Overdruk uit „TNO-Nieuws" 24 (1969) 63-67

Bibliotheek Hoofdkantoor TNO
's-Gravenhage 31 OKT. 1969

Investigations on the permeability of red bloodcell membranes to non electrolytes[1])

Onderzoekingen over de permeabiliteit van de membranen van rode bloedcellen voor niet-electrolyten

Drs. J. M. C. WESSELS*) and Dr. D. T. F. PALS
Central Laboratory TNO

Samenvatting

Over de eigenschappen van biologische membranen is nog betrekkelijk weinig bekend. Een beter inzicht in deze problematiek is wenselijk om vast te kunnen stellen aan welke eisen bepaalde biologisch aktieve stoffen moeten voldoen. Vele farmaceutica, biociden, metabolieten etc. moeten biologische membranen passeren alvorens werkzaam te kunnen zijn.
Enig onderzoek werd verricht om een bijdrage te leveren aan de oplossing van dit probleem.
Permeatie-metingen werden uitgevoerd met rode bloedlichaampjes (erythrocyten) van enkele zoogdieren. Daarnaast werd onderzoek gedaan om vast te stellen of er een correlatie bestaat tussen permeabiliteit en membraanstructuur. Bovendien werden theoretische beschouwingen over het permeatie-mechanisme getoetst aan de hand van literatuurgegevens.

Dit onderzoek heeft tot de volgende interessante resultaten geleid:

1. *De erythrocyten van 6 zoogdieren kunnen, wat hun doorlatendheid voor glycerol betreft, ingedeeld worden in twee groepen. Binnen elke groep is de spreiding gering, terwijl een aanzienlijk verschil in doorlatendheid bestaat tussen beide groepen.*
2. *Het effekt van variaties in experimentele omstandigheden (b.v. in temperatuur) op beide groepen membranen is verschillend.*
3. *Dezelfde indeling in twee groepen wordt verkregen, wanneer in oplossing gebrachte membranen van erythrocyten worden geëlektroforeerd.*
4. *Het transport van stoffen (b.v. suikers) door biologische membranen behoeft niet noodzakelijkerwijs m.b.v. carrier-moleculen plaats te vinden. Een hypothese werd opgesteld die de in de literatuur vermelde resultaten kan verklaren. Aan beide zijden van de membraan bevinden zich een aantal plaatsen die potentieel in staat zijn permeërende stoffen te adsorberen. Van deze plaatsen is op elk moment slechts een klein deel aktief.*

Summary

A better understanding of the permeability properties of biological membranes is of the utmost importance to various branches of the chemical industry (pharmaceuticals, biocides, cosmetics, etc.).

The present communication describes experiments that:

a. *elucidate the permeability behaviour of membranes of different mammalian red blood cells (erythrocytes), and*
b. *correlate membrane structure and membrane permeability.*

Furthermore formulates a hypothesis aiming at an explanation of permeation phenomena observed.
Although the problem of permeation is still far from being solved, the following encouraging results have been obtained:

1. *The erythrocytes of 6 mammalian species can be divided into two groups with respect to their permeability to glycerin. In each group the interspecific variations are small, but a considerable difference exists between these two groups.*
2. *The influence of environmental conditions, e.g. variations in temperature, on the permeability differs for these two groups of erythrocyte membranes.*
3. *Agargel electrophoresis of solubilized erythrocyte membranes yields a similar division into two groups of membranes.*
4. *From theoretical studies it is concluded that transport of matter (e.g. sugars) through biological membranes is not necessarily due to supposed carrier-molecules. Another explanation is proposed which agree well with experimental data published in the literature. Essentially, it submits that at both sides of the membrane a small, locally varying, but proportionally constant, part of the adsorbing spots will be liable to react with molecules of the permeant.*

I Introduction

Because the knowledge of the permeation behaviour of biological membranes is far from complete, the penetration rate of drugs, metabolites, biocides, etc. is hardly predictable.

Though many different studies have been carried out to elucidate the permeability behaviour of various compounds, generally their results have not been sufficiently exact to allow a fundamental

[1]) Revised version of a paper read at the 146the Conference of the "International Society for Experimental Biology" held in Wageningen, The Netherlands, April 1967.

* Present address Laboratory for Biochemistry, Catholic University, Nijmegen, The Netherlands.

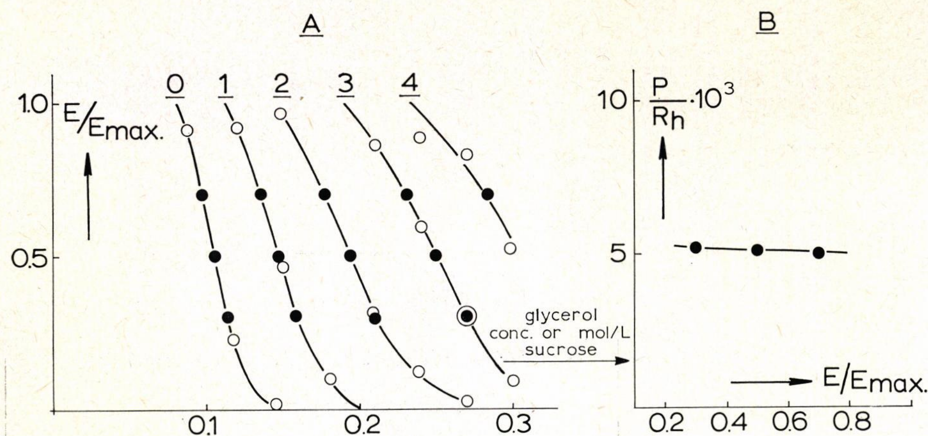

Fig. 1. Hemolysis of pig erythrