten, kunnen veroorzaakt zijn door verandering in intracellulaire pH.

K.K. Tsuboi en K. Fukunaga (1970)
Biochim. Biophys. Acta 196, 215

4

De veronderstelling van Dijck et al., dat glucagon de door secretine gestimuleerde vocht– en elektrolytsekretie in de hondepankreas remt doormiddel van stimulering van het adenylcyclase, lijkt onwaarschijnlijk.

W.P. Dijck, J. Rudick, B. Hoexter en H.D. Janowitz (1969)
Gastroenterology, 56 , 531

5

Het door Nordøy en Rødset waargenomen verband tussen de vetzuursamenstelling van plaatjesfosfolipiden en de aktiviteit van plaatjesfactor 3 is, in tegenstelling tot hun konklusie, niet in overeenstemming met de resultaten van Rouser en Schloredt.

A. Nordøy en J.M. Rødset (1971).
Acta med. scand., 190, 27
G. Rouser en D. Schloredt (1958).
Biochim. Biophys. Acta, 28, 81

6

De weinig kritische eksperimentele benadering door Girsch en Rabinovitch van de bleking van rhodopsine in het donker onder invloed van ureum, wettigt niet hun konklusies

S.J. Girsch en B. Rabinovitch (1971)
Biochem.Biophys.Res. Comm., 44, 550.

7

De bewering dat de lymphocyten in de follikelkappen van de tonsilla palatina afkomstig zijn uit de germinatieve centra van de follikel en niet uit de circulerende lymphocyten populatie, is aanvechtbaar.

E. Koburg (1967)
In: Germinal centers in immune responses.
Eds. H. Cottier, N. Odartchenko, R. Chindler en C.C. Congdon, p. 176.
Springer Verlag, Berlijn.

8

Bij onderzoek van het nephrotisch syndroom wordt te weinig aandacht geschonken aan de plasmalipiden spiegel

G.M. Berlyne en N.P. Mallick (1969)
Lancet II, 398.

9

De verlaging van het smelttraject van de kristallieten in georiënteerd polyetheen optredend na gamma–bestraling, moet worden toegeschreven aan ketenbreuk in de amorfe gebieden van het polymeer.

W. Glenz en A. Peterlin (1971).
J. Polymer Sci., A 2, 9, 1243

J.M.C. WESSELS

11 februari, 1972

C O N T E N T S

GENERAL INTRODUCTION

The red blood cell (RBC) is surrounded by a membrane separating the cell contents from the surrounding fluid. Since the composition of the extracellular fluid, plasma, differs considerably from that of the cell contents, this barrier must possess an outspoken selectivety, or mechanisms must be present which facilitate the selective uptake or release of distinct substances.

The permeability behaviour of RBC membranes of various mammalian species, as observed in isotonic solutions of distinct permeating substances, differs considerably (e.g. Jacobs et al, 1950; Hunter et al, 1965; de Gier et al, 1966; Ospina and Hunter, 1966; Moore, 1968). The lipid composition of RBC membranes shows also considerable differences in phospholipid as well as in fatty-acid composition (Hanahan et al, 1960; de Gier and van Deenen, 1961; de Gier et al, 1966; Nelson, 1967; Rouser et al, 1968).

Although some authors have suggested (Parpart and Dziemian, 1940; Kögl et al, 1960; de Gier, 1960; de Gier and van Deenen, 1964; Stein, 1967) that a correlation should exist between the permeability of RBC's to simple nonelectrolytes and the lipid composition of their membranes, no convincing correlation has been found till now. Especially the results obtained with RBC's of dog and cat do not seem to agree with this hypothesis (de Gier et al, 1966). The discrepancies between RBC permeability and lipid composition may be due to either the absence of a simple correlation, (e.g. differences in conformation on the molecular level), or to inadequate permeability measurements.

The osmotic lysis method according to Jacobs et al (1950) is used frequently in comparative studies of the permeability of RBC's (de Gier et al, 1966; Moore, 1968; Szelényi and Hollán, 1968). The use of labeled compounds would at first sight seem to be more suitable, but since the permeability of some types of RBC's to simple nonelectrolytes is fairly high and a rapid separation of cells and surrounding fluid rather complicated though not impossible (Paganelli and Solomon, 1957; Mawe and Hempling, 1965), therefore this method is less applicable in comparative studies. The photometric determination of cell membrane permeability at constant volume (Tolberg and Macey, 1966)

and the minimum volume method (Sha'afi, 1970) are also less appropriate for comparative studies.

The original lysis method (Jacobs et al, 1950) has some demerits, e.g. no allowance is made for differences in the osmotic resistance of the RBC's from different origin, and therefore the results are not quite reliable. Therefore we modified this method in some ways in order to meet the objections as far as possible. The modified lysis method permits in our opinion a more thorough physico-chemical interpretation of the permeation process. The resulting permeability data for RBC membranes of different mammals will be compared with their lipid composition.

Chapter 1 offers a brief review of RBC membrane composition and structure, together with a short survey of RBC membrane permeability to simple nonelectrolytes. The modifications in the original osmotic lysis method are described in chapter 2. Experimental results are presented which support the validity of this method under certain conditions (Chapter 3). Under more physiological conditions, however, a more complex permeability behaviour of the RBC's was observed. These complications could be explained on the basis of an exchange of anions and the buffering action of hemoglobin (Chapter 4). The permeability to glycerol of RBC membranes of eight mammalian species was determined with the modified lysis method. Data are compared with the lipid composition of these membranes (Chapter 5). In view of the observed variability of the permeability characteristics from RBC's of individual rabbits, the permeability, lipid composition and some cell characteristics of rabbit RBC's were studied in more detail (Chapter 6). The discrepancies in osmotic resistance of pig RBC's in NaCl and sucrose solutions are analysed in Chapter 7. In the last part of this chapter data on the osmotic resistance of normal and some types of pathologically altered human RBC's are presented. This study was undertaken, since preceding experiments had shown that the osmotic resistance, determined with the traditional procedure, does not present the original osmotic resistance of the RBC's.

1. SOME STRUCTURAL AND FUNCTIONAL ASPECTS OF THE RED BLOOD CELL MEMBRANE

1.1. MEMBRANE COMPOSITION

1.1.1. Introduction

The RBC membrane is composed of 46-55% protein, 35-45% lipids and 10% carbohydrate (Dodge et al, 1963; van Deenen and de Gier, 1964; Maddy, 1966; Bakerman, 1967). The composition varies somewhat with the isolation procedure and the species. Since it was our aim to compare membrane permeability and lipid composition, we will review here some of the available data on lipid composition. More detailed data will be presented in Chapter 5 and 6. Thereafter the data concerning the membrane proteins will be summarized briefly.
No attempt is made to present a complete survey of the extensive literature, rather the most relevant articles are cited.

1.1.2. Red blood cell membrane lipids

The lipid distribution in RBC membranes of different mammalian species has been studied extensively. Several reviews on this subject have been published (van Deenen and de Gier, 1964; van Deenen, 1965; Rouser et al, 1968). Large variety of phospholipids, glycolipids and glycerides, besides cholesterol, is found in the RBC membrane. Considering the variability of the fatty acid content in each lipid class, one is confronted with the fact that a great number of different lipids in rather strict proportions may be present in this membrane type.
The concentration of total lipid in mg/ml packed cells is relatively constant for all species. Conversely, total lipid per RBC is highly variable among the species, reflecting a similarly wide range in the mean corpuscular volume of the different species RBC's (Nelson, 1967). The RBC membranes of different mammalian species exhibit a molar ratio of cholesterol to phospholipids close to one (de Gier and van Deenen, 1961; Nelson, 1967). Cholesterol proved to be the dominant component

of the neutral lipid fraction, averaged about 26% of the total lipid content and showed little species variation (de Gier and van Deenen, 1961; Nelson, 1967). Besides cholesterol, the neutral lipid fraction consists of small amounts of free fatty acids, mono-, di- and triglycerides and cholesterol esters. Glycolipids, e.g. cerebrosides, globosides and gangliosides, are also found in RBC membrane extracts (Yamakawa et al, 1962; Nelson, 1967).
The phospholipids comprise about 60% of the RBC membrane lipids (van Deenen and de Gier, 1964; Nelson, 1967) and consist mainly of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin (Dawson et al, 1960; de Gier and van Deenen, 1961; Nelson, 1967; Rouser et al, 1968; Broekhuyse, 1969). Though the data presented in these papers vary considerably, a common finding is that a decrease in the relative amount of phosphatidylcholine is accompanied by an increase in sphingomyelin content. In addition to considerable variations in phospholipid composition, the RBC membrane lipids also revealed large differences in fatty acid composition (de Gier et al, 1966; Dodge and Philips, 1967; Chaffee et al, 1968; Rouser et al, 1968; Nelson, 1969). The fatty acid composition changed with the diet (Mulder et al, 1963; de Gier and van Deenen, 1964a; Walker and Kummarow, 1963 and 1964), but not the phospholipid composition (de Gier and van Deenen, 1964a).

1.1.3. Red blood cell membrane proteins

Data available on the solubility of RBC membrane proteins are controversial (Zahler, 1969). In most cases, the solubility in water at neutral pH is extremely low (Richardson et al, 1964; Rosenberg and Guidotti, 1969). To the opinion of Kaplan and Criddle (1971) Rosenberg and Guidotti (1969) have accomplished the most successful resolution of red blood cell membrane proteins to date, by combining several methods along with modification of the conventional lipid extraction and detergent solubilization of membrane proteins. The procedure involves four steps: two aqueous extractions of the ghost , a lipid extraction step, and solubilization in sodium dodecyl sulfate followed by fractionation on Sephadex. Delipidated membrane proteins are readily soluble in acid or alkaline urea (Azen et al, 1965), detergents (Lenard, 1970) and also in organic solvents (Rega et al, 1967; Zahler and Wallach, 1967). Treatment of membrane proteins with 2-chloroethanol leads to complete solubilization, the solutions giving many bands in

disc electrophoresis (Zahler and Wallach, 1967). The molecular weights found by ultracentrifugation ranged from 10.000 to 50.000.
The delipidated proteins retained their ability to recombine with membrane lipids. The low solubility in water and the ready solubility in organic solvents of membrane proteins illustrate their predominantly hydrophobic character. Hydrophobic areas of the membrane proteins are believed to be a consequence of special secondary and tertiary structures, possibly α-helical structures (Wallach and Gordon, 1968). The amino acid composition of membrane proteins or fractions thereof, is not strikingly different as compared to that of water soluble proteins (e.g. Schneiderman and Junga, 1968). Delipidated proteins could be recovered in water soluble form after treatment of RBC membranes with n-butanol (Maddy, 1966). Differences in delipidated protein composition of RBC membranes of some mammals were observed upon disc electrophoresis (Zwaal and van Deenen, 1968; Lenard, 1970).

1.2. RED BLOOD CELL MEMBRANE STRUCTURE

Many models for the structure of cell membranes have been proposed in the course of the last decades due to the increasing knowledge of their composition. Though the different theories include the interactions between lipids and between lipids and other membrane components, mainly proteins, the models appear to be more or less in conflict with each other as we shall see. Excellent reviews have been published by Zahler (1969) and by Hendler (1971).
Gorter and Grendel introduced in 1925 the hypothesis that the essential structural element of red cell membranes consists of a lipid bilayer. Results corroborating this hypothesis were obtained by Bar et al (1966). A predominant role in the model of Danielli and Davson (1935) is played by electrostatic interactions between the proteins and the polar head groups of the phospholipids, as well as by lipid-lipid interactions between the lipid hydrocarbon chains as a result of van der Waals interaction forces. The lipid bilayer is covered on both sides by proteins. In a more recent model it is perforated by protein coated pores (Stein and Danielli, 1956). X-ray studies on myelin membranes (Schmitt et al, 1965) as well as electronmicroscopical observations of Robertson (1960) leading to the unit-membrane concept, appeared to corroborate the "Danielli-model". This concept may be summarized in the following three essential points (Zahler, 1969): 1) The unit-

membrane structure represents a principle of membrane architecture basically valid for all biological membranes. 2) The main structural element of the membrane consists of a continuous bilayer of phospholipids with somewhat varying amounts of cholesterol, other neutral lipids and glycolipids, whereby the polar or hydrophilic parts of the lipids are arranged on both surfaces of the bilayer. 3) The membrane proteins on both sides of the lipid bilayer are arranged in an extended conformation (β-structure) and held in position by ionic bonds.
In all of these variants, the protein molecules are placed on the surface of the lipid bilayer, in either a globular (Danielli and Davson, 1935) or extended form (Robertson, 1960). This concept was generally accepted for several years.
More recent information, gained especially from studies on membrane proteins, seems to be in marked conflict with the essential postulates of the unit-membrane theory and in particular with the third point. Wallach and Zahler (1966) concluded from optical rotatory dispersion (ORD) and circular dichroism (CD) studies that hydrophobic interaction between membrane lipids and proteins takes place. Moreover, these spectra seemed to be characteristic of α-helical peptides. Nuclear magnetic resonance (NMR) studies demonstrated that hydrophobic bonds exist between lipids and proteins besides ionic bonds (Chapman et al, 1968). The electrical conductivity of artificial lipid bilayers is considerably less than that observed in cell membranes, indicating the existence in cell membranes of either hydrophilic pores, or a particular arrangement of the lipids due to the presence of membrane proteins (Haydon, 1968). Recombination experiments with isolated RBC membrane proteins and lipids suggest that lipids play an important role in the determination of the secondary and probably the tertiary structure of the membrane proteins (Zahler, 1969).
On the basis of these informations gained in studies of membrane proteins, membrane models involving subunits consisting of proteins and lipids were proposed (Benson, 1968; Zahler, 1969). In the "Benson-model" a predominant role is played by the hydrophobic linkage of the paraffinic chains of the lipids to certain sites on the protein moiety. The polar head groups of the phospholipids are not covered by protein, but the possibility of electrostatic interactions between lipids and proteins cannot be excluded. The fatty acid chains of the lipids may interact with each other in protein poor regions as well, resulting in a lipid bilayer. In the "Zahler-model" the subunits consist of cylindrical particles with two hydrophilic ends and a hydrophobic center containing a belt of lipids. Membrane assembly

would proceed automatically, even if the internal structure of the subunits would differ considerably. Lenard and Singer (1966), however, attributed the distortions in the ORD and CD spectra to interactions of α-helices, whereas Ji and Urry (1969) showed that light scattering from the sample itself also contributes to the distortions. The complete absence of proteins in β-conformation was moreover not proven. Furthermore, Hendler (1971) states that no evidence is presented against the existence of a protein coated bilayer with little or no β-conformation. Other recent observations support the bilayer concept. Electron spin resonance (ESR) studies revealed that nitroxide-labeled lipoidal molecules oriented themselves with their long axis perpendicular to the plane of the membrane (Hubbell and McConnell, 1969). Freeze-cleavage techniques of preparing specimens for electron microscopy have been interpreted to produce a split of the membrane along a natural plane of cleavage provided by the hydrophobic space between apolar tails in the center of a bimolecular leaflet (Branton, 1969). Moreover, the diffraction data of wide-angle and low-angle X-ray diffraction studies indicate the existence of a lamellar organization (Finean, 1969). Calorimetric studies have shown that the thermal phase transition which occurs in lipids extracted from membranes also occurs in certain intact membranes, which supports the view that the environment of most of the lipid in these membranes is the same as that in phospholipid bilayers dispersed in an aqueous medium (Steim et al, 1969). Since extraction of nearly all (more than 90%) lipid from membranes does not destroy the membranous appearance of the material, as judged electron microscopically (Cunningham et al, 1967), this clearly indicates that the protein does not simply lie on the surface of the lipid bilayer (Vanderkooi and Green, 1971). While the original unit membrane concept posed only electrostatic interactions between the polar lipid heads and the proteins, more recent versions of the unit membrane have included the provision that the nonpolar amino acid side chains penetrate into the nonaqueous interior of the lipid bilayer, implying a hydrophobic type of interaction between lipids and protein (Chapman et al, 1968). The observation that loosely and more strongly bound lipids can be distinguished when RBC membranes are extracted with organic solvents, led Parpart and Ballentine (1952) to a mosaic structure model consisting of a continuous network of protein in which islands of lipid molecules are interspersed. The loosely bound lipids might be held within the membrane only by hydrophobic bonds, whereas the more strongly bound lipids might dis-

partition coefficients. When the product of the permeability coefficient P and the square root of the molecular weight ($M^{\frac{1}{2}}$) is plotted against the partition coefficient, an even better correlation is obtained (Stein, 1967). A complication, however, is the high permeability of human RBC's to glycerol, which does not appear to obey the relationship with the oil:water partition coefficient. Stein (1962) concluded from this and other arguments that dimerization of glycerol molecules must take place, resulting in a decrease of polarity. The same hypothesis was used to explain the high permeability of human RBC's to glucose. The experimental data appeared to be insufficient precise, whereas also theoretical objections have been made (LeFevre, 1966; Miller, 1966).

In general, Stein (1967) concluded that hydrated molecules of simple nonelectrolytes entering the lipid barrier are dehydrated wholly or in part. For each hydrogen bond that has to be broken, the transfer rate is lowered 6 to 10 fold. Each $-CH_2-$ group in the permeant increases the transfer rate about twofold. It is quite likely that water and certain other small molecules could slip through transient holes in the membrane formed by spaces between the lipid chains since the fatty acids appear to be in motion (Chapman and Salsbury, 1966). Johnson and Bangham (1969) considered diffusion through a membrane as a series of successive jumps of the permeating molecules from one position with a potential free energy minimum to another. The permeating molecules must diffuse through the water phase, then penetrate into the interphase, followed by a diffusion through the hydrocarbon layer and penetration of the opposite interface. The suggestions of Stein (1967) and Johnson and Bangham (1969) are supported by recent observations of de Gier et al (1971). On the basis of the high activation energy for the penetration of some nonelectrolytes into liposomes and RBC's of some mammalian species, it seems likely that the penetration into the hydrophobic barrier is rate limiting. The independence of the activation energy of the composition of the liposomes, supports the view that for penetration into the hydrocarbon layer a similar breaking of hydrogen bonds with water molecules is necessary. The enthalpy of dehydration of glycerol will be equivalent to the energy required for the breaking of four hydrogen bonds, which corresponds to 20 kcal. This figure is close to the activation energy for penetration of liposomes. The few data on RBC membranes do not permit the conclusion that this hypotesis holds generally also for RBC membranes.

1.3.4. Facilitated diffusion

The permeability of human RBC's to glycerol appeared to be much higher than could be expected from the oil: water partition coefficient for glycerol (Stein, 1967). Furthermore it was observed many years ago that the permeability of human RBC's to glycerol is strongly inhibited by traces of copper (Jacobs and Corson, 1934; Jacobs and Stewart, 1946), by tannic acid (Hunter, 1960) and a drop in pH (Davson, 1939). The inhibition of glycerol permeability by traces of copper suggests that chemical groups on the cell surface exert a great influence,as proposed by LeFevre (1948) and Bowyer (1954). Jacobs (1954) observed in experiments with human RBC's mutual competition between ethylene glycol and glycerol, confirming a process of facilitated diffusion. Recently Hunter (1970) observed saturation in a kinetic analysis of swelling of human erythrocytes in the presence of glycerol. De Gier et al (1971) observed a lower activation energy for the penetration of glycerol than the enthalpy for dehydration indicating that the presence of polar pores or a mechanism of facilitated diffusion enables the glycerol molecules to pass the human RBC membrane without complete dehydration. Recent studies of Macey and Farmer (1970) suggest that glycerol does not pass the membrane through polar pores, or at least not through those used for water penetration. They showed that phloretin inhibits the permeability to water but not to glycerol.

1.4. SUMMARY

In the preceding review some information about composition, structure and some characteristics of the permeability of the RBC membrane to simple nonelectrolytes was presented. It was shown that none of these aspects has been fully elucidated, in spite of the efforts of many investigators. Whereas the lipid composition is now fairly well known, the knowledge concerning the protein composition is still very limited. Though it is likely that membrane proteins play an important role in the molecular arrangement of the different membrane components, their proposed role must still be considered hypothetical. As a consequence of our limited knowledge, one can hardly discriminate between the different hypothetical models, but the unit-membrane concept still seems to be the most satisfactory in the light of our present

knowledge.

The evidence presented by Stein (1967) and de Gier et al (1971) that glycerol molecules pass the RBC membrane through the hydrophobic core is based on reliable data, though the information is still rather limited. Since the composition of the hydrophobic core differs markedly with the mammalian species, it does not seem unlikely that a correlation between RBC-membrane permeability and lipid composition exists. The high permeability of RBC membranes of rat,man and rabbit, causes some difficulties and many data suggest a facilitated diffusion mechanism in these cases. The studies of Macey and Farmer (1970) made the penetration into RBC's of glycerol through membrane pores less likely. However, the permeability data from comparative studies do not seem to be fully reliable. Some important differences in properties of RBC's from different origin were not sufficiently taken into consideration. We therefore decided to perform more reliable permeability measurements. A description of the method and the results obtained with it are presented in the next chapters.

2. MATHEMATICAL DESCRIPTION OF THE LYSIS BEHAVIOUR OF RED BLOOD CELLS*

2.1. INTRODUCTION

The osmotic lysis method, proposed by Jacobs in 1934 and fully described by Jacobs et al (1950), is used frequently in comparative studies of the permeability of RBC's (de Gier et al, 1966; Moore, 1968; Szelenyi and Hollán, 1968) or of other cell types (Love, 1953; Lucké et al, 1956). Generally the turbidity change of cell suspensions is measured in this method. The turbidity of a cell suspension in an isotonic solution of permeant decreases gradually, since an ever-increasing number of cells will lyse due to diffusion of permeant through the cell membrane. The time elapsing for a certain decrease in turbidity (e.g. to 25% of the initial turbidity) often called "time of hemolysis", is taken as a relative measure of the membrane permeability. Though the time of lysis depends in some way on the permeability, it is clear that the rate of hemolysis may also be affected by the following factors which are often too little considered:

a) the surface/volume ratio, which varies with the shape of RBC's of various mammalian species (Whittam, 1964). Jacobs (1934) has taken this ratio into account in his permeability studies with ox RBC's.
b) differences in membrane elasticity. Canham and Parkinson (1970) observed no significant change in area during gradual osmotic swelling of human RBC's. Since their experimental conditions deviate considerably from normal lysis experiments - they slowly lowered the tonicity of the RBC suspension - these data cannot be taken as a proof for the absence of membrane elasticity.
c) the possible loss of osmotic material from the cells during the permeation experiments. Jacobs et al (1937) observed a rapid shrinkage of RBC's in nonelectrolyte solutions resulting in their opinion from the loss of anions. Whereas the membranes of ox RBC's are fairly impermeable to cations under these circumstances (Jacobs and Parpart, 1933), a rapid efflux of cations from human RBC's,

*The modified lysis method and the mathematical description were developed in highly appreciated cooperation with Dr. D.T.F.Pals, Central Laboratory TNO, Delft

suspended in a low ionic strength isotonic sucrose solution, has been reported by some investigators (Wilbrandt, 1940; La Celle and Rothstein, 1966; Donlon and Rothstein, 1969). La Celle and Rothstein (1966) found that a concentration of about 0.18 mM NaCl was able to suppress considerably this cation efflux. These data were confirmed by the observations of Donlon and Rothstein (1969). Cotterrell and Whittam (1971) have shown that the chloride gradient across RBC membranes and hence the membrane potential affect the sodium and potassium movements. Since the efflux of cations is relatively slow, compared with the rapid permeability measurements, little attention has been given to this phenomenon. Some data on ion fluxes will be presented in chapters 4 and 7.

d) variations in initial intracellular tonicity due to differences in plasma tonicity (Olmstead, 1966).
e) possible changes in membrane properties induced by the penetrating substances.

Jacobs et al (1950), being aware of these factors, stated that comparison of hemolysis times obtained with RBC's from different animals may lead to unreliable permeability data. The importance of these factors has been discussed in a theoretical study of Canham (1969) concerning the osmotic fragility of individual human RBC's.

The aim of our study and of the method introduced is to rule out these factors as far as possible in order to obtain a better estimation of the permeability as such. Our studies are based on the assumption that RBC's normally behave like perfect osmometers, though this is still questionable. Ege (1927) and White and Rolf (1962) observed a perfect osmometric behaviour of the RBC's in non-electrolyte solutions. A small deviation is observed in electrolyte solutions (Dick, 1971), which may be caused by differences in osmotic activity of the hemoglobin in swollen or shrunken RBC's (Adair, 1928; Gary-Bobo and Solomon, 1968). RBC ghosts, freshly prepared by one-step hemolysis and resealed at 37^{o}, behave as perfect osmometers in contrast to intact cells (Kwant and Seeman, 1970). The osmotic equilibrium of RBC's with their environment will be established almost instantaneously, since water passes the RBC membrane rapidly (Paganelli and Solomon, 1957; Blum and Forster, 1970; Farmer and Macey, 1970; Dick, 1971). At a certain low concentration of non-permeant outside the cell, the amount of water entering the RBC will surpass its maximum and lysis will take place. In this way the osmotic resistance, the maximum swelling capacity of RBC's depending on the surface/volume ratio, differences in membrane elasticity and variations in initial intra-

cellular tonicity can be determined. The ideal osmotic resistance can be taken as a time-independent quantity, since water equilibrium is established almost instantaneously. In solutions containing different concentrations of a permeating substance, the permeant and also water diffuse gradually into the cell, and an ever-increasing number of RBC's will lyse. This process is of course time-dependent. Immediately after suspending the RBC's in hypotonic solutions of permeant, when the permeant has not yet entered the cells to an appreciable extent, only water equilibrium is established. Then it must be expected that the same degree of lysis has taken place as in an analogous hypotonic solution of non-permeant. If this is true, extrapolation to t=0 of the degree of lysis, achieved at various moments in solutions of permeant must lead to the same values as observed in solutions of non-permeant. The conditions for which this prediction is true will be discussed. When this is taken into account, it appears possible to estimate RBC membrane permeability more exactly by combining measurements of osmotic resistance and permeability.

2.2. THEORY

Outside the RBC's the osmolar concentration (further called concentration) of permeant or non-permeant can be regarded as remaining constant due to the low ratio of RBC volume / medium volume (1:400). Confining ourselves to the case of one penetrating nonelectrolyte, e.g. glycerol, being present outside the cell, the concentration of this substance will be denoted by m_p. The total extracellular concentration of non-permeating substance will be assumed to be equivalent to m_n. The sum of concentrations outside the cell is thus:

$$m_p + m_n = m_c \text{ (osmol/l)} \qquad (1)$$

The quantities m_p, m_n and m_c do not depend on time, nor on the condition of the cells.

Intracellularly the situation is different. It may be presumed that, as a result of previous treatment with an isotonic solution of non-permeating substance, all permeating substances have been removed, so that at the start of the experiment only non-permeating components will be present. After a while, however, due to diffusion through the cell membrane, some penetrating substance will have entered, the intracellular concentration of which is denoted as X. Since the RBC membrane is readily permeable to water, water equilibrium is established within milliseconds. This means that the total osmotic activity on both sides of the membrane can

always be taken as equal. Since the strength of the RBC membrane is negligible small (Rand, 1964), no pressure differences will build up. In other words; the variable volume of the RBC adapts itself rapidly to each new situation, in such a way that the osmotic activities inside and outside the cell remain equal to each other. In consequence we may write for the concentration inside the cell:

$$m_c = X + \frac{N}{V} \qquad (2)$$

with X the concentration of permeating substance in the cell, depending on time; V the volume of the cell depending on time, and N the concentration of non-permeating components in the cell, which can be considered to be constant. At this point it should be remembered that N, V and X may be somewhat different for individual RBC's of the population due to variations in age and consequently in properties (O'Connell et al, 1965; Weed and Bowdler, 1967). Thus equation (2) is only valid for each fraction f of the cell population:

$$m_c = X(f) + \frac{N(f)}{V(f)} \qquad (2f)$$

For the sake of simplicity we shall omit the subscript f in the following derivations, but it should be kept in mind that these quantities depend on f. Assuming that diffusion through the cell membrane obeys the first law of Fick, which states that the amount of transported material is proportional to the difference in concentration, one obtains:

$$\frac{d(XV)}{dt} = P.S\ (m_p - X) \qquad (3)$$

in which V and S are the volume and the surface area of the RBC respectively, and P the permeability coefficient.

This equation is however overly simplified. On the basis of irreversible thermodynamics, Staverman (1951) stated that interactions must take place between solute and solvent permeating through the same channels. These interactions are represented by the reflection coefficient σ, which is defined as the ratio of the actual osmotic pressure to the theoretical van 't Hoff osmotic pressure. The reflection coefficient σ is a measure of the semipermeability of the membrane to a given solute: depending on the penetration rates of solute and solvent σ varies between zero and one. For an ideal semipermeable membrane which is impermeable to the solute, σ is equal to one. In this case equation (3), based on Fick's law, holds. In a coarse, nonselective membrane σ is zero.

Equation (3) must therefore be replaced by a more general expression, e.g. that given by Stein (1967). Using our symbols, this expression reads:

$$\frac{d(XV)}{dt} = S\left\{(1 - \sigma)\ \frac{m_p + X}{2} \cdot \frac{1}{S} \cdot \frac{dV}{dt} + P\ (m_p - X)\right\} \qquad (4)$$

The determination of the reflection coefficients for different permeants and for RBC's from different mammalian species is rather complicated (Goldstein and Solomon, 1960; Stein, 1967; Sha'afi et al, 1970). Values for the reflection coefficient of glycerol are only published for human RBC's, which have a relatively high permeability to glycerol. Goldstein and Solomon (1960) found experimentally a σ of 0.88 $\pm$ 0.02 by the "zero time method". This method depends upon a determination of the concentration of permeant required to cause the initial rate of water entrance into the cell to become zero. Stein (1967) calculated a σ value of 0.995-0.997 from permeability data which were obtained, however, in the presence of volume flow.

When the water permeability of the RBC membrane is high compared to that of the permeant, the permeability coefficient will certainly not differ by an order of magnitude in the presence or absence of water flow (Stein, 1967). Sha'afi et al (1970) obtained experimental evidence that the permeability coefficient of urea in human RBC's decreases with increasing solvent flow. Sha'afi et al (1971) concluded that there was a surprisingly good agreement between the permeability coefficients computed from hemolysis times (Höber and Ørskov, 1933) and those obtained using rapid reaction techniques. But they argued that it is not possible to combine these data, which differ by an order of magnitude, into a single ratio as was done by Stein (1967). Phenomenologically the permeability coefficient and the reflection coefficient are entirely independent parameters (Di Polo et al, 1970). The frictional treatment (Katchalsky and Curran, 1965) cannot be applied to the red blood cell membrane, because the diffusion coefficient in water does not bear a predictable relationship to the solute-water friction in the membrane (Sha'afi et al, 1970).

The following points make the assumption acceptable that the reflection coefficient may be neglected in our permeability studies:

1) As will be shown in chapter 5 , the determined permeability coefficient for glycerol is not, or only to a very small degree, dependent on the extracellular glycerol concentration. On theoretical grounds, the reflection coefficient must depend to some extent on the concentration (Stein, 1967; Sha'afi et al, 1970). Since the determined permeability coefficient is virtually independent of the concentration, one may take the reflection coefficient to be almost one. Sha'afi et al (1970) and Savitz and Solomon (1971) also suggested that the effect of solute concentration on the permeability coefficient is relatively unimportant (Sha'afi et al, 1971).
2) Since the reflection coefficient is equal to the ratio of the observed

reads, if fraction f is lysed,

$$m_n^h(f) \leqslant \frac{N(f)}{V_h(f)} \qquad (5)$$

b. Presence of only permeating substance.

Substituting equations (2) and (3) and eliminating X we find, with omission of subscript f:

$$VdV = \frac{P.S.N}{m_p}\, dt$$

Integration between the limits t = O and t = t_h, where t_h is the time of hemolysis of the fractions considered, gives:

$$V_h^2 - V_o^2 = \frac{2\ P.S.N}{m_p} \cdot t_h \qquad (6)$$

provided that S does not depend on V or on t.
Considering further that at t = O, X = O, with equation (2) $V_o = \frac{N}{m_c} = \frac{N}{m_p}$ and equation (5) $N = m_n^h \cdot V_h$ we find:

$$\frac{m_p}{m_n^h} - \frac{m_n^h}{m_p} = 6 \cdot \frac{P}{R_h} \cdot t_h \qquad (7)$$

where we have written $\frac{S}{V_h} = \frac{3}{R_h}$

Equation (7) gives a relation between the hemolysis time t_h, and m_p and m_n^h, all for one particular fraction f.

c. Presence of permeating and non-permeating substances.

In the same way, by elimination of X from equations (2) and (3) and integration, we find for a particulate fraction f:

$$\frac{m_c - m_n^h}{m_n} - \frac{m_n^h . m_c}{m_n^2} \ln \frac{m_n^h\ (m_c - m_n)}{m_c\ (m_n^h - m_n)} = 3\frac{P}{R_h} \cdot t_h \qquad (8)$$

The degree of osmotic lysis can be determined in two ways, either from turbidity measurements or from the amount of liberated hemoglobin. It is frequently supposed that decreased transmission or increased scattering automatically means particle shrinkage and the reverse particle swelling. This interpretation was suggested by some experiments and seemed to be indicated by Koch's (1961) theoretical relation. However, exceptions to this behaviour have been reported (Yamashita et al, 1968; Deamer et al, 1967) especially for cases where the particle strongly absorbs. Particle shrinkage can increase scattering at some particle sizes (chloroplasts), decrease it for others (yeast) as shown by Bryant et al (1969).

ERRATA.

Pages 24 and 25 have to change places.

Page 60. The first paragraph has to be replaced to the end of this page.

Page 70. Table IX. Read in the sixth line: standard devations.

Page 72. Read in the second line: standard deviations.

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osmotic pressure and the theoretical osmotic pressure, a given concentration of permeant molecules never exerts its full osmotic effect, even at t = 0 when no molecules have penetrated the membrane (Goldstein and Solomon, 1960). Extrapolation of our permeation data at various moments to t = 0, leads to a concentration of permeant causing a distinct degree of lysis which cannot be distinguished from the concentration of non-permeant causing the same degree of lysis (see Chapters 3 and 5). It may also be concluded from this observation that the reflection coefficient for glycerol is almost one.

3) Recent investigations of Macey and Farmer (1970) suggest that in human RBC's water and glycerol do not share the same pathway through the membrane.

4) In a theoretical study (Pals,1971) it can be shown that Fick's law holds for our permeability studies when the solvent-solute interaction does not exceed the ratio given by the volume fractions of solvent and solute. No arguments for such large interactions were found in the literature. We may, therefore, conclude that in our comparative studies of RBC membrane permeability to glycerol σ is about one and equation (3) holds.

Hemolysis of fraction f of the RBC population occurs, whenever V(f) exceeds a critical value $V_h(f)$, which value may depend on the composition of the solutions on both sides of the membrane. Our experiments indicate (Chapter 3) that the resistance towards non-permeating sucrose is quite different from that towards non-permeating NaCl. Thus in general the values for $m_n^h(f)$ and $V_h(f)$ - the concentration of non-permeating substance leading to lysis of fraction f - obtained with NaCl or sucrose, cannot be applied to permeation experiments with glycerol. The reasons for these discrepancies will be discussed in Chapters 4 and 7. It should already be mentioned here, that the observed differences are not or only to a minor degree due to variations in $V_h(f)$, but are caused by exchange of anions coupled to the buffering action of hemoglobin

We will consider three cases: a) $m_n \neq 0$, $m_p = 0$; b) $m_n = 0$, $m_p \neq 0$ and c) $m_n \neq 0$, $m_p \neq 0$.

In our experiments only the first two cases have been studied, but not combinations of permeating and non-permeating substances.

a. Presence of only non-permeating substance.

In this case, as a result of $m_p = 0$, also $X = 0$ for all cells: there is no time dependence, a fraction f is either lysed or not. Equation (2f)

The theory (Latimer et al, 1968) also predicts that scattering changes may be indicative of changes in internal structure rather than of volume changes. According to Koch (1970), reliable results with a normal spectrophotometer can be obtained in a simple way only with spherical cells not exceeding 5 μ^3. The shape of RBC's may differ considerably, especially when the circumstances are varied, and moreover the light absorption may increase when hemoglobin is liberated. We decided to determine the liberation of hemoglobin instead of following turbidity changes. Therefore, the extinction of the supernatant was determined after removing the intact cells by centrifugation. It should be mentioned here that the individual RBC's in the population may differ in their hemoglobin content (Bahr and Zeitler, 1962; Morselt and James, 1971). The consequence may be that the relative extinction (E/E_{max}) may not be considered as a measure of the percentage of lysed cells. Since we studied the lysis rate of small fractions of the total RBC population, this fact has no important consequences.

Considering equation (5) it will be clear that by plotting the relative extinction of the hemoglobin in the supernatant (E/E_{max}) as a function of m_n, we should obtain a curve which shows the total fraction of cells with values of $N/V \geq m_n$ as a function of m_n. All cells can be arranged according to the value of N/V_h, so that for a given value of E/E_{max} the same fraction of cells (those with N/V_h values just a little bit larger than the given values) hemolyse. Thus, the experimental plot of E/E_{max} versus m_n^h is at the same time a plot of percentage of hemoglobin contained in cells with $N/V > m_n^h$. Equations (7) and (8) show that from measurements of E/E_{max} as a function of time, for systems with and without permeating substances, the constant P/R_h of substances through RBC membranes can be calculated quantitatively. Since R_h can be determined from the critical cell volume, the permeability coefficient can be estimated.

3. COMPARISON OF THE MATHEMATICAL DESCRIPTION OF RED BLOOD CELL LYSIS WITH THE EXPERIMENTAL DATA

3.1. INTRODUCTION

In the preceding chapter a mathematical description of the lysis behaviour of RBC's was given. Arguments were presented favouring the proposed modified osmotic lysis method above the original one of Jacobs et al (1950). It was shown that combined estimations of the osmotic resistance in solutions of a non-permeant and of the lysis rate in solutions of a permeating substance, afforded a more reliable insight in the permeability of RBC's to a substance like glycerol. This mathematical evaluation was extensively tested with pig RBC's and, as reported in chapter 5, with RBC's of man and sheep.

3.2. MATERIALS AND METHODS

3.2.1. Isolation of red blood cells

RBC's were isolated by centrifugation (10 min 1000 x g) from fresh pig blood, which had been defibrinated with glass beads. The buffy coat was removed and the RBC's were either resuspended in serum, or treated four times with a five- to tenfold excess of 300 mM sucrose or 150 mM NaCl, both solutions being buffered with 1 mM sodium phosphate to pH 7.5. "Reagent grade" chemicals, obtained from Merck, were used.

3.2.2. Lysis experiments

With a microsyringe 0.05 ml of the RBC suspension, hematocrit 0.4-0.5, was added in duplicate to 10 ml samples of buffered solutions of isotonic or hypotonic NaCl, sucrose or the permeating substance glycerol. Buffer composition and pH were as described before. All solutions were kept at 37°C. After addition of the RBC's, the test tubes were shaken immediately and placed again into a constant temperature bath of 37°C.

After various time intervals hemolysis was stopped by adding 1 ml 1.5 M NaCl, whereafter the non-hemolysed cells were removed by centrifugation (10 min 1000 x g). The extinction of the supernatant, containing hemoglobin from the lysed cells, was measured at 540 nm and found to be proportional to the amount of hemoglobin liberated. Although not all RBC's in a population contain the same amount of hemoglobin (Morselt and James, 1971), we could neglect this point since we compared the lysis rate of distinct fractions of the RBC population. When in the following the term "50% hemolysis" is used, this means that the extinction of the supernatant relative to that of a fully hemolysed preparation (E/E_{max}) is 0.5.

3.2.3. Determination of the osmolarity of the test solutions

The osmolarity of the test solutions in mosmol/l was calculated from the specific gravity and the osmolality estimated from freezing-point depression with an Advanced Instruments osmometer.

3.2.4. Determination of the average critical cell volume (V_h)

Per ml RBC's resuspended in serum (hematocrit 0.4-0.5) was added 0.05 µC inulin-(carboxylic acid-^{14}C) obtained from Radiochemical Center Amersham, and dissolved in 0.05 ml 300 mM unbuffered sucrose. Thereafter 1 ml unbuffered sucrose of different osmolarity was added to 1 ml samples. After mixing, the samples were centrifuged at once (10 min 1000 x g). The radioactivity was determined in 0.2 ml of the supernatant, added to 10 ml Instagel (Packard Instrument Cy.), with a Packard Tri-Carb Liquid Scintillation Spectrometer Type 3380, equipped with an Absolute Activity Analyzer Model 544. The activities were compared with the activity of a standard dilution of ^{14}C-inulin. The total cellular volume was calculated from these data. The average RBC volume was calculated from the cell count made with a Coulter Counter Model B, equipped with a 50 µ aperture tube, and from the total cellular volume. The average RBC volumes were plotted against the corresponding osmolarities determined in a parallel experiment without added ^{14}C-inulin. An approximate value for the average critical cell volume (V_h) was found by extrapolation to the sucrose concentration causing 50% hemolysis. Small inaccuracies in the determination of V_h may be neglected because R_h, calculated from V_h, is inversely proportional with $V_h^{1/3}$.

3.3. EXPERIMENTAL RESULTS

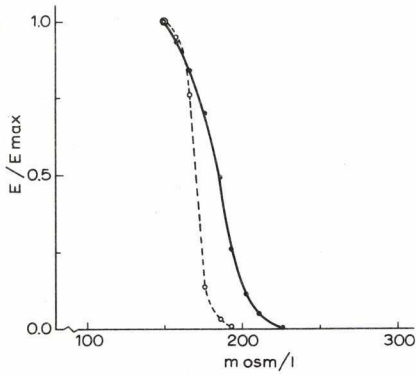
3.3.1. Introduction

The results of the lysis experiments are presented in the form of graphs in which the relative extinction (E/E_{max}) of the solutions, obtained after removal of the unhemolysed cells, is plotted against the concentration of the test solution in mosmol/l.

3.3.2. Shape of lysis curves

All lysis curves appeared to be S-shaped which means that the RBC's were not all identical. They did not all lyse at the same concentration of non-permeant instantaneously, or at a certain concentration of permeant at the same moment. This may be caused by differences in properties of the RBC's, e.g. their surface/volume ratios, probably due to variations in age (O'Connell et al, 1965; Weed and Bowdler, 1967; Canham, 1969). It therefore seems reasonable to assume that at each level of extinction a distinct fraction of the RBC population is lysed The validity of this assumption is supported by the experiments represented in Fig. 1.

Fig. 1
The osmotic resistance of untreated pig RBC's.
The closed circles represent the osmotic resistance of the total population, the open circles that of the same population after removal of the less resistant RBC fractions.



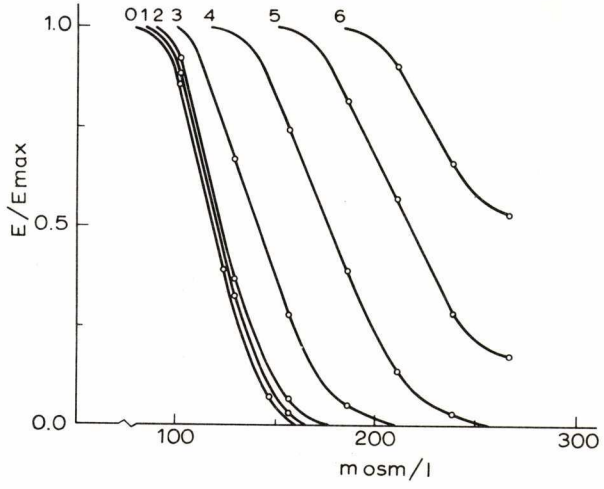
Two resistance curves are presented, showing the lysis of pig RBC's in solutions of the non-permeating substance NaCl. One refers to the total population, the other to a fraction of the population after partial lysis at 180 mosmol NaCl/l and isolation of the cells which have remained intact. The slope of the second curve is steeper, which indicates that some fractions were removed completely while others remained as a whole. It seems likely that the older RBC's were removed, just as was observed with human RBC's (Marks and Johnson, 1958).

3.3.3. Lysis experiments with untreated pig red blood cells

In this experiment pig RBC's were resuspended in serum. 0.05 ml suspension was added to 10 ml solution of the non-permeant sucrose or the permeant glycerol. The lysis curve 0 (Fig. 2) represents the osmotic resistance of the RBC population in hypotonic sucrose solutions, whereas the curves 1-6 represent the degree of lysis in glycerol solutions of varying tonicity at various moments. On account of theoretical considerations (2.2) it may be assumed that the lysis curves in hypotonic sucrose solutions near t=0 and at t=300 sec were the same since water equilibrium is established almost momentarily.

Fig. 2

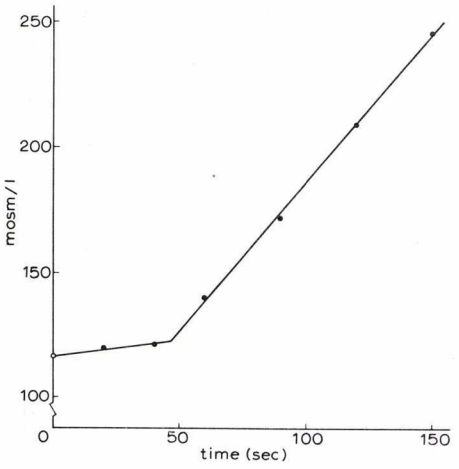
The lysis of untreated pig red blood cells in sucrose and in glycerol. Curve 0 represents the osmotic resistance in sucrose after 300 sec, curves 1-6 the lysis in glycerol after 20,40,60,90,120 and 150 sec, respectively.



Sucrose was chosen as the non-permeant, because the permeability experiments were also performed with a nonelectrolyte. The most striking fact observed was, that hardly any increase in the lysis of RBC's, due to diffusion of glycerol into the cells, was seen in a rather long period of time after t=0. The osmotic resistance curve in sucrose solutions and the lysis curves in glycerol solutions after 20 and 40 sec nearly coincided. The 50% hemolysis values, plotted against time, once more illustrate this observation (Fig. 3). Since it may be assumed that the lysis in hypotonic sucrose would be complete almost instantaneously, the 50% hemolysis value in mosmol/l for sucrose is always plotted at t=0.

Fig. 3

Fifty percent hemolysis values in mosmol/l of untreated pig red blood cells in glycerol (●) and in sucrose (o) solutions as a function of time.



According to the theoretical considerations (2.2) an ever-increasing number of RBC's should lyse due to a gradual increase of the intracellular glycerol concentration. It was observed that in sublytic glycerol concentrations the RBC population was almost instantaneously lysed to a

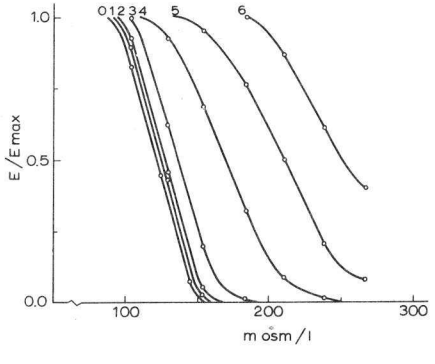
certain degree. Though it was expected that lysis would continue due to a gradual increase of the intracellular glycerol concentration, lysis appeared to be retarded considerably. After about 50 sec lysis proceeded, for which phenomenon the term "delayed lysis" is proposed. A possible explanation for the delayed lysis could be the gradual loss of osmotically active substances from the RBC's in the beginning of the experiment, compensating for the increase of the intracellular glycerol concentration. This would result in a temporary constancy of the osmotic activity in the RBC. As soon as the loss of permeating substances from the cells would be complete, the lysis would proceed due to the penetration of glycerol molecules. When this hypothesis is true, the delayed lysis in the beginning of the experiment should disappear upon treating the RBC's, prior to the lysis experiments, with 150 mM non-permeating NaCl in order to remove permeating substances from the cell contents.

3.3.4. Lysis experiments with pig red blood cells treated with 150 mM NaCl

The results of a lysis experiment with pig RBC's, treated four times with a five- to tenfold volume of 150 mM NaCl, are shown in Fig. 4.

Fig. 4

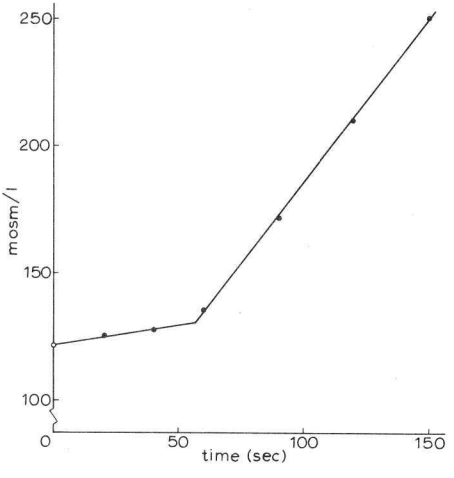
The lysis of NaCl treated RBC's in sucrose and glycerol solutions. Curve 0 represents the osmotic resistance in sucrose at t=300 sec. Curves 1-6 the lysis in glycerol after 20, 40, 60, 90, 120 and 150 sec, respectively.



Lysis curve 0 represents the osmotic resistance in sucrose solutions at t=300 sec, which is about equal to that at t=0. Curves 1-6 represent lysis in glycerol solutions at various time intervals. The delayed lysis is equal to, or even larger than in the experiment with untreated RBC's. The 50% hemolysis values as a function of time once more confirm these conclusions (Fig. 5).

Fig. 5

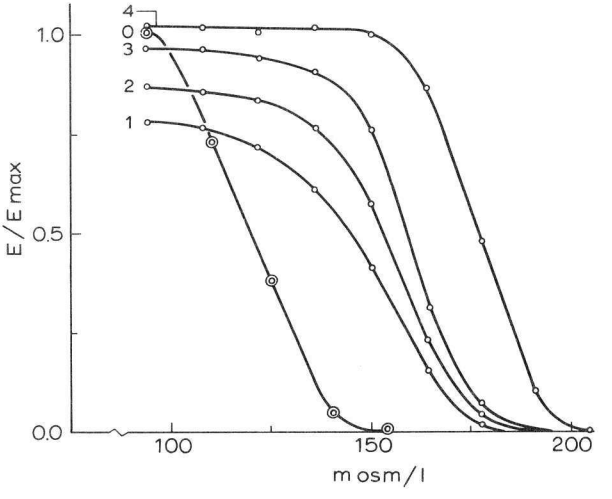
Fifty percent hemolysis values in mosmol/l of NaCl treated pig RBC's in glycerol (●) and in sucrose (o) solutions as a function of time.



One might object that generally the osmotic resistance is determined in hypotonic NaCl solutions. However, even if the osmotic resistance in NaCl solutions differs considerably from that in sucrose solutions, no explanation for the coincidence of the lysis curves in glycerol solutions after 20 and 40 sec is available. Nevertheless we made a comparative study of the osmotic resistance in hypotonic NaCl and sucrose solutions. Special attention was payed to the time-dependence. The lysis in hypotonic NaCl solutions of RBC's, treated with 150 mM NaCl, was stopped after 5,10,15 and 300 sec by the addition of hypertonic NaCl (see 3.2.2.). The results, presented by curves 1,2,3 and 4 in

Fig. 6, demonstrate that the osmotic resistance in hypotonic NaCl solutions is strongly time-dependent, an observation corroborated by the results of Bowdler and Chan (1969). This observation is,however, not in accordance with the theoretical considerations (2.2), which state that the osmotic resistance is almost time-independent. The osmotic resistance in hypotonic sucrose solutions of RBC's treated with 300 mM sucrose shows virtually no time-dependence, since curve 0 represents the degree of lysis after 5 as well as after 300 sec.

Fig. 6
Osmotic resistance of pig RBC's.
Curve 0: sucrose treated cells in hypotonic sucrose solutions after 5 and 300 sec.
Curves 1-4: NaCl treated cells in hypotonic NaCl solutions after 5, 10, 15 and 300 sec, respectively.



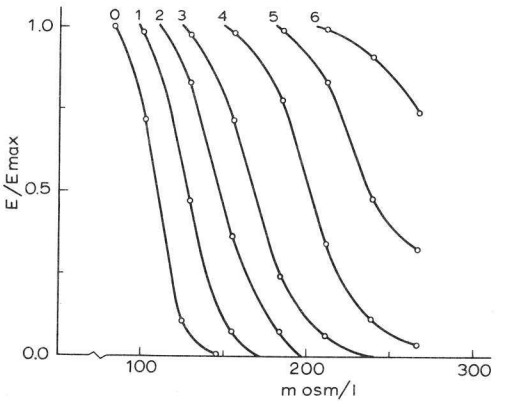
Though the osmotic resistance will be discussed in more detail in Chapter 7 some supplementary remarks must be made here. The time-dependence of the osmotic resistance in hypotonic NaCl solutions was observed also with untreated RBC's and RBC's treated with 300 mM sucrose, though the degree depended somewhat on the pretreatment. In no case was a shift of the osmotic resistance with time observed in hypotonic sucrose solutions. Furthermore, the RBC's appeared to be less resistant in NaCl than in sucrose solutions. The dependence

of the cell fragility on the osmotic properties of the medium was also observed for RBC's of six mammals by Coldman et al (1970).
As was shown, the osmotic resistance in NaCl solutions appeared to be time-dependent in contrast to that in sucrose solutions. Moreover, the delayed lysis in glycerol solutions was not only detected with untreated RBC's, but also, and even more pronounced, with RBC's treated with 150 mM NaCl. The delayed lysis therefore can not simply be explained from a gradual loss of osmotically active substances from the RBC's. It seemed likely that intracellular, osmotically active electrolytes or extracellular electrolytes, originating from serum or NaCl solution, acting in or at the cell membrane contribute to the delayed lysis. In order to test this assumption, the osmotic resistance in sucrose and the lysis rate in glycerol solutions were determined for RBC's previously treated with 300 mM sucrose.

3.3.5. Lysis experiments with pig red blood cells treated with 300 mM sucrose

The RBC's were treated four times with a five- to tenfold volume of 300 mM sucrose to remove permeating substances from the cell contents and to exclude the possible electrolyte effects. Thereafter the lysis in hypotonic sucrose and glycerol solutions was studied (Fig. 7).

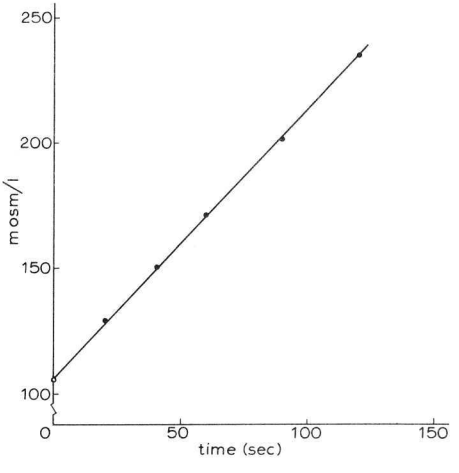
Fig. 7
The lysis of pig RBC's treated with 300 mM sucrose in respectively sucrose and glycerol.
Curve 0: osmotic resistance in sucrose at t=300 sec.
Curves 1-6 lysis in glycerol after 20, 40, 60, 90, 120 and 150 sec,resp.



Obviously the delayed lysis was absent, indicating that an ever-increasing number of RBC's lysed due to the gradual increase of the intracellular glycerol concentration. The 50% hemolysis values plotted against time, confirm the foregoing statement (fig. 8). The values in glycerol showed a straight line relationship. Extrapolation to t=0 gives a m_p value (the concentration of permeating glycerol causing, due to water flow only, 50% hemolysis at about t=0) fitting very well with the experimental m_n^h value (the concentration of non-permeating sucrose causing 50% hemolysis instantaneously, due to water flow only).
In this case all essential conditions for a mathematical analysis of the lysis data appeared to be fullfilled.

Fig. 8

Fifty percent hemolysis values in mosmol/l of pig RBC's treated with 300 mM sucrose in glycerol (●) and in sucrose (o) solutions as a function of time.



3.4. COMPARISON OF MATHEMATICAL DESCRIPTION AND LYSIS DATA

If the assumption is true that at each extinction level a distinct fraction of the RBC population is lysed, the theoretical equation (7);

$$\frac{m_p}{m_n^h} - \frac{m_n^h}{m_p} = 6\,\frac{P}{R_h}\,.\,t_h$$

must be valid for each extinction level representing a uniform fraction of RBC's. Therefore the curves 1-6 of Fig. 7, referring to lysis in glycerol solutions of RBC's treated with 300 mM sucrose, may be described by this equation, since glycerol is the only solute present extracellularly. The osmotic contribution of 1 mM sodium phosphate buffer may be neglected. Another consequence of the above assumption is that the difference in glycerol concentration between the interior of the cells and the surrounding solution must be equal to m_n^h at the moment of lysis. This concentration difference must be the same for all time curves at a distinct extinction level, provided that the same RBC suspension is used.

In order to compare theory and experimental data, pairs of points of different time curves representing the same relative extinctions, were selected from Fig. 7. The following equation can be derived for such pairs from equation (7) when $t_2 = n.t_1$:

$$n\left(\frac{m_{p_1}}{m_n^h} - \frac{m_n^h}{m_{p_1}}\right) = \left(\frac{m_{p_2}}{m_n^h} - \frac{m_n^h}{m_{p_2}}\right), \text{ and thus;}$$

$$m_n^h = \left(\frac{nm_{p_1} - m_{p_2}}{\frac{n}{m_{p_1}} - \frac{1}{m_{p_2}}}\right)^{\frac{1}{2}} \qquad (9)$$

Pairing the data of all six curves permits the calculation of a mean value for m_n^h (actually the value of m_p at t= 0) for each fraction corresponding to a given relative extinction. An example of such a calculation is given in Table I.

Table I

Comparison of experimentally found critical sucrose tonicities (m_n^h) in mosmol/l at three extinction levels, with the m_p values in glycerol solutions at t = 0 in mosmol/l calculated by means of equation (9)

E/E_{max}	m_p at t(x) sec				mean m_p at t = 0	m_n^h
	20	40	60	90	(calculated)	(exp.)
0.25	138	161	184	222	119.8 ± 1.9	119
0.50	128	147	170	204	110.8 ± 1.9	110
0.75	116	136	155	187	100.7 ± 2.9	100

The fact that the calculated m_p values at t = 0 and the experimental values for m_n^h, obtained from resistance measurements in sucrose fit the same curve, renders additional support to the theory set forth in chapter 2. Extrapolation of the 50% hemolysis values in glycerol at various times to t = 0 (Fig. 8), also confirms this statement. Since m_p at t = 0 is equal to m_n^h, the neglection of the reflection coefficient in the mathematical considerations appears to be justified (cf. 2.2).

3.5. CALCULATION OF THE PERMEABILITY COEFFICIENT

The values of P/R_h calculated from curves 0 - 6 in Fig. 7 are plotted in Fig. 9 as a function of the relative extinction

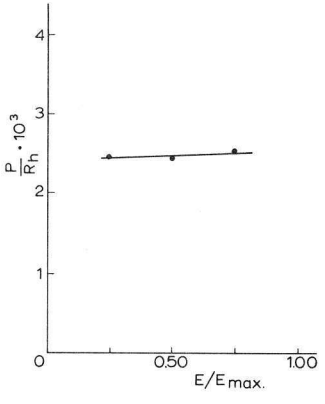


Fig. 9.

Ratio of permeability coefficient and critical radius of sucrose treated RBC's in glycerol solutions, versus the relative extinction. The ratios were calculated by means of equation (7).

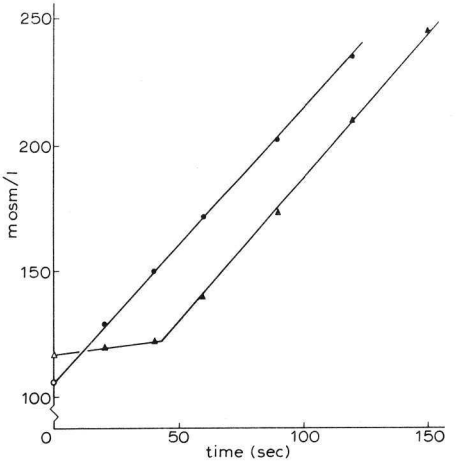
The P/R_h ratios did not appear to differ considerably for the different fractions of the RBC population. An average value for R_h can be calculated from the determined average of V_h. The value for R_h amounted to 2.85 μ ± 0.03 (n=3). The average glycerol permeability coefficient at

pH 7.5 and 37°C of pig RBC's amounts to $6.60 \cdot 10^{-7}$ cm·sec^{-1} (S.D. $\pm$ $0.66 \cdot 10^{-7}$ cm·sec^{-1}, n=8) at the 50% hemolysis level.

3.6. CONCLUSIONS

Pretreatment of RBC's appears to influence their lysis behaviour in hypotonic sucrose and glycerol solutions at pH 7.5 and 37°C. A lysis behaviour, in agreement with our theoretical considerations is observed with RBC's treated with 300 mM sucrose (Figs. 7 and 8). The m_p value at t=0, obtained through extrapolation from m_p values after different time intervals, is virtually equal to the experimentally determined m_n^h value.
The lysis behaviour of untreated RBC's, or RBC's treated with 150 mM NaCl, was more complex. During the first 40-60 sec the lysis in hypotonic glycerol solutions proceeded very slowly (Figs. 2 and 4). Thereafter, however, lysis continued at nearly the same rate as that observed with 300 mM sucrose treated RBC's (Fig. 10).

Fig. 10
Fifty percent hemolysis values in mosmol/l of untreated and 300 mM sucrose treated RBC's in glycerol and in sucrose solutions as a function of time.
Untreated RBC's in glycerol (▲) and in sucrose (Δ).
Sucrose treated RBC's in glycerol (●) and in sucrose (o).



Since after 60 sec lysis in glycerol solutions proceeded almost independently of the pretreatment, these observations suggest that RBC membrane permeability to glycerol in our circumstances hardly depends on the pretreatment. This statement is, as will be shown, supported by the permeability coefficients calculated from experimental data.

From the m_p values, obtained at $t \geqslant 60$ sec, the m_p value at t=0 can be calculated with equation (9), or estimated by extrapolation to t=0. In the ideal case the m_p value should be almost equal to m_n^h, as found for sucrose treated RBC's. This does not always hold true, as shown by Fig. 10. For the cause of this discrepancy we have to anticipate on chapter 4 in which the lysis delay will be explained from the exchange of intracellular chloride with extracellular hydroxyl ions coupled with the buffering action of hemoglobin (Fig. 13). This phenomenon results in a decrease of the intracellular osmotic activity and hence in mean cell volume and increase of osmotic resistance. Upon extensive and prolonged treatment of RBC's with 300 mM sucrose buffered at pH 7.5 with 10 mM sodium phosphate, intracellular chloride ions are principally replaced by phosphate and only to some extent by hydroxyl ions (Table VI). Intracellular osmotic activity, therefore, will be decreased, though less than in the case of complete chloride-hydroxyl ion exchange. A 10 mM sodium phosphate buffer appeared to be necessary for the almost complete removal of chloride from the RBC's. In 300 mM sucrose, buffered to pH 7.5 with 1 mM sodium phosphate, intracellular chloride was only partially replaced by hydroxyl and phosphate ions (Table IV) and therefore a small lysis delay was observed. The decrease in intracellular chloride content takes place much more rapidly than the increase in phosphate concentration (Fig. 11), especially just after mixing RBC's with sucrose solution. Chloride-phosphate ion exchange will take place preferentially at low pH since RBC's appear to be much more permeable to primary phosphate than to secondary phosphate ions (Figs. 11 and 12). A large and rapid decrease of extracellular pH is observed when RBC's are added to 300 mM sucrose buffered to pH 7.5 (Fig. 14). Especially during the pretreatment when only a low extracellular volume to total red blood cell volume ratio exists (about 4), a large decrease in extracellular pH may take place and thus a chloride-phosphate exchange.

The permeability experiments are performed with 0.05 ml samples of RBC suspensions added to 10 ml samples of glycerol solutions buffered to pH 7.5 with 1 mM sodium phosphate, conditions which do not lead to momentary lysis. The ratio extracellular volume to total RBC volume

in these cases will amount to about 400 and hence the pH of the extracellular solution will be hardly affected. In this case especially chloride-hydroxyl ion exchange will take place. Even when pH decreases favouring the chloride-phosphate exchange, this exchange will hardly take place since lysis is almost complete in 150 sec, a period of time too short for a notable chloride-phosphate exchange. Summarizing, the cause of the difference between the experimentally found m_n^h value and the m_p value at t=0 might be, that a marked substitution of chloride by phosphate has taken place in RBC's which were treated with 300 mM buffered sucrose, whereas in glycerol permeation experiments at time "zero" chloride has only exchanged from RBC's against hydroxyl.

To check the validity of the mathematical description in the case of untreated and 150 mM NaCl treated RBC's, the glycerol permeability coefficients for pig RBC's were determined in some comparative studies. The glycerol permeability was determined for untreated RBC's and RBC's treated with 300 mM sucrose or 150 mM NaCl, all solutions buffered with 1 mM sodium phosphate to pH 7.5. From the lysis curves after 60 sec and longer, the m_p value in mosmol/l at t=0 was calculated with equation (9). With this m_p value at t=0 which must be equal to m_n^h, and the m_p values after different periods of time, the permeability coefficient to critical radius ratio can be calculated with equation (7). The critical radius amounted to $2.85 \cdot 10^{-4}$ cm (3.4). The results are presented in Table II. Obviously only small variations in glycerol permeability coefficients were found after the different pretreatments.

Table II

Comparison of glycerol permeability coefficients obtained with differently treated pig red blood cells.
Pig RBC's, untreated or treated with 300 mM sucrose or 150 mM NaCl were added to glycerol solutions. All solutions were buffered with 1 mM sodium phosphate to pH 7.5. The permeability was determined at 37°. The glycerol permeability coefficient is given in $cm \cdot sec^{-1} \times 10^6$.

Number of experiment	Glycerol permeability coefficients		
	Untreated	300 mM sucrose	150 mM NaCl
1	-	0.63	0.67
2	0.68	0.65	0.68
3	0.72	0.68	0.70
4	0.55	0.55	0.51
5	0.72	-	0.70

These results suggest that it is justified to simplify the permeability determinations, since after a certain moment lysis proceeds independent

of the pretreatment. Therefore, the determination of the glycerol permeability coefficient for RBC's from different mammalian origin was generally performed with untreated RBC's (Chapter 5).

4. THE ANOMALOUS LYSIS BEHAVIOUR OF UNTREATED AND NaCl TREATED RED BLOOD CELLS

4.1. INTRODUCTION

Pig RBC's, depending on their pretreatment, display differences in osmotic resistance properties in hypotonic solutions of non-permeating sucrose and in permeability behaviour in solutions of permeating glycerol (Chapter 3).
RBC's treated with 300 mM sucrose, buffered to pH 7.5 with 10 mM sodiumphosphate, showed an increased osmotic resistance in hypotonic sucrose solutions compared to that of untreated RBC's. Their permeability behaviour was in excellent accordance with theoretical predictions. RBC's treated with buffered 150 mM NaCl were less resistant than those treated with buffered 300 mM sucrose. Their permeability behaviour towards glycerol appeared to be rather complex, since a delayed lysis of about 60 sec was observed. In experiments with untreated RBC's the same phenomena, though less pronounced, were observed as with NaCl treated RBC's.
These anomalous lysis phenomena might be caused by rapid changes in osmotic properties of the RBC's. Since water equilibrium is established rapidly (Paganelli and Solomon, 1957; Blum and Forster, 1970; Farmer and Macey, 1970; Dick, 1971), the critical RBC volume will be exceeded almost instantaneously above a certain difference in initial intra- and extracellular osmotic activity. At smaller osmotic differences, however, the relatively slowly permeating glycerol molecules, accompanied by water molecules, have to pass the membrane. Thus it will take longer before the critical RBC volume is reached. In the meantime compensatory processes may take place causing either a shrinkage of the RBC's due to efflux of osmotically active material, or an increase of membrane area and thus a decrease of the volume/surface ratio. The result of these phenomena would be a delay in lysis. We assume that the differences in osmotic resistance and in permeability behaviour are related to each other. Since shrinkage of the RBC's or increase of membrane area must take place rapidly, the following possible explanations can be considered:

a) the influence of the extracellular electrolyte concentration on membrane area due to changes in membrane charge,
b) a rapid loss of osmotic active substances from RBC's in nonelectrolyte solutions. Maizels (1935), Davson (1939), Wilbrandt (1940), La Celle and Rothstein (1966) as well as Donlon and Rothstein (1969) demonstrated that in nonelectrolyte solutions human RBC's rapidly lose a considerable amount of cations and presumably also of anions,
c) variations in intracellular content of monovalent and divalent anions due to exchange with extracellular anions,
d) the exchange of hydroxylions against other anions (e.g. chloride), coupled to the buffering action of hemoglobin. Jacobs and Parpart (1933) assumed that this process is the cause of the rapid shrinkage of RBC's in non-permeating nonelectrolyte solution.

The last hypothesis is supported by the observations of Coulter (1924) that the extracellular chloride concentration increased rapidly in non-permeating nonelectrolyte solutions. A more detailed description of the hypothesis of Jacobs and Parpart was presented by the former author in 1962. This hypothesis has not yet been proven, as far as we know. Cook (1967) observed that the intracellular chloride as well as the extracellular pH varies with the cellular volume at different non-permeant concentrations. This effect was supposed to be caused by changes in net charge of hemoglobin as the spacing between the molecules varies due to cell swelling or shrinking (Gary-Bobo and Solomon, 1968). The occurrence of this effect was proven in a recent in vitro study of Gary-Bobo and Solomon (1971).

The rapid loss of osmotically active substances or the exchange of chloride and hydroxyl ions, coupled to the buffering action of hemoglobin, may decrease the mean cellular volume, whereas the cell membrane area probably does not alter. On the other hand, variation of the membrane charge will probably not influence the mean cellular volume, but will change the critical surface area either by extending the membrane area or by increasing the membrane elasticity. In all cases the osmotic resistance and thus the permeability behaviour will be altered. The exchange of hydroxyl against chloride ions, coupled to the buffering action of hemoglobin, appears to be mainly responsible for the observed phenomena at physiological pH, though small contributions of the other mentioned possibilities cannot be excluded.

4.2. MATERIALS AND METHODS

4.2.1. Isolation and treatments of red blood cells

Pig RBC's were isolated from defibrinated blood as described before (3.2.1). Sometimes they were resuspended in serum. In most cases the RBC's were treated 3-4 times at room temperature with an excess of unbuffered 300 mM sucrose, or with buffered solutions of either 300 mM sucrose or 150 mM NaCl, followed by two successive treatments with unbuffered 300 mM sucrose to remove extracellular electrolytes. The pH and buffer concentration were varied in each experiment.

4.2.2. Determination of total cell volume and mean cell volume

The average RBC volume was calculated from the total volume, which was determined with the ^{14}C-inulin dilution method, and from the number of RBC's determined with a Coulter Counter (3.2.4). This figure was used in estimating the number of untreated RBC's in one liter without medium. The concentration of some intracellular components was assayed in equal numbers of RBC's after the various treatments. Results were expressed in concentration per liter untreated cells, because the mean RBC volume may change during treatment.

4.2.3. Determination of intracellular pH

The intracellular pH was determined in a hemolysate of the RBC's in a fivefold volume of water. Though these pH values differ slightly from the actual intracellular pH (Gleichmann et al, 1965; Paymaster and Englesson, 1966), the results represent a close approximation of the intracellular pH.

4.2.4. Determination of extracellular pH changes

Extracellular pH changes were determined with a Philips pH-meter, type PW 9408, equipped with a combined glass electrode CA 13-NS and a Servogor recorder, type RE-511.

4.2.5. Determination of cation and anion concentrations

Sodium and potassium were determined in hemolysates of the RBC's as well as in serum with an Eppendorf flamephotometer. Bicarbonate was estimated in these solutions with a Natelson microgasometer (Natelson, 1963). After precipitation of protein with trichloroacetic acid (5% final concentration), inorganic phosphate was assayed with ammonium molybdate according to Bartlett (1959), chloride titrimetrically with $Hg(NO_3)_2$ (Natelson, 1963) and sulphate turbidimetrically with $BaCl_2$ in gelatin solution according to Dodgson (1961).

4.2.6. Titrimetric determination of hemoglobin net charge

The net charge of hemoglobin at different pH values was determined by potentiometric titration of 1% oxyhemoglobin in 0.1 M KCl solution at 25° (de Bruin et al, 1969; Janssen et al, 1970) with HCl and NaOH[*]

4.2.7. Osmotic resistance and permeability determinations

The osmotic resistance was determined in hypotonic sucrose solutions and permeability studies were performed in glycerol solutions (3.2.2) at 37°. The solutions were buffered with 1 mM sodium phosphate to pH 7.5, except where noted otherwise.

4.3. RESULTS

4.3.1. The influence of extracellular electrolytes on the osmotic resistance of porcine red blood cells

We determined the osmotic resistance of untreated RBC's, of RBC's treated with buffered 300 mM sucrose and of RBC's treated with buffered 150 mM NaCl (both 1 mM sodium phosphate, pH 7.5) at 37° in buffered hypotonic sucrose solutions. All resistance curves appeared to be

[*]We wish to thank Dr. L.H.M. Janssen, Department of Biophysical Chemistry, University of Nijmegen, for performing these titrations.

parallel to each other. From the values in mosmol/l causing 50% hemolysis, shown in Table III, it appears that distinct differences in osmotic resistance exist depending on the pretreatment. It seems that in hypotonic sucrose solutions a distinct number of RBC's is lysed instantaneously. Untreated and NaCl treated RBC's will be surrounded by a layer of ions at the moment of lysis. If this ionic layer would affect the osmotic behaviour of the RBC's, the variations in osmotic resistance of the different RBC suspensions should disappear after pretreatment with unbuffered 300 mM sucrose. Though in all cases a small increase in osmotic resistance was observed (Table III), the differences in osmotic resistance appeared to be maintained.

Table III. The influence of different treatments on the osmotic resistance of pig RBC's

Treatment of RBC's	50% hemolysis values in mosmol sucrose/l
Untreated	126
Untreated + 2 x unbuffered sucrose	123
4 x buffered NaCl	145
4 x buffered NaCl + 2 x unbuffered sucrose	139
4 x buffered sucrose	114
4 x buffered sucrose + 2 x unbuffered sucrose	110

Obviously the differences in osmotic resistance, caused by the different pretreatments cannot be explained by the presence or absence of an external ionic layer at the moment of lysis.

4.3.2. The loss of osmotically active substances from red blood cells in nonelectrolyte solutions

The loss of osmotically active cations and anions from RBC's was studied after various treatments (Table IV). The number of untreated RBC's representing a one liter volume was calculated from an experimentally determined mean cell volume of 61 μ^3, a value which is in agreement with the value reported in the literature (Dittmer and Grebe, 1958).

Table IV. Ion concentrations and pH in blood, serum and porcine red blood cells after various treatments

Samples containing approximately 2 ml RBC's were used. One group was washed twice with unbuffered 300 mM sucrose only. A second group was washed 4 times with 8 ml 300 mM sucrose buffered with 1 mM sodium phosphate to pH 7.5, then 2 times with unbuffered 300 mM sucrose. A third group was washed 4 times with 8 ml 150 mM NaCl, buffered with 1 mM sodium phosphate pH 7.5, then 2 times with unbuffered 300 mM sucrose. The concentrations of the ionic components in blood and serum are given in mmol/l. The concentrations in RBC's are given in mmol per $16.4 \cdot 10^{12}$ cells, the number of cells present in 1 liter of untreated RBC's. The concentrations of the ions in untreated RBC's were calculated from the concentrations in serum and blood and the total cell volume, determined by means of ^{14}C-inulin dilution (54.4% ± 0.13). The values given are the means with standard errors for 6 analyses except for HCO_3^- which was only assayed in duplicate. The intracellular pH was determined in lysates in triplicate.

	Sodium	Potassium	Chloride	Phosphate	Bicarbonate	pH
Serum	141.9±0.8	8.4±0.1	105.7±0.1	3.5±0.1	17.1-18.3	7.59±0.02
Blood	68.5±0.6	72.4±0.3	76.8±0.1	6.3±0.1	13.7-14.3	7.61±0.01
Untreated RBC's	7.1±0.3	128.1±0.5	52.6±0.2	8.7±0.1	9.4-11.4	7.60±0.01
RBC's treated with unbuffered sucrose	8.5±0.2	133.0±0.5	51.5±0.1	8.0±0.1	6.5-6.9	7.60±0.01
RBC's treated with buffered sucrose	7.0±0.1	126.4±0.8	29.7±0.3	17.2±0.1	3.4-3.6	7.89±0.03
RBC's treated with buffered NaCl	10.2±0.1	132.4±0.4	77.5±0.2	8.1±0.1	3.3-3.7	7.41±0.02

Porcine RBC's, treated with unbuffered 300 mM sucrose only, showed approximately the same cation and anion composition and content as untreated RBC's, only the bicarbonate content being lower. Treatment with unbuffered sucrose could therefore be used to remove extracellular electrolytes after the various treatments. When RBC's were treated with buffered

solutions of sucrose or NaCl, considerable changes in anion composition were observed, while the cation composition was hardly altered. The latter observation is remarkable in view of the relatively high permeability of human RBC membranes to cations in nonelectrolyte solutions (Maizels, 1935; Davson, 1939; Wilbrandt, 1940; LaCelle and Rothstein, 1966; Donlon and Rothstein, 1969). The intracellular magnesium and calcium contents have not been studied, because their contribution to the osmotic activity is negligible in view of their low intracellular concentration (Albritton, 1955). Moreover, it seems unlikely that the RBC membrane would have been more permeable to bivalent cations than to the univalent ones. Since the loss of cations from pig RBC's after these extensive treatments is negligible, a rapid loss of cations with an equivalent efflux of anions, can not be responsible for the observed phenomena. Obviously the anion composition appeared to undergo quantitatively important changes. Compared with untreated RBC's, the chloride content increased in NaCl treated cells, but decreased in sucrose treated RBC's. The phosphate content increased markedly in sucrose treated RBC's, but not in NaCl treated cells. RBC membranes of various mammalian species earlier appeared to be very well permeable to anions like chloride (Tosteson, 1959), bicarbonate (Luckner and Lo Sing, 1938), sulphate (Passow, 1969) and phosphate (Prankerd and Altman, 1955). Exchange of chloride and phosphate may play a role in the observed lysis delay. Bicarbonate cannot contribute significantly to an exchange process, since the treatments with unbuffered and buffered solutions strongly diminished its intracellular concentration. The sulphate content appeared to be less than 1 mmol/$16.4 \cdot 10^{12}$ RBC's and its contribution can therefore be neglected.

The differences in anion composition were studied more extensively in experiments at 3^{o}, 22^{o} and 37^{o}, combined with osmotic resistance determinations in hypotonic buffered sucrose solutions, in order to determine whether there would be a correlation between the changes in chloride and phosphate content. Since the variations in cation composition appeared to be small, only changes in chloride and phosphate content are given in Table V.

Table V

Anion concentration and 50% hemolysis values of pig RBC's after various treatments at three temperatures.

Samples containing approximately 2 ml RBC's were washed at the given temperatures. One group was washed twice with 8 ml unbuffered 300 mM sucrose only. A second group was washed 4 times with 8 ml 300 mM sucrose buffered with 1 mM sodium phosphate to pH 7.5, then 2 times with unbuffered 300 mM sucrose. A third group was washed 4 times with 8 ml 150 mM NaCl, buffered with 1 mM sodium phosphate to pH 7.5, then 2 times with unbuffered 300 mM sucrose. Values of phosphate and chloride are given in mmol/$16.4 \cdot 10^{12}$ RBC's. The 50% hemolysis values, determined at corresponding temperatures in buffered sucrose, are given in mosmol sucrose/l. The chloride and phosphate values represent means with standard errors from 6 analyses.

Temperature	Treatment	Phosphate	Chloride	Changes in Phosphate	Changes in Chloride	50% lysis values
22°	sucrose, unbuffered	11.1±0.2	60.4±0.5	——	——	133
3°	sucrose	11.1±0.3	45.1±0.3	0.0	-15.3	131
3°	NaCl	10.3±0.1	71.1±1.1	− 0.8	+11.3	166
22°	sucrose	21.6±0.3	38.1±0.2	+10.5	-22.3	123
22°	NaCl	10.6±0.2	70.5±1.3	− 0.5	+10.1	154
37°	sucrose	29.4±0.5	25.4±0.1	+18.3	-35.0	114
37°	NaCl	10.4±0.3	70.0±1.1	− 0.7	+ 9.6	146

At all three temperatures marked differences in osmotic resistance of NaCl treated and sucrose treated RBC's were observed. In buffered NaCl solutions a considerable increase in chloride without change in phosphate content took place at all three temperatures. In buffered sucrose solution the intracellular phosphate content was increased at 22° and 37°, but not at 3°. This effect was also observed with RBC's of man (Prankerd and Altman, 1955). At all three temperatures we observed however a decrease in chloride content. This indicates that intracellular chloride is not exchanged against extracellular phosphate only. Since the osmotic resistance and the permeability measurements were performed in sodium phosphate buffered nonelectrolyte solutions at 37°, the decrease in chloride content as well as the increase in phosphate content should be studied in more detail in order to determine which kind of exchange is taking place. The rate of exchange should be of particular importance, since only rapid changes in osmotic activity can play a role as was concluded from the lysis delay.

4.3.3. Variations in intracellular content of monovalent and divalent anions

Assuming that at 37° in buffered sucrose (and glycerol solutions) phosphate was taken up as secondary phosphate, the loss of nearly the double amount of chloride as observed at 22° and 37°, but not at 3° (Table V). could be interpreted as an exchange phenomenon. In this case the osmotic activity in the RBC's would decrease since the number of osmotically active particles in the cell would decrease. A shrinkage of the cells would be the result. This explanation could however be ruled out by exchange experiments at two different pH values.

RBC's, originating from a suspension in serum, were treated with unbuffered sucrose and subsequently suspended in a sucrose solution, buffered with 10 mM sodium phosphate at pH 7.5 and 5.5. The high buffer concentration was chosen in order to assure a nearly complete exchange of anions. After various time intervals samples were taken, and after treatment of the RBC's with ice-cold unbuffered sucrose, the intracellular chloride and phosphate content determined. We preferred addition to ice-cold unbuffered sucrose since it was observed that at low temperature no uptake of phosphate takes place (Table V).

The results obtained at pH 7.5 (Fig. 11) indicate that the chloride content decreased by 32 mmoles in 10 sec or less, while the phosphate content increased only by 1.9 mmoles per $16.4 \cdot 10^{12}$ RBC's during this period. This rules out an equivalent exchange of chloride against secondary phosphate. After this short period, however, a nearly one to one exchange on molar basis takes place, suggesting a substitution of intracellular chloride by primary phosphate, rather than by secondary phosphate.

The results obtained in a parallel experiment at pH 5.5 showed a less drastic decrease in intracellular chloride and an increased uptake of phosphate, as was also observed for human RBC's at low pH (Holton, 1952). In this case a nearly one to one exchange of chloride and phosphate on molar basis was observed (Fig. 12).

From Table V and Fig. 11 it may be concluded that at physiological temperature and pH in buffered sucrose the decrease in intracellular chloride content is only partially coupled to an exchange with phosphate. RBC's suspended in NaCl solutions showed an increased chloride content together with an unchanged phosphate content (Table V). Therefore, it seems likely that another process, possibly an exchange of chloride and hydroxyl ions coupled to the buffering action of hemoglobin as

proposed by Jacobs (1962), is involved in the anomalous lysis behaviour of untreated RBC's and RBC's treated with buffered 150 mM NaCl.

Fig. 11

Uptake of phosphate (●) and the loss of chloride (o) by pig RBC's a· pH 7.5 and 37°.
To 48 ml buffered 300 mM sucrose (10 mM sodium phosphate) 12 ml RBC suspension in unbuffered 300 mM sucrose, was added. At various times 2 ml samples were added to 8 ml ice-cold 300 mM unbuffered sucrose and the RBC's were spun down at once. The RBC's were treated once more with ice-cold unbuffered sucrose and then lysed with water.

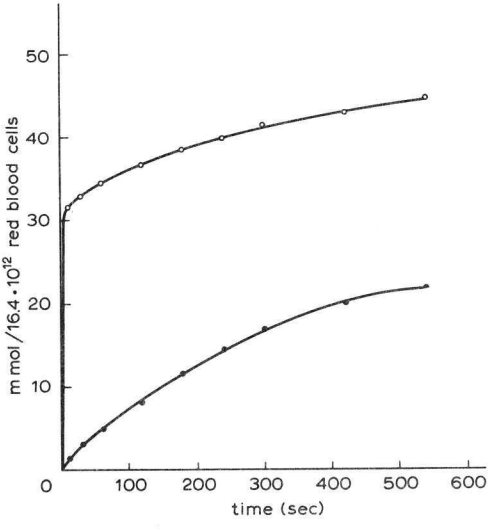
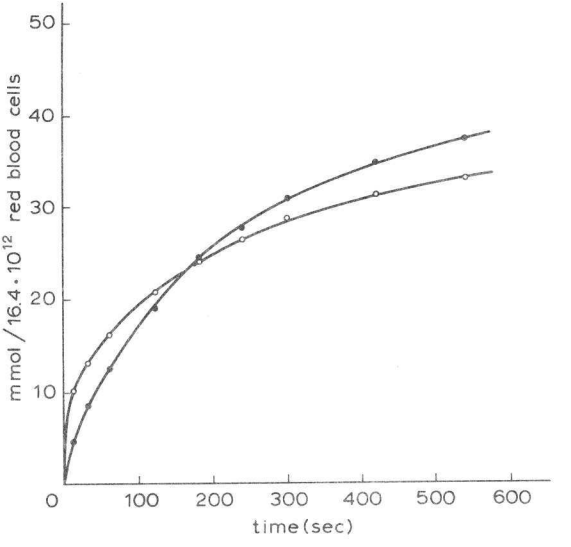


Fig. 12

Uptake of phosphate (•) and loss of chloride (o) by porcine RBC's at pH 5.5 and 37°.
The same method was used as in the experiment of Fig. 11.



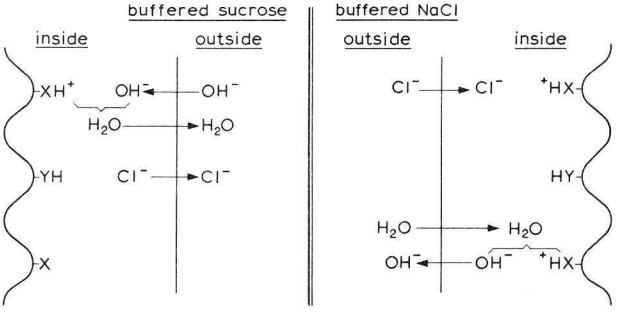
4.3.4. The exchange of hydroxyl ions against other anions and the buffering action of hemoglobin

In non-permeating nonelectrolyte solutions a chloride-hydroxyl ion exchange, coupled to the buffering action of hemoglobin, was proposed by some investigators (Coulter, 1924; Jacobs and Parpart, 1933) on the basis of indirect evidence. We attemped to prove that this may also occur in solutions of the permeating substance glycerol, and that this could explain the observed delayed lysis. A direct proof appeared however difficult. We therefore extensively studied the different aspects of this hypothesis in solutions of the non-permeating nonelectrolyte sucrose and the electrolyte NaCl. Though only indirect proof could be obtained, it is assumed from the data that this hypothesis also holds for RBC's in solutions of the permeating substance glycerol. Since the work of Van Slyke et al (1923) it has been accepted that the distribution of bicarbonate, chloride and hydroxyl ions between the

intra- and extracellular solutions obeys the Gibbs-Donnan equilibrium. From the concentration ratios can be calculated that in RBC's suspended in plasma about 60% of the intracellular cations are electrically balanced by chloride and bicarbonate, the remainder being accounted for mainly by hemoglobin and to a lesser extent by inorganic phosphate and phosphate esters. Upon addition to buffered sucrose or NaCl solutions a redistribution of anions will take place. In Fig. 13 a schematic representation of this exchange process in buffered sucrose and NaCl solutions is given.

Fig. 13

Schematic representation of chloride-hydroxyl exchange, coupled to the buffering action of hemoglobin.
X represents an imidazole or amino group and Y a carboxylic group.



In buffered sucrose solutions (pH 7.5) an exchange of intracellular chloride against extracellular hydroxyl ions will increase the intracellular pH resulting in a concomitant liberation of protons from hemoglobin. The extracellular pH will decrease. These processes cause a decrease of the intracellular osmotic activity, since the inorganic anion concentration is lowered. Treatment with buffered NaCl solution (pH 7.5) causes an influx of chloride ions, an efflux of hydroxyl ions

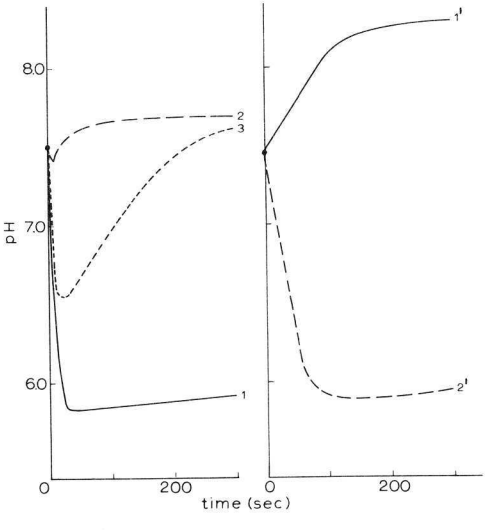
and a protonation of hemoglobin, together resulting in an increase of the intracellular osmotic value. It follows that the initial composition, the pH and the buffer capacity of the extracellular solution and to a lesser degree the duration of the treatment, will affect the following, closely connected parameters:

a) the direction and extent of chloride-hydroxyl exchange,
b) the extent of chloride-phosphate exchange in buffered nonelectrolyte solution,
c) the intracellular and extracellular pH,
d) the net charge of hemoglobin,
e) the mean cellular volume.

We investigated the change in extracellular pH upon addition of RBC's to buffered solutions containing sucrose, NaCl or Na_2SO_4 (Fig. 14).

Fig. 14

Changes in extracellular pH after addition of red blood cells to different solutions.
RBC's were treated twice with an excess of unbuffered sucrose to remove extracellular serum, and resuspended in unbuffered sucrose to a hematocrit of about 0.5. To 48 ml buffered solution of 300 mM sucrose (curve 1), 150 mM NaCl (curve 2) or 100 mM Na_2SO_4 (curve 3) respectively, 12 ml RBC suspension was added. All solutions were buffered with 1 mM sodium phosphate, pH 7.5 at 37°. Sucrose suspended RBC's were spun down and added again to buffered NaCl, pH 7.5 (curve 1'). NaCl suspended RBC's were added after centrifugation to buffered sucrose, pH 7.5 (curve 2'). The pH was recorded continuously (4.2.4).



In 300 mM buffered sucrose, a rapid and sharp decrease in extracellular pH to about 5.8, being almost complete in 30 sec, was observed (curve 1). Whereas the extracellular and intracellular hydroxyl ratio at the start was about one, the intracellular and extracellular chloride ratio was near infinity, probably resulting in a considerable exchange of extracellular hydroxyl against intracellular chloride ions. After 30 sec a slow increase in extracellular pH is seen, which may be due to a gradual loss of cations and anions (Passow, 1964) or to an exchange of intracellular hydroxyl against extracellular secondary phosphate ions. The latter pass the membrane much more slowly than chloride ions, as was shown in Fig. 11. In curve 1' the extracellular pH change is shown which occurs when these RBC's, after removal of the sucrose solution, are added to NaCl solution. An increase of the extracellular pH to about pH 8.3 was seen. This pH increase was probably caused by a considerable exchange of intracellular hydroxyl against extracellular chloride ions.

When RBC's suspended in unbuffered sucrose were added to the buffered NaCl solution, a small and rapid decrease of the extracellular pH to about 7.4 was observed (curve 2), followed by a more pronounced but slower increase to 7.7. The rapid and small pH decrease was probably caused by the low pH of the RBC suspension in unbuffered sucrose. The slow increase in extracellular pH might be caused by an exchange of intracellular hydroxyl against extracellular chloride ions since the extracellular/intracellular chloride ratio was nearly 1.9 at the start, assuming a chloride content of 52 mmol/l cells (Table IV) and a water content of 65% (Dittmer and Grebe, 1958). Since the intra- and extracellular pH were nearly equal, the initial ratio of extracellular and intracellular hydroxyl ion concentration was approximately one. When RBC's were treated first with buffered sucrose, a more pronounced increase in extracellular pH in NaCl solutions was observed (curve 1'). Here the extra- and intracellular chloride ratio was about 3.4, the intracellular chloride content being about 29 mmol/l RBC's (Table IV). Though the permeability to small anions like chloride and hydroxyl is high (Tosteson, 1959 and Crandall et al, 1971, respectively) equilibrium is reached only after a rather long period of time. Possibly, this is caused by the decreasing ratio of extracellular/intracellular chloride. When RBC's, first treated with buffered NaCl, were added to buffered sucrose, a rapid decrease in extracellular pH to about 5.9 was observed (curve 2'). The small differences in intracellular pH of RBC's treated with unbuffered sucrose and those treated with buffered

NaCl (Table IV) possibly can explain the differences between curve 1 and 2' in the rate of decrease of the extracellular pH. In both cases the ratio of intracellular versus extracellular chloride approaches infinity. Since the permeability of the RBC membrane to sulphate ions is much lower than that to chloride and hydroxyl ions (Passow, 1964), the sharp decrease in extracellular pH (curve 3) probably originated from the exchange of intracellular chloride against extracellular hydroxyl ions, and the slow increase of extracellular pH from the exchange of extracellular sulphate against intracellular hydroxyl ions (Passow, 1964).

The exchange phenomena should also influence the intracellular pH, the anionic composition and charge, the net charge of hemoglobin and the cell volume. Since the extracellular pH and the composition of the solution of non-permeant will affect the parameters mentioned, we analysed RBC's after treatment with sucrose and NaCl solutions at various pH values (Table VI).

The net charge of hemoglobin could be calculated from the titration curve of porcine oxyhemoglobin (Fig. 15), assuming a hemoglobin content of pig RBC's of 35% w/v (Dittmer and Grebe, 1958).

Fig. 15

Titration curve of pig oxyhemoglobin.
Abscis: pH. Ordinate: net charge of hemoglobin in equiv./mole.
Protein concentration 10 mg/ml. Ionic strength 0.1. Temperature 25°.

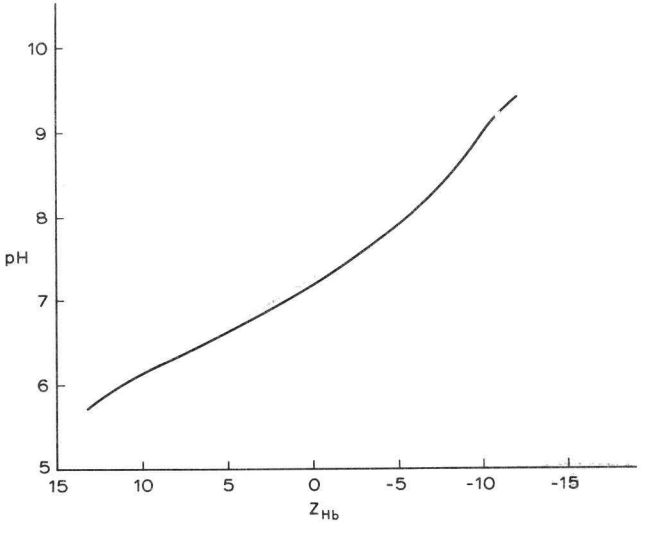


Table VI

Correlation of changes in intracellular pH, total anionic charge, net charge of hemoglobin and cell volume due to differences in composition and pH of washing solutions.

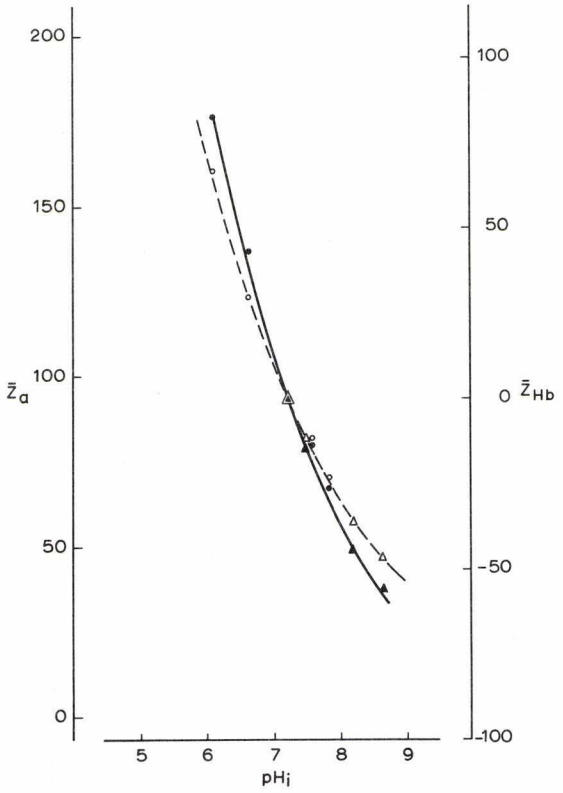
Samples containing approximately 2 ml RBC's were used. One group was washed twice with 8 ml 300 mM unbuffered sucrose only. A second group was washed 4 times with 8 ml 300 mM sucrose buffered with 10 mM sodium phosphate to different pH values, then 2 times with 300 mM unbuffered sucrose. A third group was washed 4 times with 8 ml 150 mM NaCl, buffered with 10 mM sodium phosphate to different pH values, then 2 times with 300 mM unbuffered sucrose. The total anionic charge was calculated from chloride and phosphate concentrations and the intracellular pH. The net charge of hemoglobin was calculated from hemoglobin content (36%) and intracellular pH by means of the titration curve of Fig. 15. Both charges are given in meq/$16.4 \cdot 10^{12}$ RBC's. The cell volume was determined from ^{14}C-inulin dilution and compared to that found for RBC's treated with unbuffered sucrose (100%). The values for chloride, phosphate and cell volume are means with standard errors for 6 determinations.

Treatment	Extracellular pH	Intracellular pH	Chloride	Phosphate	Total anionic charge	Net charge of hemoglobin	Cellular volume in %
Sucrose unbuffered	5.9	7.8	50.6±0.4	6.7±0.1	62.6	- 25.0	100.0±0.4
NaCl	8.5	7.8	56.4±0.1	5.6±0.1	66.5	- 25.0	99.4±0.4
NaCl	7.5	7.5	73.0±0.1	5.6±0.1	82.5	- 14.1	103.4±0.3
NaCl	6.5	6.6	129.8±0.9	6.3±0.1	137.4	+ 28.0	108.2±0.4
NaCl	5.5	6.1	169.6±0.7	6.3±0.1	176.4	+ 64.9	114.2±0.8
Sucrose	8.5	8.6	5.6±0.3	15.3±0.1	35.6	- 47.5	91.9±0.2
Sucrose	7.5	8.2	5.5±0.1	22.0±0.1	47.5	- 37.7	93.0±0.3
Sucrose	6.5	7.5	6.5±0.2	42.6±0.2	77.4	- 13.3	94.0±0.1
Sucrose	5.5	7.2	20.6±0.3	48.3±0.3	93.1	- 0.5	98.2±0.6

The variations in anion composition, and thus in total anionic charge were considerable and appeared to be correlated with the changes in the net charge of hemoglobin (Fig. 16), as previously assumed by Jacobs (1962).

Fig. 16

Relation between intracellular pH (pH_i), net hemoglobin charge ($\bar{Z}_{Hb}$ Δ , o) and total anionic charge ($\bar{Z}_a$ ▲ , ●), both in meq/16.4·10^{12} red blood cells. Data were obtained from Table VI. The points (Δ) and (▲) are derived from RBC's treated with buffered sucrose, the points (o) and (●) from RBC's treated with buffered NaCl.



A substantial cation efflux was observed in buffered 300 mM sucrose at all pH values. This was also observed by Maizels (1935), Davson (1939), Wilbrandt (1940), La Celle and Rothstein (1966) and Donlon and Rothstein (1969). The changes in chloride content and pH were comparable with those observed in pig erythrocytes (Table VI).

The observed deviations at higher and lower pH values may be caused by inaccuracies in the determination of the intracellular pH. Since the degree of oxygenation of hemoglobin will be somewhat different at the various intracellular pH values and small differences in net charge between oxyhemoglobin and hemoglobin exist (Janssen et al, 1970), this phenomenon may contribute to the small discrepancy between the net charge of hemoglobin and the anionic charge. Studies of Gary-Bobo and Solomon (1968 and 1971) indicate that the net charge of hemoglobin decreases with increasing intracellular concentration. This would be the case at higher intracellular pH values, when the RBC's have shrunk due to the chloride-hydroxyl ion exchange. The curves for anionic charge and net charge of hemoglobin have been vertically displaced with regard to each other so as to make them coincide at pH 7.2 where $\bar{Z}_{Hb}$ is zero and the net anionic charge is 93.1 meq/$16.4 \cdot 10^{12}$ RBC's.

Changes in anion content and hemoglobin charge proved to be accompanied by changes in cellular volume (Table VI). Since particularly the chloride content of RBC's varied with pH and the half time for chloride exchange is approximately 0.2 sec (Tosteson, 1959), a rapid chloride-hydroxyl ion exchange, coupled to variations in mean RBC volume, appeared to be likely. Furthermore, in buffered NaCl solutions only slight changes in phosphate content were observed, whereas the chloride content increased with decreasing pH values of the NaCl solutions. In buffered sucrose solutions, however, a more complex situation exists, since besides the decline of the chloride content at all pH values, an increased phosphate content was observed at decreasing pH values of the sucrose solutions.

The same experiment as reported in Table VI was performed with human RBC's at 3^{o}, since at room temperature considerable lysis takes place in isotonic sucrose solutions at neutral and alkaline pH values. The most pronounced changes in ion content in buffered sucrose and NaCl solutions, compared with unbuffered sucrose, are presented in Table VII.

Table VII

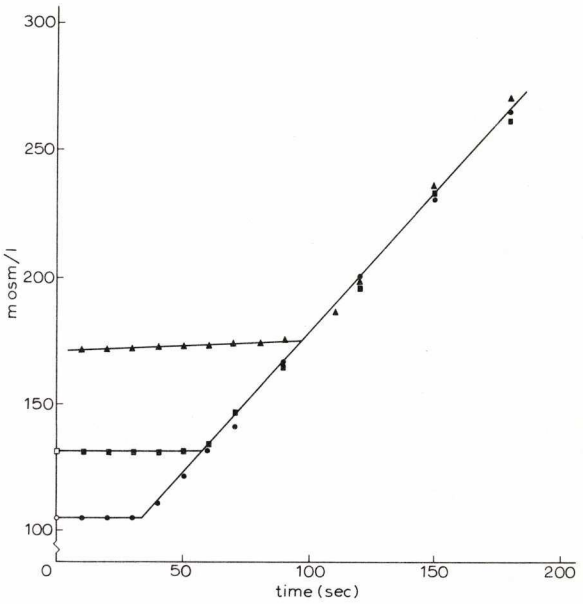
Changes in intracellular pH and in cation and anion composition of human RBC's after different treatments.
RBC's were treated at 3° with unbuffered sucrose, buffered sucrose pH 8.5 and buffered NaCl pH 5.5.
The same method was used as in Table VI. Determinations were performed in duplicate.

Treatment	Extra-cellular pH	Intra-cellular pH	Cation content	Chloride	Phosphate
Sucrose unbuffered	5.8	7.5	121.7	63.6	3.2
NaCl	5.5	6.1	126.7	170.7	2.8
Sucrose	8.5	8.6	101.4	8.4	5.0

Proof that the chloride-hydroxyl ion exchange influences the osmotic resistance in buffered sucrose as well as the delayed lysis in buffered glycerol solutions, was obtained with porcine RBC's containing different chloride concentrations. RBC's were used either untreated, or treated with 150 mM NaCl, buffered with 10 mM sodium phosphate at pH 6.0 and 7.5. Then they were treated with unbuffered sucrose. In comparison with untreated RBC's, only the chloride content was changed in NaCl treated RBC's. It was increased by a factor 1.8 at pH 7.5 and a factor 3.4 at pH 6.0. The retardation of the lysis in buffered glycerol solutions (5 mM sodium phosphate, pH 7.5) appeared to be larger at higher intracellular chloride content (Fig. 17).
The osmotic resistance in hypotonic buffered sucrose (5 mM sodium phosphate, pH 7.5) was also influenced and appeared to be lower at higher intracellular chloride content. After treatment with NaCl at pH 6.0 the experimentally determined resistance in hypotonic buffered sucrose solutions appeared to be lower than the value obtained by extrapolation of the line, which represents the delayed 50% hemolysis values in glycerol, to t = 0. The cause of this phenomenon is still unclear.

Fig. 17

Fifty percent hemolysis values in mosmol/l at 37° of pig red blood cells in buffered solutions of glycerol and sucrose (5 mM sodium phosphate, pH 7.5).
Untreated RBC's in sucrose (o) and in glycerol (●).
RBC's treated with NaCl, pH 7.5 in sucrose (□) and in glycerol (■).
RBC's treated with NaCl, pH 6.0 in glycerol (▲).



4.4. CONCLUSIONS

An explanation for the delay of lysis of NaCl treated or untreated RBC's in glycerol solutions can now be given. In strongly hypotonic glycerol solutions the RBC's lyse almost instantaneously due to water transport only. At sufficiently high concentrations of glycerol, however, two processes take place acting in opposite directions. Glycerol molecules pass the membrane leading to an increase of the mean cell volume. In the meantime a rather rapid chloride-hydroxyl ion exchange takes place, which is probably complete within seconds and leads to a decrease of the mean cell volume. It follows that when the buffer capacity of the glycerol solutions is sufficiently high and lysis does not take place at once, the RBC's will shrink rapidly to a certain minimum volume, almost independent of the initial intracellular chloride con-

tent. The buffer capacity of 1 mM sodium phosphate, pH 7.5, will generally be sufficient, since the ratio of the total RBC volume and the volume of the test solution is about 1 : 400. Moreover the lysis rate of the RBC's will be equal after the disappearance of the differences in chloride content, which could be experimentally established (Fig. 17). Now we have shown that a delay of lysis may take place in solutions of permeating nonelectrolytes, the question arises to what extent, besides the factors described in chapter III, this phenomenon affects the time of lysis. In general the osmotic lysis method is performed at physiological pH values in isotonic solutions of permeant with untreated or NaCl treated RBC's. Hence a normal or increased amount of intracellular chloride ions will be present. When the RBC's are suspended in an excess of buffered, permeating nonelectrolyte, a rapid chloride-hydroxyl ion exchange will take place resulting in a delay of lysis. Prevention of this lysis delay would lead to a decrease of the lysis time. The lysis time determined in this way will represent the lysis rate due to permeant diffusion only. It can be concluded from Fig. 17, for instance, that whereas in 300 mM glycerol 50% lysis of untreated pig RBC's would be found after approximately 240 sec, the same 50% lysis value in 300 mM glycerol due to glycerol diffusion only would be found after approximately 200 sec since a lysis delay of about 40 sec was observed. The extent of the delay depends primarily on intracellular pH and chloride content, and also on extracellular pH and buffer capacity. Probably the intracellular pH of untreated RBC's of different mammalian species varies somewhat, whereas also differences in the intracellular chloride content exist (Albritton, 1955). Since, however, the chloride-hydroxyl ion exchange is complete in a short period of time, this phenomenon does not affect the permeability coefficient for glycerol when this is determined with the modified lysis method as shown in chapter 3.

5. COMPARISON OF GLYCEROL PERMEABILITY AND LIPID COMPOSITION OF RED BLOOD CELL MEMBRANES FROM EIGHT MAMMALIAN SPECIES

5.1. INTRODUCTION

In chapter 2 we explained that the original lysis method of Jacobs et al (1950) has some shortcomings in comparative permeability studies of RBC membranes to simple nonelectrolytes. We therefore introduced a modification of the original lysis method. In contrast to the assumptions in chapter 2, a complex lysis behaviour in glycerol solutions was observed with untreated pig RBC's as well as with pig RBC's pretreated with buffered 150 mM NaCl pH 7.5 (3.3.3 and 3.3.4). After pretreatment of pig RBC's with buffered 300 mM sucrose of pH 7.5, the lysis behaviour of the RBC's in glycerol solutions appeared to be in accordance with the mathematical description of the lysis process presented in chapter 2 (3.3.5). In chapter 4 it was shown that the observed lysis delay with untreated and NaCl treated RBC's is probably due to chloride-hydroxyl ion exchange, coupled to the buffering action of hemoglobin. Since this exchange takes place rapidly, resulting in shrinkage of the RBC's, an extra amount of permeant has to enter the RBC in order to cancel this effect. Thereafter, lysis proceeds at the same rate as observed with RBC's pretreated with buffered 300 mM sucrose (3.6). Hence it appeared to be possible to calculate permeability coefficients from the more complex lysis data obtained with untreated RBC's. In this chapter the glycerol permeability coefficients of RBC membranes from different mammalian species at 37° and pH 7.5 are compared with the times of hemolysis. The latter seemed less reliable on theoretical grounds. Although in general osmotic lysis experiments are performed at room temperature (Jacobs et al, 1950; Szelényi and Hollán, 1968; Moore, 1968), it seemed to us to be preferable to perform such experiments at 37°. Our reason for this is that at low temperature the observed marked differences in times of lysis might be due to pre-lytic loss of potassium and anions and to differences in hemoglobin release. The former authors did not rule out these phenomena in their experiments.

In the second part of this chapter the values for glycerol permeability are compared with literature data on the lipid composition.

5.2. MATERIALS AND METHODS

5.2.1. Blood sampling

Fresh blood, with heparin as anticoagulant (50 I.U. per 10 ml of blood), was used. Pig and ox blood were defibrinated with glass beads. Whole blood, without pretreatment, was used throughout the experiments. In some cases human and sheep RBC's were pretreated once, with 300 mM sucrose, buffered to pH 7,5 with 5 mM sodium phosphate at room temperature.

5.2.2. Permeability measurements

The permeability of RBC's to glycerol was determined at 37° in hypotonic and isotonic glycerol solutions buffered to pH 7,5 with 1 mM sodium phosphate. At different moments lysis was stopped by the addition of 1.5 M NaCl. Technical details are given in 3.2.2. In the case of rat, man and rabbit the permeability to glycerol was also studied in hypertonic glycerol solutions due to high permeability of their RBC's for this substance.

5.2.3. Determination of mean cell volume and critical cell volume

Mean cell volume in plasma or serum was determined with the ^{14}C-inulin dilution method (4.2.2). The mean critical cell volume was determined by means of a modification of the same method (3.2.4).

5.3. RESULTS

5.3.1. Comparison of red blood cells from defibrinated and heparinized blood

The osmotic resistance in buffered sucrose solutions of pig and human RBC's obtained from defibrinated and heparinized blood appeared to be equal, in accordance with the results of Dacie and Lewis (1968).

Moreover, the lysis rates in 300 mM glycerol solutions did not differ significantly. Therefore, it was justified to use heparinized blood in our experiments.

5.3.2. Concentration dependence of the permeability coefficient

It was shown in chapter 3 (3.3.5) that the lysis behaviour of pig RBC's in glycerol and in sucrose solutions after pretreatment with buffered 300 mM sucrose pH 7.5 was in good accordance with the mathematical description of lysis given in chapter 2. These data supported the assumption that the reflection coefficient for glycerol does not differ very much from one. It therefore seemed to be justified to neglect in our studies this reflection coefficient and to use the simplified mathematical description of lysis. Pig RBC's, however, are rather poorly permeable to glycerol in contrast to those of man, rat and rabbit. No proof was given that the reflection coefficient may be neglected in all cases. In this section it will be shown that under comparable circumstances also the lysis behaviour of sheep and human RBC's is in agreement with theoretical predictions, and that the reflection coefficient does not deviate very much from one.
In order to remove intracellular chloride, human RBC's were treated with an excess of buffered sucrose. The pretreatment was not repeated, since it was observed that human RBC's in nonelectrolyte solutions loose cations and anions (Maizels, 1935; Davson, 1939; Wilbrandt, 1940; La Celle and Rothstein, 1966; Donlon and Rothstein, 1969).
The lysis behaviour of these pretreated RBC's was studied in buffered sucrose and glycerol solutions. In Fig. 18 the results are presented. Lysis curve 0 represents the osmotic resistance of the RBC population in sucrose solutions, whereas curves 1-7 represent the degree of lysis in glycerol solutions of varying tonicity and at various moments. Obviously no lysis delay takes place, as was also observed for pig RBC's (3.3.5). The data indicate that an ever-increasing number of RBC's lysed due to the gradual increase of the intracellular glycerol concentration. The 50% hemolysis values plotted versus time confirm the foregoing statement (Fig. 19).

Fig. 18.

Lysis of sucrose treated human red blood cells in sucrose and in glycerol solutions.
Human RBC's were pretreated once with a tenfold volume of 300 mM sucrose, buffered with 5 mM sodium phosphate to pH 7.5. Lysis was determined at 37° in sucrose and in glycerol solutions, both buffered with 1 mM sodium phosphate to pH 7.5.
Curve 0 represents the osmotic resistance in sucrose after 300 sec, curves 1-7 the lysis in glycerol after 5, 10, 15, 20, 25, 30 and 35 sec, respectively.

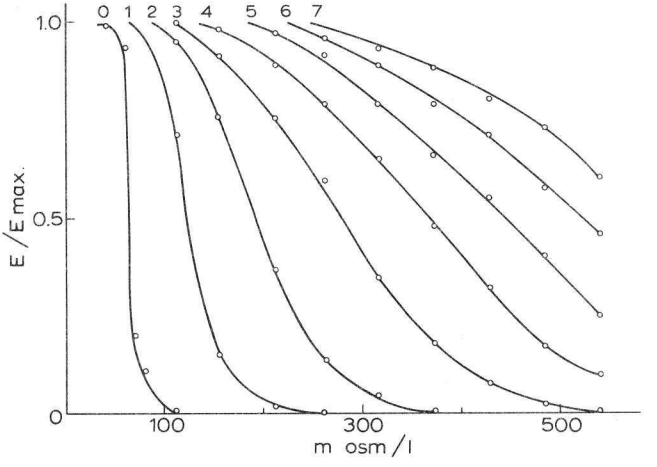
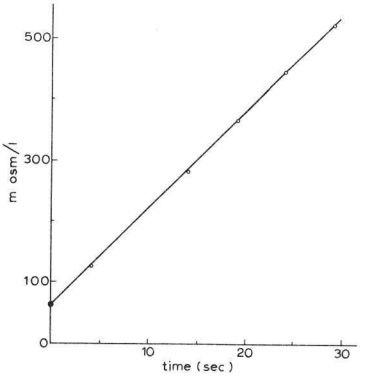


Fig. 19.

Fifty percent hemolysis values of sucrose treated human red blood cells. Red blood cells were treated once with 300 mM sucrose buffered to pH 7.5 with 5 mM sodium phosphate. Fifty percent hemolysis values are expressed in mosmol/l for glycerol (o) and sucrose (●) solutions, buffered to pH 7.5 with 1 mM sodium phosphate, as a function of time.



Since addition of RBC's and mixing take about one second, the data are plotted against the times given in Fig. 18, minus one second. The values in glycerol solutions showed a nearly linear relationship. Extrapolation to t=0 gives a m_p value (the concentration of permeating substance which causes, due to waterflow only, 50% hemolysis), fitting very well with the experimentally determined m_n^h value (the concentration of non-permeating sucrose which causes momentary 50% hemolysis due to waterflow). Since the reflection coefficient is equal to the ratio of the observed osmotic pressure and the theoretical osmotic pressure, a given concentration of permeant molecules never exerts its full osmotic effect, even at t=0 when no molecules have penetrated the membrane (Goldstein and Solomon, 1960). Extrapolation of our permeation data at various times to t=0 leads to a concentration of permeant causing a certain degree of lysis which cannot be distinguished from the concentration of non-permeant causing the same degree of lysis.
On theoretical grounds, the reflection coefficient must depend to some extent on the concentration of extracellular permeant (Stein, 1967; Sha'afi et al, 1970). The permeability coefficients of human and sheep RBC's, calculated by means of equations (7) and (9) from m_p at t=0 and the m_p values after various time intervals, appeared to be nearly independent of concentration (Table VIII).

Table VIII

Concentration dependence of the glycerol permeability coefficient. The data are obtained with RBC's from one man and one sheep, after treatment with 300 mM sucrose buffered to pH 7.5 with 5 mM sodium phosphate. The P/R_h ratio was calculated with equation (7) from the glycerol concentrations in mosmol/l causing 50% hemolysis at different times (m_p), taking m_n^h equal to m_p at t=0 (calculated with equation (9)). The permeability coefficient for glycerol (P in cm. $sec^{-1} \times 10^6$) was calculated from this ratio with R_h equal to 3.20×10^{-4} and 2.34×10^{-4} cm, respectively for man and sheep (Table X).

Human RBC's				Sheep RBC's			
Time (sec)	m_p	P/R_h	P	Time (sec)	m_p	P/R_h	P
0	64	-	-	0	127	-	-
4	125	6.0	19.2	30	147	0.16	0.37
9	195	5.0	16.0	60	164	0.14	0.33
14	284	5.0	16.0	90	184	0.14	0.33
19	365	4.8	15.5	120	208	0.14	0.33
24	445	4.8	15.5	150	235	0.15	0.35
				180	262	0.15	0.35
				210	288	0.15	0.35
				240	316	0.14	0.33

The resulting P glycerol values were constant, except for a somewhat higher P glycerol in the first time interval. This means that the reflection coefficient for glycerol is nearly one for extremes in glycerol permeability like in human and sheep RBC's. Thus, it seems reasonable to conclude that for all mammalian RBC's the reflection coefficient will not deviate much from one. This conclusion is in good agreement with the glycerol reflection coefficient for human RBC's of Stein (1967), who calculated a value of 0.995-0.9997, but it deviates markedly from the results of Goldstein and Solomon (1960), who obtained a value of 0.88 ± 0.02 for human RBC's.

5.3.3. Lysis behaviour of untreated mammalian red blood cells in glycerol solutions

As stated in chapter 3, the permeability coefficients of untreated pig RBC's for glycerol could be calculated from the lysis data obtained in glycerol solutions of different tonicity and at various moments, since after a certain period of delayed lysis, lysis proceeds at a rate, which is independent of the pretreatment. We assume that this is also true for RBC's from other mammalian species.
The ratio of the permeability coefficient (P) and the critical cell radius (R_h) were calculated from the lysis curves in glycerol (37^{o} and pH 7.5) of untreated RBC's from rat, man, rabbit, pig, horse, dog, ox and sheep with equations (7) and (9). The critical cell radius was determined by extrapolating the mean cell volume found at different tonicities to the tonicity causing 50% lysis (3.2.4). From the P/R_h ratios and the determined R_h values (Table X), the permeability coefficients at the 50% hemolysis level were calculated. These data are compared with the times of hemolysis, that is the time elapsing before 50% hemolysis has occurred in 300 mM glycerol.
It is theoretically possible to determine the permeability coefficient at different levels of hemolysis, which represent different fractions of the RBC population. However, this appeared to be impossible in this study, since only the mean critical radius is known. The permeability coefficient at the 50% hemolysis level represents the average permeability of the RBC populations. The data obtained for eight mammalian species are presented in Table IX.

Table IX

Comparison of glycerol permeability coefficients and hemolysis times in eight mammalian species.
Times of hemolysis were determined in 300 mM glycerol and permeability coefficients in hypotonic and isotonic glycerol solutions. All solutions were buffered with 1 mM sodium phosphate to pH 7.5. Permeability coefficients and times of hemolysis with their standard ~~errors~~ deviations were determined at the 50% hemolysis level.
In parentheses the number of subjects is given for each species.

Species	P glycerol ($cm.sec^{-1}x10^{6}$)	Time of hemolysis (sec)
Rat(6)	19.96±3.28	5±1
Man(7)	18.27±4.23	13±2
Rabbit(10)	3.65±2.62	57±30
Dog(3)	1.37±0.20	164±8
Horse(8)	1.02±0.12	114±12
Pig(9)	0.70±0.05	177±11
Ox(7)	0.43±0.09	562±50
Sheep(8)	0.32±0.01	258±21

It can be concluded from Table IX that, although the time of hemolysis gives an impression of the RBC membrane permeability, the glycerol permeability coefficients determined with the modified osmotic lysis method are more reliable. The hemolysis times suggest that dog RBC membranes are less permeable to glycerol than those of horse and the same can be said for ox and sheep RBC membranes. The permeability coefficients for glycerol, however, demonstrate the reverse.
The variability in the permeability coefficient and the time of hemolysis do not seem to be due to variations in the P/R_h ratios and to inaccuracies of the determination of R_h, since the reproducibility of the determinations appeared to be sufficiently precise (Table X). Since few values of the critical cell volume are given in the literature, the mean cell volumes found in plasma or serum are compared with data from the literature in order to check the cell volume determination with ^{14}C-inulin.

Table X

Reproducibility of permeability coefficient determinations. Comparison of the ratio of the glycerol permeability coefficient and critical radius (P/R_h), the critical cell volume (V_h), the critical radius (R_h), the mean cell volume in plasma or serum (MCV) and the MCV's from literature. V_h, R_h and MCV were determined in duplicate for three subjects. P/R_h ratios were determined for the number of animals given between brackets, V_h and MCV are given in μ^3, R_h in 10^{-4} cm. ± SD.

Species	P/R_h	V_h	R_h	MCV	MCV (Literature) W	D&G	D&P
Rat (6)	69.08±11.36	100.2±3.3	2.89±0.07	62.7±3.7	-	61	41
Man (7)	57.11±13.20	137.6±3.6	3.20±0.10	87.7±5.7	87	87	86
Rabbit (10)	12.42±8.97	104.3±1.1	2.92±0.01	64.3±2.3	57	61	60
Dog (3)	5.14±0.76	79.8±2.1	2.67±0.05	65.1±1.3	67	66	68
Horse (8)	4.27±0.49	58.6±2.5	2.40±0.06	49.7±2.5	-	-	48
Pig (9)	2.36±0.23	93.5±1.5	2.85±0.05	61.5±2.3	58	61	58
Ox (7)	1.26±0.22	99.8±0.9	2.88±0.02	51.1±3.6	58	50	48
Sheep (8)	1.36±0.05	55.4±0.4	2.34±0.01	35.3±2.5	30	31	32

References: W:Whittam (1964), D&G:Dittmer and Grebe (1958), D&P:Dunker and Passow (1953).

The determination of the critical volume appears to be reliable, since the figure obtained for human RBC's comes close to the values given by Canham (1969) and Seeman et al (1969). Jacobs (1934) presented some arguments for an increase of the ox RBC volume by a factor 1.7 before lysis. In our case this value is almost 1.9. The mean cell volumes appeared to be in accordance with data in literature. Obviously the discrepancies in sequence of the glycerol permeability coefficients and the times of hemolysis are real and are probably due to variations in osmotic resistance and lysis delay. Coldman et al (1970) also observed considerable variations in osmotic resistance for RBC's from some mammalian species. When RBC's are suspended in glycerol solutions, lysis does not take place instantaneously. In the meantime a considerable chloride-hydroxyl ion exchange, coupled with a buffering action of hemoglobin, takes place, which results in shrinkage of the RBC's. The osmotic resistance of these RBC's will, therefore, be increased. From the lysis curves in glycerol after different time-intervals the lysis curve at t=0 can be calculated with equation (9) (3.4). The 50% hemolysis values in mosmol/l, obtained in this way, amounted to nearly 65 for rat, 89 for man and 126 for sheep RBC's. With untreated human and rat RBC's hardly any lysis delay was observed, whereas with untreated pig

RBC's a lysis delay of 40-50 sec was observed (3.3.3).

Whereas in general within each species the standard ~~errors~~ deviations for the ratio of permeability coefficient to critical radius (P/R_h), as well as the permeability coefficient ranged from 10 to 20%, much higher standard ~~errors~~ deviations (up to 72%) were observed for rabbit RBC's. This led us to study the permeability behaviour of rabbit RBC's to glycerol in more detail. The results will be presented in chapter 6. It is impossible to compare our glycerol permeability coefficients and the times of hemolysis with data from the literature, since the published experiments were performed at undefined room temperatures (Jacobs et al, 1950; Szelényi and Hollán, 1968; Moore, 1968). Though de Gier et al (1966) carried out some experiments at different temperatures, no comparable times of hemolysis from their publication can be obtained.

5.4. COMPARISON OF LIPID COMPOSITION AND GLYCEROL PERMEABILITY OF MAMMALIAN RED BLOOD CELLS

As shown in the preceding section, the glycerol permeability of RBC's from some mammalian species differs markedly. It is known that their lipid composition also varies considerably (de Gier and van Deenen, 1964; Nelson, 1967; Rouser et al, 1968). A comparison of the glycerol permeability coefficients with these data will be presented in this section.

a. Phospholipid, cholesterol and glycolipid content of red blood cell membranes

De Gier et al (1968) observed that the glycerol permeability of liposomes was correlated to some extent with cholesterol content and with phospholipid composition. Although it would be useful to know the number of lipid molecules per unit of membrane area, this information is scarcely given in the literature. Only Gercken and Brockman (1969) presented data on the number of phospholipid molecules per rabbit RBC.

In Table XI the permeability coefficients for glycerol are compared with the amounts of phospholipid, cholesterol and glycolipids in red blood cell membranes. Data on lipid composition were taken from Rouser et al (1968).

Table XI.

Relative amounts of phospholipids, cholesterol and glycolipids in red blood cell membranes compared with their glycerol permeability coefficients.
Lipid components are given in percentages of total lipid. Moreover, the molar ratio of phospholipids and cholesterol is presented. The glycerol permeability coefficient (P glycerol) at pH 7.5 and 37° is given in $cm.sec^{-1} \times 10^6$.

Species	Glycolipid	Phospholipid	Cholesterol	$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	P glycerol
Rat	8.3	67.0	24.7	0.74	20.0
Man	-	58.0	23.0	0.79	18.3
Rabbit	5.3	65.8	28.9	0.88	3.6
Dog	22.7	52.6	24.7	0.94	1.4
Horse	23.5	52.0	24.5	0.94	1.0
Pig	13.4	59.8	26.8	0.90	0.7
Ox	7.7	64.8	27.5	0.85	0.4
Sheep	10.3	63.2	26.5	0.84	0.3

Obviously, neither a correlation of P glycerol with the cholesterol / phospholipid ratio, nor with the glycolipid content of the RBC membranes exist.

b. Phospholipid composition

Although among others de Gier and van Deenen (1960) suggested a correlation between phospholipid composition and permeability of RBC membranes, de Gier et al (1966) stated that such a correlation is less probable since, for instance, dog RBC membranes which are poorly permeable to glycerol, showed a phospholipid composition comparable to that of the much more permeable rat RBC membranes. In Table XII the phospholipid composition of mammalian RBC membranes is presented. Data were taken from Nelson (1967) except those for human RBC's which are from Rouser et al (1968).
No clear correlation between phospholipid composition and RBC membrane permeability to glycerol seems to exist. The data on phospholipid composition of Nelson (1967) and Rouser et al (1968) can be considered reliable, since for human RBC membranes essentially the same results were obtained by Dodge and Phillips (1967) and by Broekhuyse (1969), and for rabbit RBC membranes by Gercken and Brockmann (1969) and ourselves (chapter 6).

Table XII.

Phospholipid composition and glycerol permeability of mammalian red blood cells.
Data from Nelson (1967) for all animal species and from Rouser et al (1968) for human RBC's were compared with the glycerol permeability coefficients (P glycerol) in $cm.sec^{-1} \times 10^{6}$.

Species	LPC	S	PC	PI	PS	PE	PA	U	P glycerol
Rat	3.8	12.8	47.8	3.5	10.8	21.5	0.3	-	20.0
Man	-	26.9	28.9	1.3	13.0	27.2	2.2	-	18.3
Rabbit	0.3	19.0	33.9	1.6	12.2	31.9	1.6	-	3.6
Dog	1.8	10.8	46.9	2.2	15.4	22.4	0.5	-	1.4
Horse	1.7	13.5	42.4	0.3	18.0	24.3	0.3	-	1.0
Pig	0.9	26.5	23.3	1.8	17.8	29.7	0.3	-	0.7
Ox	-	46.2	-	3.7	19.3	29.1	0.3	1.7	0.4
Sheep	-	51.0	-	2.9	14.1	26.2	0.3	4.8	0.3

Abbreviations: LPC: lysophosphatidyl choline; S: sphingomyelin, PC: phosphatidyl choline, PI: phosphatidyl inositol, PS: phosphatidyl serine, PE: phosphatidyl ethanolamine, PA: phosphatidic acid, U: unidentified.

c. Fatty acid composition

The fatty acid composition of RBC membrane lipids, determined by different authors (compare Rouser et al, 1968), shows considerable variation. Many investigators, notably de Gier and van Deenen (1964a), Walker and Kummerow (1963, 1964) and Jager and Houtsmuller (1970) reported that changes in fatty acid composition can be caused by differences in dietary composition, whereas the phospholipid composition remains unchanged (de Gier and van Deenen, 1964a). Walker and Kummerow (1964), de Gier et al (1966) and Jager and Houtsmuller (1970) showed that considerable variations in fatty acid composition hardly affected the permeability of rat RBC's to glycerol. In Table XIII the fatty acid composition of RBC membranes, reported by de Gier et al (1966) is compared with the glycerol permeability coefficient at 37° and pH 7.5, determined by us. Again, there is no clear correlation.

Table XIII

Fatty acid composition of red blood cell membrane lipids compared with glycerol permeabiltty coefficients. (P glycerol) in $cm.sec^{-1}x10^{6}$. Data on fatty acid composition were taken from de Gier et al (1966).

	Fatty acid						
Species	16:0	16:1	18:0	18:1	18:2	20:4	P glycerol
Rat	28.7	1.8	12.1	14.5	6.4	33.8	20.3
Man	27.1	3.4	9.4	19.5	16.5	19.5	18.3
Rabbit	22.3	3.3	10.5	11.8	32.0	6.6	3.6
Dog	16.9	1.7	19.0	14.2	12.9	30.8	1.4
Pig	21.4	2.4	10.4	32.1	23.2	6.4	0.7
Ox	12.1	2.7	14.1	34.5	21.1	4.8	0.4
Sheep	15.7	1.6	9.6	52.3	14.6	2.9	0.3

5.5. CONCLUSIONS

In the first part of this chapter it was shown that human RBC membranes with a high permeability to glycerol have a reflection coefficient of nearly one. The same can be stated for sheep RBC's and pig RBC's which have a very low glycerol permeability (3.3.5). Obviously, the glycerol reflection coefficient is nearly one for all mammalian RBC's studied.

Whereas for each species the glycerol permeability coefficient at 37° and pH 7.5 showed a relatively small variability (10-20%), there was a very large variability (70%) in this parameter for rabbit RBC's. Rabbit RBC's were, therefore, studied in more detail (chapter 6).

Although the time of hemolysis is an indication of the RBC membrane permeability to glycerol, the permeability coefficients obtained with the modified osmotic lysis method are preferable since, for instance, the effect of lysis delay is eliminated and hence more exact permeability data are obtained. The sequence of the glycerol permeability coefficient appeared to differ from that of the times of hemolysis in the group of animals with low permeability to glycerol.

No correlation between lipid composition, including phospholipid and fatty acid composition, and glycerol permeability coefficients could be shown.

6. THE PERMEABILITY OF RABBIT RED BLOOD CELLS TO GLYCEROL

6.1. INTRODUCTION

In the preceding chapter it was shown that, whereas in general the permeability to glycerol of RBC populations within each mammalian species studied differed only slightly, the permeability of RBC populations from individual rabbits varied considerably. This was in contrast with the observations of Jacobs et al (1935) that the high lysis rates in 300 mM glycerol of RBC's from seven rabbits were almost equal. The RBC's from some rabbits were therefore investigated in more detail. Special attention was given to the lipid composition of the cell membranes[*] and to the composition of the cell contents.
In the opinion of some authors (Jacobs and Corson, 1934; Jacobs and Stewart, 1946; Hunter et al, 1965; Ospina and Hunter, 1966) the glycerol permeability of RBC's from certain mammalian species, e.g. man, rat, mouse and rabbit, is due to a facilitated diffusion system present in the cell membrane. Since the high glycerol permeability of human RBC's is strongly inhibited by copper ions, some preliminary investigations with rabbit RBC's in copper-containing glycerol solutions were performed in order to investigate whether facilitated diffusion plays a role.

6.2. MATERIALS AND METHODS

6.2.1. Isolation of red blood cells

2-3 Months old rabbits (New Zealand White) were used. Blood was obtained from the ear vein. Heparin was added as anticoagulant (50 I.U. per 10 ml of blood).

[*] We wish to thank Dr.R.M. Broekhuyse, Department of Ophthalmology, and Dr. J.H. Veerkamp, Department of Biochemistry, University of Nijmegen, for performing the lipid analyses.

6.2.2. Determination of permeability and osmotic resistance

Permeability experiments were generally performed at 37° as described before (3.2.2). To determine whether the permeability differences were due to extracellular factors, the degree of lysis at different moments was determined in buffered 300 mM glycerol only in some cases. All solutions were buffered with 1 mM sodium phosphate to pH 7.5. To detect the presence of a Cu^{2+}-sensitive facilitated diffusion system, 10^{-6}M $CuSO_4$ was added to the 300 mM buffered glycerol solution. The osmotic resistance was determined in 1 mM sodium phosphate buffered sucrose solutions pH 7.5.

6.2.3. Lipid extraction

RBC's were treated three times with 2 vol. 0.9% NaCl after removal of the buffy coat and resuspended in 0.9% NaCl to a hematocrit reading of about 0.5. After mixing, aliquots were taken for RBC counting with a Coulter Counter and 5 ml samples for lipid extraction. Lipids were extracted with methanol and chloroform according to method I described by Broekhuyse (1969). The lipid extracts were evaporated in vacuo at 30° and the lipids were dissolved in 1 ml benzene-methanol (4:1, by vol.) and stored at -25°.

6.2.4. Chromatographic separation of phospholipids

Quantitative two-dimensional thin-layer chromatography was performed according to Broekhuyse (1969) on plates prepared from purified silica gel containing 1% alkaline magnesium silicate (Woelm). The chromatograms were developed in the first direction with chloroform-methanol-7M ammonia (90:54:11, by vol.) and in the second direction with chloroform-methanol-acetic acid-water (90:40:12:2, by vol.). After chromatography the spots were coloured with iodine vapour.

6.2.5. Quantitative determinations of phospholipids and cholesterol

The total lipid phosphorus content of extracts and of the separated phospholipids was determined according to Broekhuyse (1969[a]). Spots

were scratched from the chromatograms and transferred to pyrex tubes. Then 0.4 ml conc. sulfuric acid H_2SO_4-70% $HClO_4$ (8:2, by vol.) was added. The tubes were heated at 180° for 30 min. After cooling 9.6 ml Fiske-Subbarow reagent was added. The tubes were then heated at 90° for 20 min. Upon cooling and spinning down the silica gel, the extinction was measured at 820 nm.
Cholesterol was determined in extracts according to Abell et al (1952) with the modified Liebermann-Burchard reagent composed of a mixture of acetic anhydride, conc. sulfuric acid and glacial acetic acid (20:1:10, by vol.). The phospholipid and cholesterol content per RBC was calculated from the number of RBC's extracted, and the phospholipid and cholesterol recovery.

6.2.6. Determination of fatty acid composition

Aliquots of 0.3 ml of lipid solution in benzene-methanol were used. After evaporation at 25° in vacuo, 0.5 ml of hexane was added. Fatty acid methyl esters were prepared by methanolysis with 1 ml of BF_3-methanol (10%, w/v) at 100° for 15 min according to Morrison and Smith (1964). The fatty acids were determined on a column of 15% diethylene glycol succinate on 60-80 mesh Gas-Chrom P beads at 180° in a Packard model 7821 gas chromatograph. They were identified by comparison of their relative retention volumes with those of standard methyl esters of saturated and unsaturated fatty acids, before and after hydrogenation on polar and apolar columns (Veerkamp, 1970).

6.3. RESULTS

6.3.1. Reproducibility of permeability experiments

The mean glycerol permeability coefficient of rabbit RBC's at 37°, pH 7.5, appeared to amount to $3.63 (\pm 2.62) \times 10^{-6} cm.sec^{-1}$ as shown in chapter 5. The large variation might be caused by inaccuracies of the method used, e.g. traces of heavy metals may have been present occasionally in the solutions (Jacobs et al, 1935). Age and sex might also play a role. In order to investigate these possibilities, the lysis rate of RBC's from four different rabbits was determined in buffered 300 mM glycerol at four week intervals. The results, expressed as relative

extinctions of the supernatant, are listed in Table XIV.

Table XIV

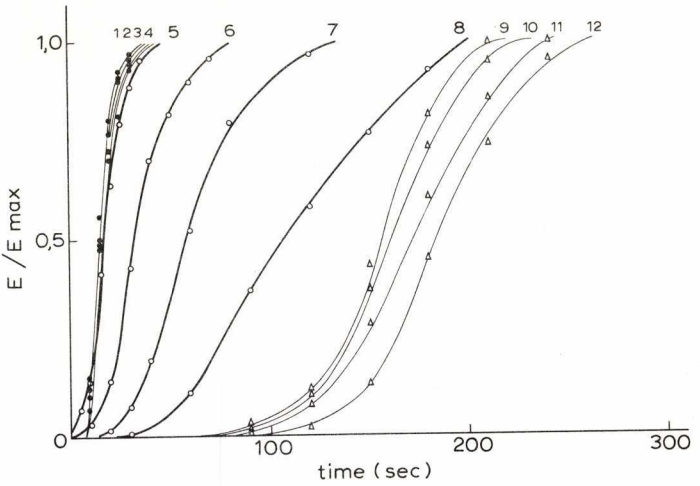
Degree of lysis of rabbit red blood cells in 300 mM glycerol at different time intervals.
Lysis was stopped with hypertonic NaCl (1.5 mM). Experiments were repeated three times (a, b and c) at 4-week intervals. The results are expressed in relative extinction of the supernatant (E/E_{max}) x 1000.

Time	Rabbit 1 (♀)			Rabbit 2 (♂)			Rabbit 9 (♂)			Rabbit 10 (♀)		
in sec	a	b	c	a	b	c	a	b	c	a	b	c
5	9	17	37	32	35	11	-	-	-	-	-	-
10	77	99	165	144	162	66	-	-	-	-	-	-
15	274	226	338	416	406	217	-	-	-	-	-	-
20	448	439	497	647	609	482	45	37	6	-	-	-
25	666	624	673	805	748	658	-	-	-	-	-	-
30	824	725	788	905	855	808	-	-	-	5	23	9
40	928	888	968	960	953	908	294	193	57	-	-	-
50	-	987	-	-	-	949	-	-	-	-	-	-
60	-	-	-	-	-	-	639	451	287	121	115	50
80	-	-	-	-	-	-	817	654	535	-	-	-
90	-	-	-	-	-	-	-	-	-	405	274	171
100	-	-	-	-	-	-	895	829	746	-	-	-
120	-	-	-	-	-	-	-	-	-	611	432	380
140	-	-	-	-	-	-	958	897	869	-	-	-
150	-	-	-	-	-	-	-	-	-	796	662	573
180	-	-	-	-	-	-	-	979	905	-	-	-
180	-	-	-	-	-	-	1000	1000	941	917	841	800
210	-	-	-	-	-	-	-	-	-	950	922	929

The reproducibility of the measurements excludes the possibility that the large differences in lysis rate are caused by random inaccuracies of the method used. Sex and small age differences do not appear to play a role. Table XIV presents only animals with extremely high (nrs. 1 and 2) and low (nrs. 9 and 10) lysis rates, but the other animals in a group of 18 animals gave intermediate lysis rates. It seems, therefore, less probably that genetic differences cause these variations, unless different genes play a role.
In some cases the lysis rate of rabbit RBC's was almost equal to that of human RBC's, in others nearly to that of pig RBC's (Fig.20).

Fig. 20

Lysis rates of red blood cells of three different mammalian species in 300 mM buffered glycerol.
Curves 1-4 represent the individual data obtained with RBC's from men, curves 5-8 that obtained with rabbit RBC's and curves 9-12 that obtained with pig RBC's.



The following characteristics are represented in this figure:

a) while the lysis rates for individual humans and for individual pigs show little variability, there is great variability among individual rabbits

b) in the case of rabbit of curve 5 a certain fraction (about 20%) of the RBC population from this rabbit is more permeable to glycerol than human RBC's.

c) in a case like rabbit of curve 8 individual RBC's appear to have different lysis rates as indicated by the small slope of the curve. Less variability among the RBC's of individual humans and pigs seems to exist.

These differences in lysis rate of individual RBC's could be due to variations in osmotic resistance, due to differences in their surface/volume ratio. Such differences would not show up in the ratio (P/R_h) of the permeability coefficient to the radius of the RBC at the moment of lysis, since osmotic resistance is discounted in these calculations (2.2). In Table XV the P/R_h ratio at 25, 50 and 75% hemolysis is given for four rabbits, men and pigs. The ratio of the P/R_h values at 25 and

75% hemolysis is also shown. It would be more accurate to give the permeability coefficients for the different RBC fractions present in the population, but the radius at lysis is not known for the different RBC fractions. It seems unlikely that R_h would vary considerably, since both the osmotic resistance in sucrose solutions as well as the mean cell volume were nearly constant for RBC's from different rabbits (Table XX). The P/R_h ratios should, therefore, yield reliable indications of the glycerol permeability of the different RBC fractions.

Table XV

Ratios of the glycerol permeability coefficient and critical radius at three levels of hemolysis

	P/R_h ratios x 10^3			Ratio 25%
Species	25%	50%	75%	Ratio 75%
Rabbit 1	8.6	5.6	2.7	3.23
Rabbit 8	16.4	12.2	7.4	2.22
Rabbit 9	32.3	23.1	19.7	1.65
Rabbit 10	34.9	32.6	27.9	1.25
Man 1	41.7	36.7	30.3	1.37
Man 2	47.0	39.3	31.3	1.50
Man 3	46.7	38.7	32.0	1.46
Man 4	61.3	48.7	41.0	1.50
Pig 1	2.9	3.6	3.1	0.95
Pig 2	2.8	2.6	2.3	1.18
Pig 3	3.2	3.0	2.5	1.27
Pig 4	3.0	2.8	2.9	1.05

These data indicate that the glycerol permeability of different fractions of human and pig RBC populations varies relatively little, while that for fractions of rabbit RBC populations, especially for rabbit 1, varies much more clearly. Moreover, the individual differences are rather small for men and pig, but are considerable in rabbit.

6.3.2. Lipid composition

Should a simple correlation exist between the glycerol permeability of RBC membranes and their lipid composition (Parpart and Dziemian, 1940; Kögl et al, 1960; de Gier, 1960; de Gier and van Deenen, 1964;

Stein, 1967), then a considerable variability in the lipid composition of RBC populations from different rabbits would be expected.

a) Phospholipid composition and cholesterol content.

The lipid of RBC membranes consists mainly of phospholipids and cholesterol (de Gier and van Deenen, 1964). We determined the phospholipid composition and the cholesterol content of RBC's from ten rabbits with a wide range of differing glycerol permeability. In Table XVI the lipid composition is compared with the glycerol permeability coefficient.

Table XVI.

Lipid phosphorus and cholesterol content in rabbit red blood cells. Lipid phosphorus and cholesterol are expressed per ml packed cells and per red blood cell in μmoles and amoles (10^{-18} moles), respectively. These data are compared with the glycerol permeability coefficients expressed in $cm.sec^{-1} \times 10^{6}$.

Rabbit	Lipid-P	Cholesterol	Lipid-P per RBC	Cholesterol per RBC	Cholesterol / Lipid-P	P glycerc
1	2.76	3.19	280	323	1.15	1.64
2	3.34	3.70	296	329	1.11	1.67
3	3.33	3.95	315	374	1.19	1.87
4	3.29	3.60	297	325	1.09	1.98
5	3.50	3.91	302	338	1.12	2.12
6	3.04	3.30	305	332	1.09	2.77
7	2.85	3.24	309	349	1.14	3.56
8	3.72	4.13	314	349	1.11	4.39
9	2.82	3.42	289	352	1.22	6.75
10	3.20	3.33	309	322	1.04	9.53
mean $\pm$ SD			302$\pm$11.2	339$\pm$16.6	1.13$\pm$0.04	3.63$\pm$2.62

Apparently the amounts of phospholipid and cholesterol per RBC and their ratio do not vary significantly. The total phospholipid content (302$\pm$11 amoles) per RBC agrees with the value of Gercken and Brockmann (1969) (313$\pm$27 amoles). The molar ratio of cholesterol to phospholipids is close to one which is in accordance with data reported by de Gier and van Deenen (1961 and 1964) and by Nelson (1967). Obviously the observed differences in glycerol permeability are not correlated with a difference in the number of lipid molecules per RBC or in the cholesterol/phospholipid ratio.

The composition of the phospholipids is given in Table XVII. The data are compared with those earlier reported for rabbit RBC membranes.

Table XVII

Phospholipid composition and glycerol coefficients for rabbit red blood cells.
Phospholipids are expressed in mole % of lipid phosphorus recovered, the glycerol permeability coefficient in $cm \cdot sec^{-1} \times 10^6$.

	Component								
Rabbit	LPC	S	PC	PI	PS	PE	PA	U	P glycerol
1	2.7	18.5	31.1	3.5	12.6	29.8	1.5	0.5	1.64
2	1.7	19.3	32.2	1.7	11.7	31.2	1.7	0.4	1.67
3	2.6	20.9	27.3	1.6	12.8	31.9	2.9	-	1.87
4	1.7	20.7	31.4	2.0	12.0	30.2	1.3	0.6	1.98
5	1.5	20.3	32.8	2.0	12.4	29.6	2.3	0.1	2.12
6	1.6	21.3	21.3	2.2	11.6	32.3	0.7	0.2	2.77
7	1.2	18.3	32.1	2.9	11.8	31.9	0.9	0.8	3.56
8	1.9	20.9	32.7	1.3	13.1	28.6	1.2	0.4	4.39
9	2.5	18.8	35.3	2.4	10.3	27.9	1.8	1.2	6.75
10	1.9	17.4	33.1	1.9	12.0	32.0	2.0	0.7	9.53
Mean	1.9	19.7	31.8	2.2	12.0	30.5	1.6	0.5	3.63
±SD	0.5	1.4	2.1	0.6	0.8	1.6	0.7	0.1	2.62
de G	-	29	44	-	27		-	-	
N	0.3	19.0	33.9	1.6	12.2	31.9	1.6	-	
G	0.1	15.7	36.3	1.3	12.4	32.6	1.6 z	-	

LPC: lysophosphatidyl choline, S: sphingomyelin, PC: phosphatidyl choline; PI: phosphatidyl inositol, PS: phosphatidyl serine, PE: phosphatidyl ethanolamine, PA: phosphatidic acid, U: unidentified.
References; de G: de Gier and van Deenen, 1961; N: Nelson, 1967; G: Gercken and Brockmann, 1969.

No clear differences in phospholipid composition were observed. The data appeared to agree rather well with those reported by other investigators (Nelson, 1967; Gercken and Brockman, 1969). Thus, we must conclude that the observed permeability differences are not accompanied by significant variations in phospholipid composition. Since variations in fatty acid composition could also affect the membrane permeability, the fatty acid composition of the lipids was determined.

b) Fatty acid composition.

De Gier and van Deenen (1964a) demonstrated that the fatty acid composition of RBC membranes from certain mammalian species varied with the diet, while the ratio between the different phospholipid classes remained unaltered. Variations in fatty acid composition appeared to affect the glycerol permeability of liposomes (de Gier et al, 1968). Although the times of hemolysis of rat RBC's in glycerol solutions did not appear to be significantly affected by the observed changes in fatty acid composition (Walker and Kummerow, 1964; de Gier et al, 1966), this does not prove that no differences in permeability exist, as was set forth in Chapter 2. Therefore, we determined the fatty acid composition of the lipids of the RBC membrane from the various rabbits in comparison to the permeability coefficients for glycerol (Table XVIII).

Table XVIII

Fatty acid composition of rabbit red blood cell lipids.
The data are compared with the glycerol permeability coefficients ($cm \cdot sec^{-1} \times 10^6$). The fatty acid composition is given as percentages of total fatty acids. tr: denotes less than 0.5%.

Fatty acid	Rabbit 1	2	3	4	5	6	7	8	9	10	Mean±SD
14:0	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
16:0	19.3	19.2	21.4	18.8	19.4	19.1	20.4	19.0	21.2	18.7	19.6±1.0
16:1	4.0	5.1	5.8	6.2	6.5	5.1	5.5	5.4	5.1	5.3	5.5±0.7
18:0	18.5	17.3	17.4	18.0	18.5	17.9	16.4	18.4	19.9	17.4	17.9±0.9
18:1	11.2	10.2	9.7	10.8	10.5	10.6	11.0	10.2	10.9	12.2	10.7±0.7
18:2	25.7	27.8	26.7	26.1	24.6	26.9	27.4	26.9	26.1	27.3	26.5±0.9
18:3	2.4	2.3	1.7	2.7.	1.9	1.9	1.9	2.9	2.9	1.8	2.2±0.5
20:3	1.0	0.6	0.6	0.6	0.7	0.8	2.2	0.6	0.9	1.4	0.9±0.5
20:4	3.8	5.9	6.6	5.5	5.3	5.9	4.0	4.7	3.5	4.8	5.0±1.0
22:4	3.7	2.5	2.2	2.6	2.5	2.5	2.8	2.7	2.4	2.6	2.6±0.4
22:5	1.7	1.5	1.5	1.3	1.2	1.8	0.7	1.0	0.9	1.0	1.2±0.4
22:6	4.5	4.3	3.7	4.6	4.9	4.7	3.5	4.6	3.1	4.2	4.2±0.5
24:0	0.8	1.2	1.3	1.0	1.5	1.1	1.6	1.1	1.3	1.5	1.2±0.3
P glycerol	1.64	1.67	1.87	1.98	2.12	2.77	3.56	4.39	6.75	9.53	3.63±2.62

The variations in fatty acid composition between the different animals appeared to be rather small, and there seemed to be no correlation with the glycerol permeability coefficient.

Compared with the data from the literature (Gercken and Brockmann, 1969; de Gier et al, 1966), certain differences in fatty acid composition were

observed (Table XIX), which may be due to variations in the fatty acid composition of the diet.

Table XIX
Fatty acid composition of rabbit red blood cells

Fatty acid	Data from Table XVIII	Gercken and Brockmann (1969)	de Gier et al. (1966)
16:0	19.6	23.5	22.3
16:1	5.5	1.8	3.3
18:0	17.9	11.7	10.5
18:1	10.7	17.9	11.8
18:2	26.5	25.5	32.0
18:3	2.2	1.7	-
20:4	5.0	7.6	6.6
22:4	2.6	1.0	-
22:5	1.2	0.5	-
22:6	4.2	2.4	-
Other	4.6	6.4	13.5

It can be concluded that differences in phospholipid composition, cholesterol/phospholipid ratio and fatty acid composition cannot explain the observed differences in glycerol permeability of RBC's from individual rabbits.

6.3.3. Osmotic resistance, mean cell volume, cation and anion composition and glycerol permeability of rabbit red blood cells

Differences in osmotic resistance, reflecting chiefly the volume/surface ratios of RBC's, markedly affect the rate of hemolysis of red blood cells (chapter 2). We, therefore, determined osmotic resistance in buffered sucrose and mean cell volumes of RBC's from some rabbits with considerable differences in glycerol permeability (Table XX). The osmotic resistance in sucrose and the mean cell volume differed little for these three rabbits, suggesting that the mean cell surface areas were also nearly equal. This suggestion is supported by the observation that the amounts of cholesterol and phospholipid per RBC were nearly equal (Table XVI).
Analysis of the quantitatively most prominent cations and anions showed no noteworthy differences (Table XX) except for chloride in rabbit nr. 10.

The latter differences may reflect differences in extracellular and/or intracellular pH according to the Gibbs-Donnan law. A close correspondence between the distribution ratios for hydrogen and chloride ions in rabbit RBC's was experimentally observed by Calvey (1970). This author reported that the intracellular pH of rabbit RBC's, suspended in phosphate buffered isotonic NaCl solution at pH 7.4, amounted to 7.27 (90% range 7.19-7.34, n=40). The effects of these differences in chloride concentration on membrane permeability to glycerol are still obscure and should be studied more extensively.

Table XX.

Comparison of mean cell volume, composition of cell content and osmotic resistance with the glycerol permeability of untreated rabbit red blood cells.
The cation and anion composition was determined in sixfold and the mean cell volume in triplicate in the ways described earlier (4.2). The data (±SE) are expressed in mmoles/l RBC's and in μ^3/RBC, respectively. The osmotic resistance in sucrose was determined in duplicate and is given as the 50% hemolysis value in mosmol/l. The data were compared with the permeability coefficient of glycerol expressed in $cm.sec^{-1}x10^6$.

	Rabbit 1	Rabbit 9	Rabbit 10
Sodium	12.0±0.3	10.8±0.3	13.7±0.5
Potassium	105.5±0.6	107.1±0.4	101.7±0.5
Chloride	45.9±0.8	44.4±0.8	55.2±1.0
Phosphate	8.9±0.1	8.5±0.2	7.9±0.1
Mean cell volume	64.1±0.5	66.2±1.4	65.7±0.6
50% Hemolysis	107	101	108
P glycerol	1.64	6.75	9.53

6.3.4. Facilitated glycerol diffusion into rabbit red blood cells

Since a facilitated diffusion system has been suggested for rapid glycerol transport (Jacobs and Corson, 1934; Jacobs and Stewart, 1946; Hunter et al, 1965; Ospina and Hunter, 1966; Hunter,1970), differences in glycerol permeability for the RBC's from various rabbits may be related to a variable capacity of this system.
Jacobs and Stewart (1946) showed that the lysis rate of human RBC's in isotonic glycerol solutions at pH 6.5 and at room temperature was inhibited by about 90% after addition of $6.4 \cdot 10^{-7}$M copper chloride. It was assumed from these observations that copper ions exert their inhibitory effect at the membrane surface, strongly suggesting a

facilitated diffusion system. The effect of the same concentration of copper ions on the lysis rate of rabbit RBC's, however, appeared to be very small (Jacobs et al, 1935).

Whereas Jacobs et al (1935) observed that the lysis rate of RBC's in isotonic glycerol was reproducibly high, we observed considerable differences in lysis rate with RBC's from different rabbits. It therefore seemed useful to reinvestigate the copper effect. We compared the lysis rate of RBC's from some rabbits in buffered 300 mM glycerol without and with 10^{-6}M $CuSO_4$. Both untreated RBC's and RBC's treated with unbuffered 300 mM sucrose to remove plasma were used. In Table XXI the data obtained with RBC's treated with unbuffered 300 mM sucrose are shown. These data are equivalent to those obtained with untreated RBC's.

Table XXI

Effect of cupri ions on the lysis rate of rabbit red blood cells in buffered 300 mM glycerol.
The rate of hemolysis is given as the relative extinction (E/E_{max}) x 1000.
Glycerol solutions without (-) and with (+) 10^{-6}M $CuSO_4$ were used.

Time in sec	Rabbit 1		Rabbit 2		Rabbit 9		Rabbit 10	
	+	-	+	-	+	-	+	-
5	8	11	2	1	-	-	-	-
10	58	66	53	37	-	-	-	-
15	231	217	174	165	-	-	-	-
20	487	482	347	338	10	6	-	-
25	649	658	527	497	-	-	-	-
30	781	808	686	673	-	-	7	9
40	898	908	808	788	84	57	-	-
50	969	949	949	968	-	-	-	-
60	-	-	-	-	301	287	59	50
80	-	-	-	-	566	535	-	-
90	-	-	-	-	-	-	194	171
100	-	-	-	-	729	746	-	-
120	-	-	-	-	869	869	358	380
140	-	-	-	-	891	895	-	-
150	-	-	-	-	-	-	573	584
160	-	-	-	-	952	941	-	-
180	-	-	-	-	-	-	794	800
210	-	-	-	-	-	-	910	929
240	-	-	-	-	-	-	950	972

No effect of copper ions was observed, neither with RBC's with a high lysis rate, nor with those with a rather low lysis rate. Since it is not known whether the copper ion itself and at the concentration used is a general inhibitor of a facilitated diffusion system for glycerol, more investigations with other substances that are believed to inhibit this system, e.g. the aspecific inhibition by tannic acid (Hunter et al, 1965; Ospina and Hunter, 1966) and the more specific inhibition by phloretin (Macey and Farmer, 1970) will be necessary.

6.4. CONCLUSIONS

The observed differences in glycerol permeability of red blood cells from different rabbits did not appear to be correlated with differences in lipid composition, nor with differences in sex or age. Osmotic resistance, cell content and mean cell volume appeared to be equal for rabbit RBC's which differed markedly in glycerol permeability. Though copper ions strongly inhibit the permeability of human RBC's to glycerol suggesting the existence of a facilitated diffusion system, these ions had no effect on the glycerol permeability of rabbit RBC's in some preliminary experiments. A more detailed study thus will be necessary.

7. THE OSMOTIC RESISTANCE OF RED BLOOD CELLS IN SODIUM CHLORIDE AND IN SUCROSE SOLUTIONS

7.1. INTRODUCTION

Since water equilibrium is established almost instantaneously, the ideal osmotic resistance of RBC's can be taken as a time-independent quantity as was stated before (2.1). In chapter 3 it was shown that this obviously holds in hypotonic solutions of sucrose, but not in hypotonic solutions of NaCl. Moreover, the osmotic resistance in NaCl solutions was considerably lower than in sucrose solutions. Though the osmotic resistance curve in sucrose solutions always coincides with that found by extrapolation of the curves representing the delayed lysis in glycerol solutions (3.3.5), this does not prove that the osmotic resistance in sucrose solutions really represents the original osmotic resistance of RBC's.

Differences in osmotic resistance in NaCl and in nonelectrolyte solutions have been observed for RBC's of six mammals (Coldman et al, 1970). These authors have shown that RBC fragility depends almost entirely on the osmotic properties of the suspending medium. When allowance is made for the relative impermeability of RBC's to sodium ions, their free permeability to chloride, and the unequal distribution of chloride between RBC's and suspending medium, the osmotic effects of NaCl and the nonelectrolyte mannitol are virtually identical.

Bowdler and Chan (1969) concluded from studies of osmotic lysis of human RBC's in NaCl and KCl solutions, that besides the intracellular/extracellular chloride ratio the increase in cation permeability by certain RBC's plays a role too. These authors observed a biphasic lysis process in NaCl solutions under conditions, which do not lead to complete lysis. The rapid lysis would to some extent be due to the intracellular/extracellular chloride ratio, the slower part of the lysis process to cation permeability of cells swollen to a volume close to that which is critical for lysis.

Our own observations and those of Coldman et al (1970) and of Bowdler and Chan (1969) indicate that the osmotic resistance in NaCl solutions is anomalous and does not represent the original osmotic resistance of RBC's. The osmotic resistance in sucrose solutions appears to be closest

approximation of the original osmotic resistance.
In the first part of this chapter a more detailed study of the osmotic resistance of pig RBC's in hypotonic NaCl and sucrose solutions will be presented. The results support the suggestion that the osmotic resistance in sucrose solutions, comes closest to the original osmotic resistance. We studied in more detail the time-dependency and the pH-dependency of the osmotic resistance in hypotonic NaCl solutions. The osmotic resistance was also determined in sucrose, choline chloride and KCl solutions. The RBC's were pretreated in most cases either with isotonic solutions of non-permeating electrolytes or nonelectrolytes. The pH changes in the extracellular solution were recorded in some cases. In clinical practice the osmotic resistance is generally determined in hypotonic NaCl solutions (Dacie and Lewis, 1968). In the second part of this chapter data will be presented of comparative studies of the osmotic resistance in NaCl and in sucrose solutions of normal and pathologically altered human RBC's. Attempts were made to explain the observed differences in osmotic resistance in pathological cases from variations in intracellular composition and the surface/volume ratio.

7.2. MATERIALS AND METHODS

7.2.1. Isolation of pig red blood cells and determination of their osmotic resistance

Pig RBC's were isolated from defibrinated blood as described (3.2.1). In some cases the RBC's were treated 4 times with an excess of buffered 300 mM sucrose, 150 mM NaCl or 2 times with unbuffered 300 mM sucrose. When not otherwise mentioned, a 1 mM sodium phosphate buffer of pH 7.5 was used. All centrifugations were performed at 1000 x g during 10 min. The osmotic resistance of pig RBC's in hypotonic NaCl and sucrose solutions was determined as described before (3.2.2). Since pH and buffer strength of the hypotonic NaCl and sucrose solutions varied, these values will be presented at the appropriate places. Extracellular pH changes were recorded as described before (4.2).

7.2.2. Determination of the amount of sedimented pig red blood cell membrane material.

After addition of 0.05 ml RBC suspension to 10 ml hypotonic NaCl solu-

tion lysis was stopped at different moments by the addition of 1 ml 1.5 M NaCl. The more or less lysed RBC's were spun down at 1000 x g during 10 min. The sediment was treated three times at 4° with carbondioxide saturated water according to Parpart (1942). The flocculant material visually appeared to be nearly free of hemoglobin and could be spun down easily. The sediment was dried overnight at 80° and digested for 1.5 h at 180° in 0.2 ml of 70% perchloric acid. Thereafter, phosphate was determined by the method of Bartlett (1959), to serve as a measure for the amount of membrane material.

7.2.3. Isolation of human red blood cells

Humans RBC's were isolated as described (3.2.1) from blood obtained by venapuncture. Generally 50 I.U. of heparin were added to 10 ml of blood in order to prevent coagulation. Blood was obtained from normal donors and from some patients suffering from sidero achrestic anemia, hereditary hemolytic anemia, hereditary thrombocytopathic thrombocytopenia or an undefined anisocytosis.
Short anamnesis of patients studied:

J.H. (1-2-1903) Sideroachrestic anemia (SAA-1)
A hepatosplenomegalie was observed. The hemoglobin content was 8.0 mmol/l blood. The RBC's appeared to be hypochromic and anicytosis was observed. The reticulocyte number was normal. Mean RBC volume decreased.

M.v.d. K. (5-5-1937). Sideroachrestic anemia (SAA-1)
Besides hepatosplenomegalie, a low hemoglobin value (5.7 mmol/l blood) was observed. The RBC's appeared to be hypochromic and anisocytosis was observed. Mean RBC volume decreased.

J.B. (19-5-1910). Undefined anisocytosis (A)
The hemoglobin content was 4-5 mmol/l blood. Anisocytosis was observed. Mean RBC volume appeared to be nearly normal. The number of reticulocytes was increased.

H.A.M. de P. (19-7-1909). Hereditary hemolytic anemia (HHA)
The hemoglobin content amounted to 8.1 mmol/l blood. It was concluded from lactate dehydrogenase determinations (240 I.U.), the Coombs's test and some other informations that this was a case of hemolytic anemia.

J.P. (19-10-1941). Hereditary thrombocytopathic thrombocytopenia (HTT)
A thrombocytopenia was observed (80 x 10^9/thrombocytes/l blood).
After splenectomy the thrombocyte number was almost normal.

The hemoglobin content was 8.6 mmol/l,a normal value. Moreover an anisocytosis and poikilocytosis was observed. The mean cell volume was somewhat increased. Based on Daniels biopsy the diagnosis was Morbus Besnier Boeck.

7.2.4. Determination of size distribution and number of human red blood cells

The size distributions were obtained with a Particle Size Distribution Analyzer (model J) connected to a Coulter Counter (model B). Internal machine settings were: Vernier-90; Matching Switch-32H. Amplification was set at 1, aperture current was 0.707. The RBC's were counted with the same instrument. Blood was previously diluted 1:16000 with Isoton (Coulter Electronics).

7.2.5. Determination of the osmotic resistance of human red blood cells

The osmotic resistance of human RBC's in NaCl solutions at 37^{o} after 300 sec was determined according to the clinical method (Dacie and Lewis, 1968) based on that of Parpart et al (1947). The various NaCl tonicities were obtained by diluting a standard solution of 153 mM NaCl and 10 mM sodium phosphate, pH 7.3, with water. It should be noted that an error is introduced by dilution with water, since the pH changes with the ionic strength (Mortensen, 1963). The osmolarities were determined as described (3.2.3). The osmotic resistance determination in sucrose solutions, bufferd with 1 mM sodium phosphate to pH 7.3, was also performed at 37^{o} during 300 sec. Technical details are given in 3.2.2.

7.2.6. Determination of total cell volume and mean cell volume of human red blood cells

To 6 ml aliquots of blood 0.3 ml 300 mM sucrose, containing 0.3 μC ^{14}C-inulin, was added. After vigorous shaking, 1 ml samples were transferred to glass tubes (length: 115 mm, inside diameter: 3 mm). After

centrifugation the hematocrit was read and the radioactivity was determined in the plasma layer (4.2.2). The packed RBC column was cut just below the buffy coat and 0.2 ml of packed RBC's were resuspended in 4 ml Isoton. After centrifugation the radioactivity in the supernatant was determined. The total cellular volume was calculated from the radioactivity in plasma, compared to that of a standard. The volume of trapped plasma was computed from the radioactivity of the Isoton supernatant and that found in plasma. Another representative value for the total cell volume was obtained from the hematocrit value, corrected for the amount of trapped plasma. The mean cell volume (MCV) was calculated from the total cell volume, determined in two ways as described above, and the number of RBC's present in the blood.

7.2.7. Determination of intracellular cation and anion composition of human red blood cells

Sodium, potassium, chloride and phosphate contents of the cells was determined in lysates of packed RBC's (0.2 ml cells + 1.8 ml water) as previously described (4.2.5). The concentration of these components per liter of RBC's was found after correction for the contribution from trapped plasma.

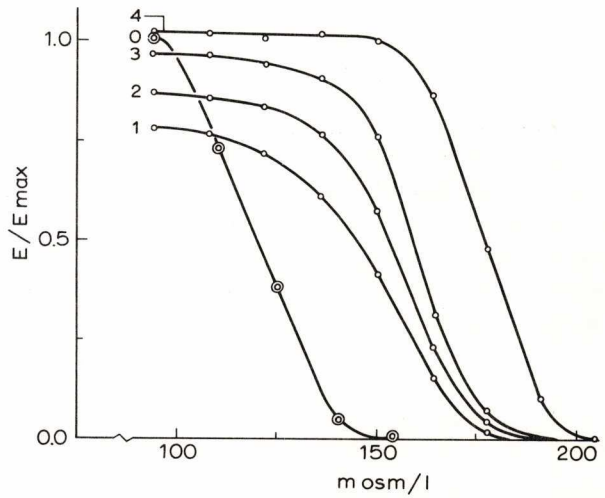
7.3. ANALYSIS OF THE DISCREPANCIES IN OSMOTIC RESISTANCE OF PIG RED BLOOD CELLS IN NaCl AND IN SUCROSE SOLUTIONS

7.3.1. The time-dependent and tonicity-independent lysis of pig red blood cells in hypotonic NaCl solutions

As shown before (3.3.4), the osmotic resistance of NaCl treated RBC's appeared to be strongly time-dependent at pH 7.5 and 37^{o} in hypotonic NaCl solutions (Fig. 21).
A more detailed study revealed that this time-dependency is not restricted to a slow decrease of the osmotic resistance, but is also time-

Fig. 21

Osmotic resistance of pig red blood cells.
RBC's were treated 4 times with 300 mM sucrose or 150 mM NaCl, both solutions buffered with 1 mM sodium phosphate to pH 7.5. The osmotic resistance was determined in hypotonic NaCl and sucrose solutions, buffered in the same way.
Curve O: sucrose treated cells in hypotonic sucrose solutions after 5 and 300 sec.
Curves 1-4: NaCl treated cells in hypotonic NaCl solutions after 5, 10, 15 and 300 sec, respectively.



dependent below a certain tonicity, which causes in the longer run complete lysis (e.g. 120 mosmol NaCl/l). Below 120 mosmol/l the lysis rate obviously hardly depends on the tonicity. Possible explanations for this time-dependent and tonicity-independent lysis of pig RBC's in hypotonic NaCl solutions are: 1) different fractions of the RBC population lyse at a different rate, 2) all RBC's lose relatively slowly their hemoglobin content, 3) a combination of both.
In order to discriminate between the possibilities, it is important to know the amount of membrane material present after different time intervals in the sediment. From investigations of Dodge et al (1963) it is known that for the sedimentation of hemoglobin free RBC membranes a centrifugal force of about 20.000 x g (20 min) is required. If, however, RBC's have partly lost their hemoglobin content, a centrifugal force of 1000 x g appeared to be sufficient. In our experiments the RBC

suspensions in hypotonic NaCl solutions were centrifuged at 1000 x g (10 min) after various times of lysis. The sediment was analyzed for membrane material.

After 5 sec at tonicities ranging from 59 to 121 mosmol/l the relative extinction (E/E_{max}) was about 0.7 (Fig. 21). If the various fractions of the RBC population lyse at different rates, we would expect to find 30% of the membrane material in the sediment, since about 70% of the RBC's would be lysed completely.

NaCl treated RBC's were subjected to lysis in hypotonic NaCl solutions for various periods of time. After stopping lysis by addition of 1.5 M NaCl the suspensions were centrifuged and the relative extinction of the supernatant determined to serve as an estimate of percentage lysis. After repeated treatment of the sediment with carbondioxide saturated water, the phosphorus content of these sediments was determined as a measure for the amount of membrane material. The phosphorus content in the sediment was expressed as a percentage of the original membrane phosphorus. A comparison of the percentage of the relative extinction (liberated hemoglobin) and the relative amount of membrane phosphorus sedimented, is given in Table XXII.

Table XXII

Comparison of percentage liberated hemoglobin (%H) and relative amount of membrane material in the sediment (%P) upon lysis in NaCl solutions.

NaCl treated RBC's were added in duplicate to hypotonic NaCl solutions (pH 7.5), ranging from 59 to 291 mosmol NaCl/l. Lysis was stopped after 5, 10, 15 and 300 sec by making the solutions slightly hypertonic with a concentrated NaCl solution. The sediment obtained after centrifugation at 1000 x g (10 min) was treated with carbondioxide saturated water and thereafter the membrane phosphorus content determined. For further details see 7.2.2.

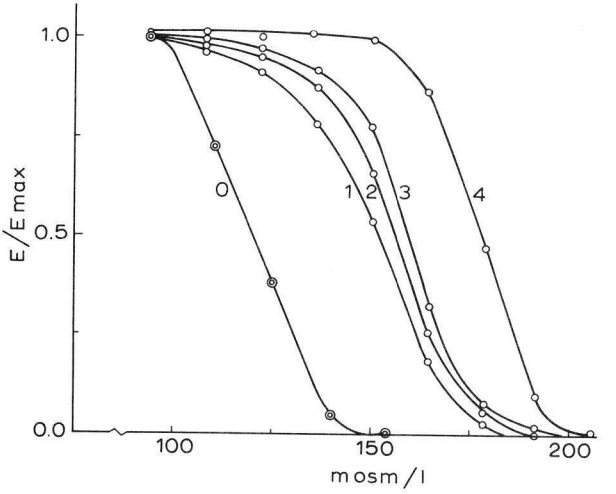
Tonicity in mosmol/l	Lysis time in sec: 5		10		15		300	
	%H	%P	%H	%P	%H	%P	%H	%P
59	75	70	82	84	95	48	98	10
89	71	69	84	74	94	59	100	12
121	70	71	80	74	90	66	98	12
152	58	72	75	76	85	57	98	21
183	15	93	29	93	37	85	93	29
200	1	101	1	105	2	102	16	89
291	0	98	0	102	0	98	0	102

After 10 sec, when about 75-84% of the hemoglobin was liberated in the 59-152 mosmol/l range, 74-84% of the membrane phosphorus was recovered in the sediment representing the non-lysed cells. Even after 15 sec when 85-95% hemoglobin release was observed, still 50% of the membrane material was present in the sediment. After 300 sec, below 152 mosmol/l hemoglobin release was almost complete and only about 10% of the membrane phosphorus was recovered in a hardly visible sediment. This experiment suggests that the tonicity-independent but time-dependent lysis rate below 152 mosmol/l is mainly due to a gradual loss of hemoglobin from all RBC's, though small differences in hemoglobin release between different fractions of the RBC population cannot be excluded.
A possible reason for this tonicity-independent but time-dependent lysis behaviour observed in NaCl solutions, but not in sucrose solutions, may be that the intracellular pH of RBC's changes upon addition to buffered NaCl or sucrose solutions as was shown in chapter 4 (4.3.2). In RBC's treated with buffered 300 mM sucrose (pH 7.5) the intracellular pH amounted to 7.9 and in those treated with buffered 150 mM NaCl (pH 7.5) to 7.4. The latter value is close to the isoelectric point of hemoglobin which appears to be about pH 7.2 (4.3.4). Perhaps these differences in intracellular pH affect the release of hemoglobin.

In the preceding lysis experiment the values of the relative extinction (E/E_{max}) after different times were calculated from the observed extinctions in NaCl solutions and the extinction upon complete lysis in buffer solution. Since however, in strongly hypotonic NaCl solutions a time-dependent lysis was observed, it seems not justified to calculate the relative extinction (E/E_{max}) with as E_{max} the extinction upon complete lysis in buffer solution. To our opinion the tonicity-independent extinction found at different times in very hypotonic NaCl solutions should be taken as E_{max}. The curves presented in Fig. 22 were recalculated in this way from the curves presented in Fig. 21. This figure shows that the time-dependent shift to lower osmotic resistance values is still maintained. It may be concluded that in hypotonic NaCl solutions the lysis pattern is composed of a fairly rapid and tonicity-independent lysis process, and a rather slow tonicity-dependent lysis process.

Fig. 22

Corrected osmotic resistance of pig red blood cells in NaCl solutions. The data presented in Fig. 21 were recalculated by taking the extinctions after 5, 10, 15 and 300 sec, respectively, in 90 mosmol NaCl/l as the total amount of hemoglobin that could be liberated in the corresponding periods of time (E_{max}). curve 0; sucrose treated cells in sucrose solutions after 5 and 300 sec, curves 1-4; NaCl treated cells in NaCl solutions after 5,10,15 and 300 sec, respectively.



7.3.2. The tonicity- and time-dependent lysis of pig red blood cells in hypotonic NaCl solutions

The osmotic resistance curves of RBC's treated with buffered 150 mM NaCl, determined in NaCl solutions at t=300 and t=900 sec, were found to coincide. This means that an equilibrium situation is reached within 300 sec. The lysis rate between 0 and 300 sec was studied in more detail at three tonicities, 150, 165 and 180 mosmol/l, and at time intervals varying from 5-30 sec. A biphasic pattern of the tonicity dependent lysis was obtained, in accordance with the observations of Bowdler and Chan (1969) on human RBC's. First a rapid lysis phase was observed, which after about 60 sec shaded off into a slow lysis phase. Maximum lysis was reached after nearly 240 sec. Since the lysis rate slowed down in the longer run, it was difficult to determine the time at which the equilibrium situation was reached.

Before attempting an explanation of the observed tonicity- and time-dependent lysis in buffered hypotonic NaCl solutions, we shall present some more data concerning the osmotic resistance in buffered NaCl and sucrose solutions of pig RBC's, treated in different ways. In Fig. 21. it was shown that the osmotic resistance in buffered sucrose solutions of RBC's treated with buffered isotonic sucrose differed considerably from that obtained in buffered NaCl solutions with RBC's treated with buffered isotonic NaCl solution. The lysis curves appeared to parallel each other. Whereas the 50% hemolysis value in sucrose amounted to 120 mosmol/l, this value was in NaCl solutions about 175 mosmol/l. When, however, NaCl treated RBC's were added to buffered sucrose solutions and sucrose treated RBC's to buffered hypotonic NaCl solutions, a different picture was obtained. After 300 sec the lysis curve of sucrose treated RBC's in hypotonic NaCl solutions almost coincided with that obtained with NaCl treated RBC's. The lysis curve of NaCl treated RBC's in sucrose differed considerably from that of sucrose treated RBC's in hypotonic sucrose. Comparative 50% hemolysis values of differently treated RBC's in hypotonic NaCl and sucrose solutions are presented in Table XXIII.

In chapter 4 it was shown that obviously the chloride-hydroxyl ion exchange coupled to the buffering action of hemoglobin, markedly affects the osmotic resistance of RBC's. RBC's treated with buffered 150 mM NaCl, pH 7.5, contained an increased amount of chloride ions, were swollen and therefore were less resistant. RBC's treated with buffered 300 mM sucrose, pH 7.5, showed a decrease of the chloride content, were shrunken and appeared to possess an increased osmotic resistance. The resistance experiments described above strongly suggest that this exchange phenomenon also plays a role in the anomalous osmotic resistance behaviour in NaCl solutions.
Since the RBC membrane is very permeable to monovalent anions (Luckner and Lo Sing, 1938; Tosteson, 1959; Crandall et al, 1971), the slow shift to lower osmotic resistance values in NaCl solutions is apparently in contradiction with the exchange hypothesis. Measurement of extracellular pH changes in isotonic NaCl solutions showed that these

changes take place rather slowly, being complete after about 200-300 sec (Fig. 14). A possible explanation for the slow chloride-hydroxyl ion exchange in this case may be the low extracellular/intracellular chloride ratio of about 1.44. If this exchange process takes place slowly, the osmotic resistance curve in NaCl solutions after 5 sec will not differ much from that obtained due to the flow of water only. This suggestion is supported by the observed agreement between the osmotic resistance of NaCl treated RBC's in NaCl solutions at t = 5 sec (152 mosmol/l) and that of the same RBC's in sucrose solutions (149 mosmol/l).

Table XXIII

Comparison of 50% hemolysis values of differently treated pig red blood cells in buffered sucrose and NaCl solutions as a function of time.
RBC's, untreated or treated with either 300 mM sucrose or 150 mM NaCl, both buffered with 1 mM sodium phosphate to pH 7.5, were added to buffered hypotonic NaCl or sucrose solutions. The 50% hemolysis values are given in mosmol/l.

Treatment	50% hemolysis in sucrose			50% hemolysis in NaCl			
	5 sec	15 sec	300 sec	5 sec	10 sec	15 sec	300 sec
Buffered sucrose pH 7.5	120	120	120	130	143	---	170
Buffered NaCl pH 7.5	---	149	149	152	156	159	176
Untreated	---	130	130	---	---	---	172

Furthermore it was observed that only small differences exist in the osmotic resistance of the RBC's in choline chloride, KCl and NaCl solutions buffered with 1 mM sodium phosphate to pH 7.5. The osmotic resistance in choline chloride was slightly increased (nearly 5 mosmol/l) and that in KCl slightly decreased (about 3 mosmol/l) compared to that in NaCl solutions. This observation is in apparent contradiction to the data of Bowdler and Chan (1969), who observed, in addition to the biphasic lysis process, a more pronounced lysis in KCl than in NaCl solutions. Since, however, the osmotic resistance curves are fairly steep, an increase of lysis with 10-20% due to cation permeability only causes a small shift of the osmotic resistance curve to lower resistance values (about 5 mosmol/l). Obviously, the slow shift to lower osmotic resistance values in NaCl solutions (pH 7.5) is largely due to chloride-hydroxyl ion exchange coupled to the buffering action of hemoglobin.

This suggestion is further supported by measurements of the osmotic resistance in hypotonic NaCl solutions of different pH values. In hypotonic NaCl solutions of pH 8.5 no decrease with time of the osmotic resistance of untreated RBC's was observed, whereas in hypotonic NaCl solutions of pH 6.5 a considerable decrease was seen. At equilibrium the 50% hemolysis value amounted to 142 mosmol/l at pH 8.5 and to 196 mosmol/l at pH 6.5. In Table VI it was seen that the chloride content of RBC's treated with 150 mM NaCl solutions of various pH values, showed considerably variations with the pH of these solutions. With decreasing extracellular pH the intracellular chloride content increased.

The tonicity-independent but time-dependent lysis (7.3.1) was observed in NaCl solutions of pH 8.5 as well as in NaCl solutions of pH 6.5. A complication, which cannot be explained at the moment, is the tonicity- - and time-dependent lysis in buffered hypotonic NaCl solutions (pH 7.5) upon treatment of RBC's with an excess of buffered 150 mM NaCl (pH 7.5). It was expected that after this extensive treatment the intracellular chloride content would have reached a maximum value. Since the net charge of hemoglobin and thus its buffering action apparently depend on its intracellular concentration (Gary-Bobo and Solomon, 1968 and 1971) we also treated RBC's with an excess of buffered 110 mM NaCl, pH 7.5. Upon extensive treatment with 110 mM NaCl, however, the same time-dependent osmotic resistance was observed as with RBC's treated with buffered 150 mM NaCl.

Since the osmotic resistance in NaCl solutions at physiological pH values proved to be strongly dependent on time, which was not the case in sucrose solutions, it may be concluded that the osmotic resistance of RBC's is probably best represented by the osmotic resistance curve in sucrose. The validity of this conclusion will be argumented in the next section.

7.3.3. The validity of the osmotic resistance of pig red blood cells determined in sucrose solutions

In Table IV it was shown that the intracellular cation and anion composition of the RBC's was virtually unchanged upon treatment with unbuffered 300 mM sucrose. Upon treatment with buffered 300 mM sucrose (pH 7.5) a considerable number of osmotically active chloride ions were lost, reflected in shrinkage of the RBC's. If this chloride-hydroxyl

ion exchange in hypotonic sucrose solutions takes place much more rapidly than the release of hemoglobin, then this will result in a rapid shrinkage of the RBC's prior to lysis. The shrunken RBC's would be able to withstand a higher difference in osmotic activity and would thus lyse at lower tonicities, resulting in an increase in osmotic resistance. Since in unbuffered sucrose solutions this exchange phenomenon and the resulting RBC shrinkage do not take place, the osmotic resistance curve in this case should diverge from that obtained in buffered sucrose solutions. However, the osmotic resistance curves of untreated RBC's in unbuffered hypotonic sucrose solutions and in hypotonic sucrose solutions buffered with 1 mM or 5 mM sodium phosphate to pH 7.5, almost coincided. The 50% hemolysis values in a representative experiment, using RBC's pretreated with buffered 150 mM NaCl, amounted to 152, 149 and 146 mosmol/l, respectively. Thus lysis of RBC's in sucrose solutions must occur much more rapidly than the chloride-hydroxyl ion exchange.

A complication that might also cause a small increase in osmotic resistance, is the pre-lytic loss of potassium and an equivalent number of anions as observed for human RBC's by Kwant and van Steveninck (1968), Seeman et al (1969) and Livne and Raz (1971). We could show, however, that a pre-lytic loss of potassium and anions scarcely occurs in pig RBC's. RBC's, treated with unbuffered 300 mM sucrose in order to remove extracellular serum, were added to hypotonic unbuffered sucrose solutions. After 300 sec the solutions were made slightly hypertonic with a concentrated sucrose solution. After centrifugation the released amounts of hemoglobin and of potassium were determined in the supernatant. The data, relative to the values in a completely lysed preparation, were plotted versus the osmolarity. The obtained hemoglobin and potassium curves almost coincided,the difference being less than 2-3 mosmol/l.

The differences in osmotic properties of pig RBC's under the circumstances presented above also apply to human RBC's in general. Two minor differences were observed in human RBC's: a slight time-dependence of the lysis process in hypotonic sucrose solutions which was absent in pig RBC's, and a significant difference between the "potassium" osmotic resistance curve and the "hemoglobin" osmotic resistance curve, amounting to about 8 mosmol/l after 300 sec. Since lysis is complete nearly instantaneously, it is still questionable whether the potassium loss, which was measured after a considerably longer time, plays a role or not.

The momentary lysis of RBC's in sucrose solutions, independent of the presence of buffer, and the negligible loss of cations, justify the conclusion that the original resistance of RBC's is best represented by the osmotic resistance in sucrose solutions.

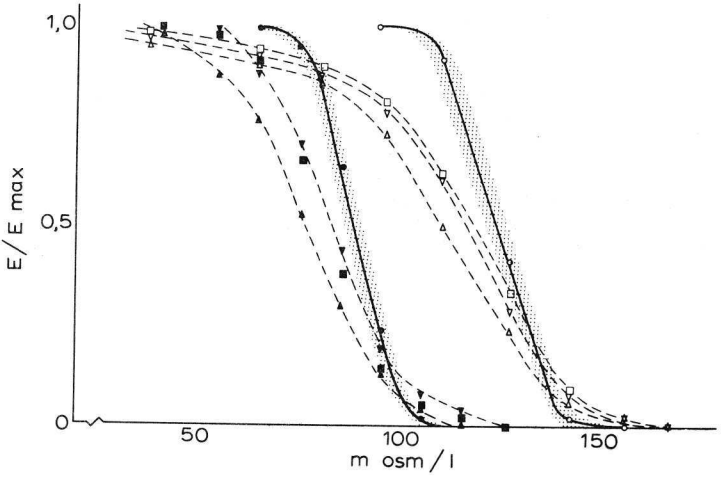
7.4. COMPARISON OF THE OSMOTIC RESISTANCE OF NORMAL AND PATHOLOGICALLY ALTERED HUMAN RED BLOOD CELLS IN NaCl AND SUCROSE SOLUTIONS

7.4.1. Osmotic resistance determinations in sucrose and in NaCl solutions

The osmotic resistance of untreated human RBC's, obtained from 16 normal donors (further called "controls") was determined after 300 sec in hypotonic NaCl and sucrose solutions, buffered with sodium phosphate to pH 7.3.

Fig. 23

Osmotic resistance of human red blood cells in sucrose and in NaCl solutions.
The osmotic resistance was determined in hypotonic NaCl and sucrose, buffered with sodium phosphate to pH 7.3.
Curves of controls (n = 10) with standard deviations (shaded areas) in sucrose (●) and NaCl (o). Curves of two patients suffering from sidero-achrestic anemia in sucrose (▲ and ▼) and in NaCl (Δ and ▽). Curves of a patient suffering from an undefined anisocytosis in sucrose (■) and in NaCl (□).



The osmotic resistance curves in each solution appeared to parallel each other. Moreover, the curves in sucrose solutions were almost parallel to those found in NaCl solutions. From the tonicities, giving rise to relative extinctions of 0.25, 0.50 and 0.75, a good impression of the variability of the osmotic resistance in sucrose and in NaCl solutions was obtained. The osmotic resistance of controls lies between narrow limits in both solutions (Table XXIV).
The average osmotic resistance curves for the controls were compared with those of untreated RBC's from some pathological cases.
In Fig. 23 the average osmotic resistance curves of controls in sucrose and in NaCl solutions are shown together with the osmotic resistance curves of RBC's obtained from two patients with sideroachrestic anemia and one patient with an undefined anisocytosis.
In Fig. 24 the same curves of controls are compared with osmotic resistance curves of RBC's obtained from a patient suffering from hereditary hemolytic anemia and one patient with hereditary thrombocytopathic thrombocytopenia. The tonicities representing levels of hemolysis of 0.25, 0.50 and 0.75, respectively, are listed in Table XXIV.

Fig. 24

Osmotic resistance of human red blood cells in sucrose and NaCl solutions. The osmotic resistance was determined in hypotonic NaCl and sucrose, buffered with sodium phosphate to pH 7.3.
Curves of controls (n = 10) with standard deviations (shaded areas) in sucrose (●) and NaCl (o). Curves of a patient suffering from hereditary hemolytic anemia in sucrose (▲) and in NaCl (△). Curves of a patient suffering from hereditary thrombocytopathic thrombocytopenia in sucrose (▼) and in NaCl (▽).

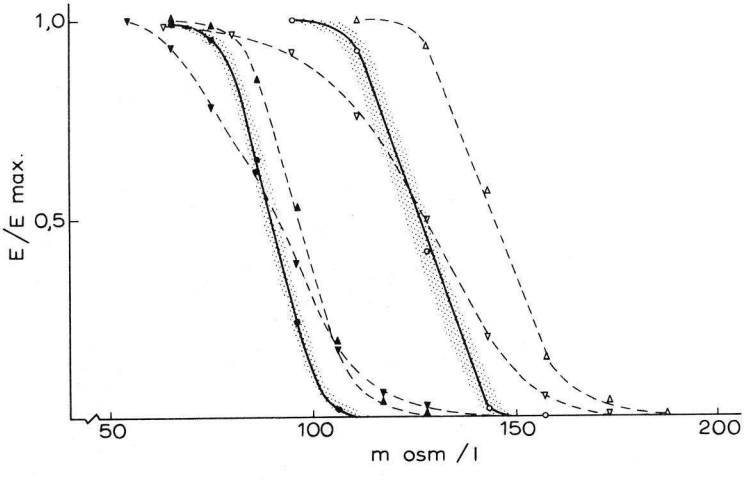


Table XXIV

Comparison of tonicities causing certain levels of hemolysis of human red blood cells in NaCl and in sucrose solutions. Tonicities are given in mosmol/l. NaCl and sucrose solutions were buffered with sodium phosphate to pH 7.3. Abbreviations for pathological cases are: SAA, sideroachrestic anemia; A, undefined anisocytosis; HHA, hereditary hemolytic anemia; HTT, hereditary thrombocytopathic thrombocytopenia.

Donors	Sucrose				NaCl			
	25%	50%	75%	Δ25-75%	25%	50%	75%	Δ25-75%
Controls (n = 16)	96±1.7	90±1.6	83±1.8	13	132±2.5	126±3.4	119±3.1	13
SAA-1	89	78	66	23	124	108	92	32
SAA-2	92	83	73	19	127	116	99	28
A	91	82	72	19	130	118	102	28
HHA	104	98	90	14	148	142	133	15
HTT	102	91	78	24	140	126	112	28

From the curves and the Δ25-75% values which indicate their slopes, one can conclude that only the osmotic resistance curves of RBC's obtained from a patient with hereditary hemolytic anemia run parallel to those found for the controls, but they are shifted to lower osmotic resistance values in both solutions. The resistance curves for the other patients were less steep, as indicated by the higher Δ 25-75% values. This implies a greater range in osmotic properties of the fractions of their RBC population. In the cases of sideroachrestic anemia and anisocytosis the osmotic resistance of nearly the entire RBC population was increased, compared to that of controls. In the case of hereditary thrombocytopathic thrombocytopenia the osmotic resistance curves were less steep than those of the controls, almost half of the RBC population being more resistant and half being less resistant. In all pathological cases the most pronounced deviations of the osmotic resistance curves from the control curves occurred in NaCl solutions. Hence, from a diagnostic point of view, osmotic resistance determinations in NaCl solutions are preferable. Since differences in composition of the cell contents and/or differences in mean cell volume and size distributions could cause the deviations from the control curves, these parameters were investigated and their results are described in the following sections.

7.4.2. Analysis of the composition of the cell content

In Table XXV the analytical data of plasma and RBC's of controls as well as of patients are presented. The standard error of the determinations with RBC's of controls was of the same order as observed with RBC's of patients.

Table XXV

Cation and anion concentrations of human red blood cells. The values are given in mmol/l RBC's for ten controls (± S.D.) and five patients (± S.E.). All determinations, performed as described in 4.2, were carried out in fivefold. The abbreviations for the pathological cases are given in Table XXIV

Donor	Sodium	Potassium	Total	Chloride	Phosphate	Total
Controls	10.3±2.7	98.1±4.6	108.5±3.9	56.2±1.9	4.3±1.0	60.5±1.8
SAA-1	11.5±0.2	104.8±0.5	116.3	62.1±0.3	2.6±0.1	64.7
SAA-2	12.6±0.7	111.3±0.8	123.9	62.5±0.1	4.6±0.1	67.1
A	8.3±0.5	113.6±0.7	121.9	59.4±0.6	1.8±0.1	61.2
HHA	7.2±1.1	95.1±0.6	102.3	44.9±0.5	8.1±0.1	52.9
HTT	24.1±0.3	89.0±0.2	113.0	59.5±0.4	3.5±0.1	63.0

Some significant deviations from the control values were observed in the RBC's of patients.

a) In the cases of sideroachrestic anemia and anisocytosis a small increase in the potassium concentration as well as a small increase in chloride concentration was observed.
b) In the patient with hereditary hemolytic anemia a decreased chloride content and an increased phosphate content was found.
c) In the case of hereditary thrombocytopathic thrombocytopenia the sodium concentration was increased and the potassium concentration decreased.

No significant differences in the plasma concentrations of sodium, potassium, chloride and phosphate were observed.

7.4.3. Determination of mean cell volume, size distribution and relative hemoglobin content

In Table XXVI are presented the number of RBC's per ml blood, the total cell volume determined from ^{14}C-inulin dilution as well as from hematocrit

readings corrected for trapped plasma and calculated from these two parameters the mean cell volume (MCV). There was a good agreement between the two values obtained for the total cell volume, therefore the mean value is presented.

Table XXVI

Number of human red blood cells per ml blood, total cell volume, mean cell volume (MCV) and relative hemoglobin concentration.
The relative hemoglobin concentration was calculated by multiplying the extinction at 540 nm of 0.05 ml blood lysed in 10^3 ml buffer solution, by 10^{11} and dividing by the total RBC volume in μ^3. The value obtained for controls was arbitrarily taken as 1.00. All determinations were performed in triplicate. For controls the mean with standard deviation is given. Abbreviations for pathological cases are given in Table XXIV.

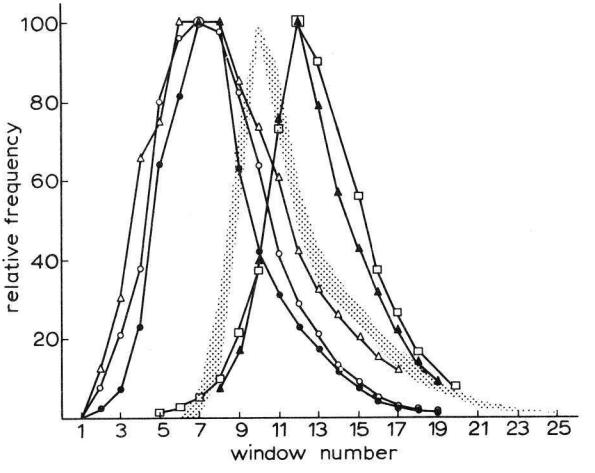
Donors	RBC's x 10^{-9} per ml blood	Total Cell Volume %		MCV in μ^3	Rel.Hb Conc.
		^{14}C-inulin	Hematocrit		
Controls	4.66±0.44	40.6±3.8	41.5±2.6	87.6±5.9	1.00±0.04
SAA-1	5.37	40.2	38.8	73.6	0.76
SAA-2	5.22	35.1	35.2	66.6	0.84
A	3.44	30.0	28.0	84.3	0.74
HHA	3.23	26.5	26.5	81.5	1.10
HHT	3.87	37.9	38.2	98.4	0.97

The relative hemoglobin concentrations obtained from the extinctions appeared to be reliable, since in one experiment with RBC's of two controls and two patients we obtained results equal within the experimental error with a hemoglobin determination by the hemiglobin-cyanide reaction (van Kampen and Zijlstra, 1964).
From the data presented in Table XXVI we may conclude that:

a) The mean cell volume of RBC's from patients suffering from sideroachrestic anemia was lower than that of controls, and the same was true for the mean hemoglobin concentration.
b) Whereas the mean cell volume of RBC's from a patient suffering from an undefined anisocytosis, was normal, the hemoglobin concentration was lowered.
c) The mean cell volume of RBC's from the patient with hereditary hemolytic anemia was equal to that of controls, while the mean hemoglobin concentration was higher than that of the controls.
d) RBC's of a patient suffering from hereditary thrombocytopathic thrombocytopenia contained a normal hemoglobin concentration, but their MCV was increased.

The size distribution curves of pathological RBC's were compared with the distribution range from three controls (Fig. 25). An increase in window number corresponds with an increase in cellular volume.

Fig. 25
Size distribution curves of normal and pathological RBC's.
SAA-1 o—o, SAA-2 ●—●, A Δ—Δ , HHA Δ—Δ, HTT □—□ and shaded area for three controls.
Abbreviations are given in Table XXIV.



The distribution curves of RBC's from patients with sideroachrestic anemia and an undefined anisocytosis show that these RBC's are generally smaller than those of controls. The distribution curves of RBC's from patients suffering from hereditary hemolytic anemia and hereditary thrombocytopathic thrombocytopenia indicate that these RBC's are generally somewhat larger than those from controls.

7.4.4. Discussion of the differences in osmotic resistance of normal and pathologically altered human RBC's in NaCl and sucrose solutions

As shown in Fig. 23 and Table XXIV the difference in osmotic resistance of control RBC's in NaCl and sucrose solutions amounted to 36±1.6 mosmol/l at three levels of hemolysis. The osmotic resistance was lower

in NaCl compared to that in sucrose solutions. As was stated in 7.2, this difference apparently originates from chloride-hydroxyl ion exchange, coupled to the buffering action of hemoglobin, taking place in NaCl solutions. Since the clinical method is performed at pH 7.3 and the intracellular pH amounts to about 7.5, a marked anion exchange may be expected in view of the increasing chloride uptake at lower extracellular pH.

We shall now discuss the observations with RBC's originating from the pathological cases and compare these informations with the data obtained with control RBC's. This discussion is somewhat speculative, since the data of pathological RBC's are rather scarce.

A) RBC's obtained from patients suffering from sideroachrestic anemia. It was observed that in sucrose as well as in NaCl solutions almost the total RBC population was more resistant than that of controls (Fig.23). The deviation in NaCl solutions appeared to be more pronounced than in sucrose solutions. The nearer the osmotic resistance of certain RBC fractions to that of control RBC fractions is, the larger is the difference in osmotic resistance in NaCl and in sucrose solutions. Obviously a certain fraction of the RBC population is almost normal. The small increase from controls in mean cation and anion concentration implies a negligible difference in intracellular osmotic activity (Table XXV). Possibly this increase compensates to some extent the decreased hemoglobin content (Table XXVI). The mean cell volume and the hemoglobin concentration are decreased. The size distributions (Fig.25) are rather wide. These changes suggest that the hemoglobin concentration of the individual RBC's varied considerably. This suggestion is confirmed by the decrease in osmotic resistance difference in NaCl and in sucrose solutions upon increased resistance of the fractions of the RBC population. The chloride-hydroxyl ion exchange will decrease with decreasing hemoglobin concentration, since this exchange depends on the hemoglobin concentration. Though the cell volumes differ considerably, no indications for differences in membrane area were obtained.

B) RBC's from a patient with an undefined anisocytosis.
The increased osmotic resistance in NaCl and in sucrose solutions (Fig. 23), the slightly increased cation content (Table XXV) and the decreased hemoglobin concentration (Table XXVI) are comparable to those observed in sideroachrestic anemia. The osmotic resistance curves in NaCl and in sucrose solutions are not parallel to each other, as

was also observed in sideroachrestic anemia. Whereas the size distribution curve (Fig. 25) is almost equal to that of RBC's in sideroachrestic anemia, the mean cell volume appeared to be almost equal to that of controls. The decreased hemoglobin concentration, the size distribution curve, the increased osmotic resistance in sucrose and in NaCl solutions and the different slopes of these two resistance curves, suggest that the cell volume of the RBC's differs considerably, whereas the membrane area appears to be nearly normal.

C) RBC's from a patient with hereditary hemolytic anemia.
Whereas the mean cell volume was almost normal (Table XXVI)a markedly decreased osmotic resistance was observed in NaCl solutions (Fig. 24). The osmotic resistance curves appeared to be almost parallel to each other and to those of control RBC's. The differences between the osmotic resistance in sucrose and that in NaCl solutions was 43 mosmol/l, which is larger than for the controls (36 ± 1.3 mosmol/l). Though the cation content was relatively normal, the anion composition appeared to differ markedly (Table XXV). However, since at an intracellular pH of nearly 7.5 phosphate will be present mainly as secondary phosphate, the net anionic charge will be almost equal to that of the controls (59 msomol/l RBC's). The ratio of intra- and extracellular chloride is lower than in the controls, leading to a slightly higher exchange of intracellular hydroxyl and extracellular chloride ions, and thus to a more pronounced decrease of the osmotic resistance in NaCl solutions. The exchange phenomenon will, moreover, be intensified by the higher hemoglobin concentration in view of its buffering action. The same phenomenon probably underlies the shifted size distribution curve in Isoton, a solution with a high chloride concentration (Fig. 25). This curve suggests an increased mean cell volume, whereas a normal value was obtained with the ^{14}C-inulin method (Table XXVI).

D) RBC's from a patient suffering from hereditary thrombocytopathic thrombocytopenia.
The RBC population was partly less resistant and partly more resistant than those of controls (Fig. 24). The curves in sucrose and in NaCl solutions are nearly parallel to each other. The mean cell volume was somewhat increased, whereas the mean hemoglobin concentration was almost normal (Table XXVI).The size distribution curve was somewhat shifted to higher cell volumes (Fig. 25), which agrees with the increased mean cell volume. The total cation concentration was almost normal, but the

potassium content was diminished and that of sodium increased. The anionic concentration was within the normal range.
These data suggest that the surface/volume ratio differs for the various fractions of the RBC population. Hence, the less resistant cells were probably closer to spherical, the more resistant ones somewhat flattened.

7.5. CONCLUSIONS

In the first part of this chapter it was shown that the osmotic resistance of porcine and human RBC's in NaCl solutions (pH 7.5 and 37^{o}) is time-dependent and decreases slowly in time. After about 300 sec equilibrium is reached. The time-dependent lysis in NaCl solutions appeared to be rather complex. In very hypotonic NaCl solutions an almost tonicity-independent but time-dependent lysis process was observed. It was suggested that this process probably is due to a fairly slow release of hemoglobin from the RBC's. A pH decrease may affect the hemoglobin release, since in NaCl solutions the intracellular pH is lowered. At higher NaCl concentrations both a tonicity-dependency as well as a time-dependency of the lysis process were observed. Experiments performed at different pH values and in solutions of different chloride salts strongly suggest that this phenomenon is mainly caused by chloride-hydroxyl ion exchange coupled to the buffering action of hemoglobin. A small contribution is probably supplied by the cation permeability in cells swollen to a volume close to that critical for hemolysis. It is concluded that the osmotic resistance curves in sucrose solutions provide the best approximation of the original osmotic properties of RBC's. Lysis in buffered sucrose must take place much more rapidly than chloride-hydroxyl ion exchange, since almost the same osmotic resistance data were obtained in unbuffered sucrose in which no exchange is observed. A pre-lytic loss of potassium appeared to be unimportant, since the osmotic resistance curves of pig RBC's, obtained from the release of hemoglobin and potassium, almost coincided. With human RBC's only small differences were observed.
In the second part of this chapter values for the osmotic resistance of normal and pathologically altered human RBC's were obtained and discussed. Though the osmotic resistance determined in sucrose solutions apparently leads to the most reliable data, for diagnostic purposes the osmotic resistance determined in NaCl solutions at pH 7.3, or slightly less is

recommended. The more pronounced differences in NaCl solutions are probably caused by changes in hemoglobin concentration, which affect the degree of chloride-hydroxyl ion exchange in normal and pathologically altered RBC's.

8. GENERAL SUMMARY

The permeability properties and composition of red blood cell membranes, originating from different mammalian species, display considerably differences. Although various authors have suggested that there would be a correlation between lipid composition and membrane permeability, such a correlation has not been demonstrated convincingly. Since the lipid composition can be determined with high accuracy, lack of demonstration could be due either to non-existence of correlation, or to wrong way of determining membrane permeability. The shortcomings of the determination of membrane permeability by means of the osmotic lysis method according to Jacobs have led to the investigations described in this thesis. Comparative permeability studies of mammalian red blood cells have been performed with glycerol as permeant. The osmotic resistance of human and pig RBC's in electrolyte and nonelectrolyte solutions has been studied in more detail.

In chapter 1 a short survey of current knowledge of composition and structure of the RBC membrane is presented. Special attention is devoted to lipid and protein composition and some models of membrane structure are discussed. Most recent investigations lend support to the unit-membrane concept. Next, a short survey of present insights in membrane permeability to simple nonelectrolytes is given. Special attention is given to diffusion through pores, diffusion though the lipid bilayer and facilitated diffusion. No evidence is available so far to indicate which one of these phenomena regulates membrane permeability for simple nonelectrolytes like glycerol.

In chapter 2 the osmotic lysis method, commonly used in permeability studies with red blood cells, is discussed critically. This method is based on the observation that RBC's suspended in an isotonic solution of a permeating substance swell upon penetration of permeant and water. When the maximum cell volume is exceeded, lysis occurs. The time elapsing until a certain degree of lysis has occurred (time of hemolysis) is taken as a relative measure of membrane permeability. Since time of hemolysis depends not only on membrane permeability, but also on the surface/volume ratio, differences in this ratio affect the time of hemolysis. Differences in surface/volume ratio are reflected in osmotic

resistance, i.e. the maximum swelling capacity of the red blood cells due to osmotic water flow as a result of differences in extracellular tonicity of solutions of non-permeating substances.
It is possible to eliminate the influence of differences in surface/volume ratio on permeability determinations by combining estimations of the hemolysis rates in solutions of permeating substance ranging from very hypotonic to isotonic, and the osmotic resistance in hypotonic solutions of a non-permeating substance. Equations are given which describe the lysis process and which can be used to calculate the permeability coefficient. Simplification of the equations by neglecting the reflection coefficient appear to be justified on the basis of the experimental results described in Chapters 3 and 5.

In Chapter 3 the validity of the mathematical description of the osmotic lysis is proven. The lysis behaviour of pig red blood cells, pretreated with isotonic solution (pH 7.5) appears to be in accordance with theoretical predictions. The glycerol permeability coefficients have been calculated from these data. Neither with untreated RBC's, nor with RBC's treated with isotonic NaCl solution is an ideal lysis pattern obtained. In both cases a lysis delay has been observed in glycerol solutions. After a certain period of time lysis proceeds at a nearly normal rate. The glycerol permeability coefficients can also be calculated in these cases.

In Chapter 4 it is shown that the lysis delay is mainly due to a rapid chloride-hydroxyl ion exchange coupled to the buffering action of hemoglobin. Due to this exchange process the amount of osmotically active substances in the red blood cell diminishes rapidly resulting in shrinkage. This means that an extra number of permeating molecules must pass the cell membrane before lysis can proceed.

In Chapter 5 it is shown that RBC membrane permeability is quantitatively best represented by the permeability coefficient determined with the modified osmotic lysis method. In a group of mammalian species with low glycerol permeability the glycerol permeability coefficient shows a different sequence than is found for the times of hemolysis in isotonic glycerol. Neither the times of hemolysis nor the glycerol permeability coefficients appear to be correlated with published data on the phospholipid/cholesterol ratio, the phospholipid composition and the fatty acid pattern. Whereas for all other species the glycerol permeability coefficient shows a relatively small variability (10-20%), there was a very large variability (70%) in this parameter for rabbit RBC's.

In the studies presented in Chapter 6 it is shown that RBC's obtained from individual rabbits, differ markedly in glycerol permeability. Red blood cells from ten rabbits have been studied in more detail. Although marked individual differences in glycerol permeability have been observed, no differences in cholesterol/phospholipid ratio, phospholipid composition and fatty acid pattern can be detected. These data once again confirm that no simple correlation between lipid composition and glycerol permeability exists. Mean cell volume, osmotic resistance and composition of the cell content do not show notable variations. Preliminary experiments do not reveal a facilitated diffusion system for glycerol in rabbit red blood cell membranes with a high glycerol permeability.

The osmotic resistance in NaCl solutions is strongly time-dependent, whereas in sucrose solutions the osmotic resistance is time-independent, as is shown in Chapter 3. In Chapter 7 investigations are presented which suggest that the time-dependence in NaCl solutions is due to a delayed hemoglobin release and to chloride-hydroxyl ion exchange. Moreover, it is shown that the osmotic resistance in sucrose solutions gives the best approximation of the original osmotic resistance of RBC's. A comparative study of the osmotic resistance of normal and pathologically altered human RBC's in NaCl and in sucrose solutions demonstrates that from a diagnostic point of view the best results are obtained in NaCl solutions, which is the method commonly used in clinical studies.

Our findings indicate that more accurate and reliable data on nonelectrolyte permeability can be obtained with the modified lysis method. In studies under variable conditions, e.g. of temperature and pH, this method eliminates accompanying changes like pre-lytic loss of ions, retardation of lysis due to ion-exchange and differences in release of hemoglobin. Such accurate permeability data are essential for further studies of the relation between permeability characteristics and membrane structure.

A more detailed study of the differences in glycerol permeability of RBC's from individual rabbits seems to be of interest, since their lipid composition appeared to be not significantly different, while there was a remarkable individual variation in permeability in this species.

9. SAMENVATTING

Zowel de permeabiliteits-eigenschappen als de samenstelling van rode bloedcel-membranen van verschillende zoogdier species blijken aanzienlijk te verschillen. Ofschoon verschillende auteurs gesteld hebben dat er een korrelatie moet bestaan tussen de samenstelling van de lipiden en de permeabiliteit van de rode bloedcel-membraan, kon een dergelijk verband niet overtuigend aangetoond worden. Aangezien de samenstelling van de lipiden met een hoge graad van nauwkeurigheid bepaald kan worden, moet het feit dat voornoemd verband niet aangetoond kon worden het gevolg zijn van óf de afwezigheid van een dergelijke korrelatie, óf van de onjuiste wijze van bepalen van de membraanpermeabiliteit. De tekortkomingen van de bepaling van de membraanpermeabiliteit m.b.v. de osmotische lysis methode volgens Jacobs, vormden de aanleiding tot het onderzoek beschreven in dit proefschrift. Vergelijkend permeabiliteits-onderzoek werd uitgevoerd met rode bloedcellen van verschillende zoogdieren. Als permeërende stof werd glycerol gebruikt. Daarnaast is de osmotische resistentie van rode bloedcellen van mens en varken in electrolyt en niet-electrolyt oplossingen uitvoerig bestudeerd.

In hoofdstuk 1 is een kort overzicht gegeven van de huidige kennis van de samenstelling en bouw van de rode bloedcel-membraan. Vooral aan de samenstelling van de lipiden en proteïnen is aandacht besteed. Daarnaast zijn enige membraan-modellen besproken. Ook de meest recente onderzoekingen blijken in overeenstemming te zijn met het "unit-membrane" model. Bovendien is een kort overzicht gegeven van de opvattingen betreffende de permeabiliteit van rode bloedcel-membranen voor eenvoudige niet-electrolyten. Vooral aan diffusie door poriën, aan diffusie door de lipiden dubbellaag en aan "facilitated" diffusie werd aandacht besteed.

In hoofdstuk 2 worden kritisch de verschillende facetten van de osmotische lysis methode besproken. Deze methode is gebaseerd op het feit dat rode bloedcellen zwellen tengevolge van de penetratie van molekulen permeërende stof en water. Wordt het maximale volume overschreden, dan treedt lysis op. De tijd die verloopt totdat een bepaalde mate van lysis heeft plaats gevonden (hemolyse tijd) wordt beschouwd als een relatieve maat voor membraan-permeabiliteit. De hemolysetijd hangt echter niet alleen af van de membraan-permeabiliteit, maar onder andere ook van de

verhouding oppervlakte/volume. Verschillen in de verhouding oppervlakte/volume komen tot uiting in de verschillen in osmotische resistentie, de maximale zwelbaarheid van rode bloedcellen tengevolge van watertransport dat veroorzaakt wordt door verschillen in extracellulaire toniciteit van oplossingen van niet-permeërende stoffen.
Door bepalingen van de hemolysesnelheden in hypotone en isotone oplossingen van permeërende stoffen te kombineren met osmotische resistentie metingen, zijn verschillen in de verhouding oppervlakte/volume te elimineren. Formules zijn afgeleid die het lysis proces beschrijven en die gebruikt kunnen worden voor de berekening van de permeabiliteits-coefficient. Eksperimenteel is aangetoond (hoofdstukken 3 en 5) dat de reflectie coefficient verwaarloosd mag worden.

In hoofdstuk 3 is aangetoond dat de mathematische beschrijving van de osmotische lysis opgaat. Het lysis gedrag van rode bloedcellen van het varken, die voorbehandeld zijn met isotone saccharose oplossing (pH 7.5), blijkt in overeenstemming te zijn met theoretische voorspellingen. Uit de eksperimentele resultaten is de permeabiliteits-coefficient berekend. Noch met onbehandelde rode bloedcellen, noch met rode bloedcellen die tevoren behandeld zijn met isotone NaCl oplossing is een ideaal lysis patroon verkregen. In beide gevallen vindt een vertraagde lysis in glycerol plaats. Na een bepaalde tijd gaat de lysis met een normale snelheid verder. Ook in deze gevallen blijkt de permeabiliteits-coefficient voor glycerol berekend te kunnen worden.

In hoofdstuk 4 is aangetoond dat de vertraagde lysis voornamelijk veroorzaakt wordt door een snelle uitwisseling van chloride en hydroxyl ionen gekoppeld aan de bufferende werking van hemoglobine. Tengevolge van de uitwisseling van ionen vermindert de hoeveelheid osmotisch aktief materiaal hetgeen krimpen van de rode bloedcellen tot gevolg heeft. Daarom zal een ekstra hoeveelheid permeërende stof de celmembraan moeten passeren voordat lysis verder kan gaan.

In hoofdstuk 5 is aangetoond dat de permeabiliteit van rode bloedcelmembranen kwantitatief het best weergegeven wordt door de permeabiliteits-coefficient bepaald met de osmotische lysis methode. Bij zoogdier species met een geringe permeabiliteit voor glycerol, blijkt de volgorde van de permeabiliteits-coefficienten anders te zijn dan die van de hemolyse tijden in isotone glycerol. Noch de hemolyse tijden, noch de permeabiliteits-coefficienten blijken te korreleren met gepubliceerde

verhoudingen van fosfolipiden en cholesterol, samenstellingen van de fosfolipiden en de vetzuurpatronen. Terwijl voor alle andere diersoorten de permeabiliteits-coefficient voor glycerol betrekkelijk kleine variaties vertoont (10-20%), is de variatie van deze parameter bij rode bloedcellen van konijnen aanzienlijk groter (70%).

Uit de in hoofdstuk 6 beschreven eksperimenten blijkt dat de permeabiliteit voor glycerol van rode bloedcellen van verschillende konijnen sterk uiteen loopt. Rode bloedcellen van tien konijnen zijn uitvoeriger bestudeerd. Ofschoon aanzienlijke individuele verschillen in glycerol permeabiliteit zijn waargenomen, blijken de verschillen in de verhouding van cholesterol en fosfolipiden, in de samenstelling van de fosfolipiden en de vetzuurpatronen zeer gering te zijn. Deze gegevens bevestigen nogmaals dat er geen eenvoudig verband bestaat tussen de samenstelling van de lipiden en de glycerol permeabiliteit.
Het gemiddelde celvolume, de osmotische resistentie en de samenstelling van de celinhoud blijken niet noemenswaardig te verschillen. Voorlopige eksperimenten hebben geen "facilitated" diffusie systeem aangetoond in rode bloedcel membranen van konijnen.

De osmotische resistentie in NaCl oplossingen is sterk tijdsafhankelijk, terwijl in saccharose oplossingen de osmotische resistentie tijdsonafhankelijk blijkt te zijn. In hoofdstuk 7 zijn eksperimenten beschreven die erop wijzen dat de tijdsafhankelijkheid in NaCl oplossingen het gevolg is van een vertraagd vrijkomen van hemoglobine, en van chloride-hydroxyl ionen uitwisseling. Bovendien is aangetoond dat de osmotische resistentie in saccharose oplossing de beste benadering is van de oorspronkelijke osmotische resistentie van de rode bloedcellen. Een vergelijkende studie van de osmotische resistentie van normale en pathologische rode bloedcellen van de mens in NaCl en in saccharose oplossing toont aan dat voor diagnostische doeleinden de beste resultaten verkregen worden in NaCl oplossingen, zoals gebruikelijk in klinische studies.

Onze resultaten tonen aan dat met de gemodificeerde lysis methode nauwkeurige en betrouwbare gegevens over de permeabiliteit voor niet-electrolyten verkregen kunnen worden.
Wijzigingen in pre-lytische lek van ionen, in vertraagde lysis tengevolge van ionenuitwisseling en in het vrijkomen van hemoglobine, die kunnen

optreden bij eksperimenten uitgevoerd bij verschillende temperaturen of pH waarden, worden met de beschreven methode ondervangen. Nauwkeurig bepaalde permeabiliteits-coefficienten zijn van essentieël belang voor toekomstig onderzoek naar het verband tussen permeabiliteits-eigenschappen en membraan struktuur.
Een nadere bestudering van de verschillen in permeabiliteit van rode bloedcellen van individuele konijnen is van belang aangezien de samenstelling van de lipiden nauwelijks blijkt te verschillen, terwijl de permeabiliteit voor glycerol sterk uiteen loopt.

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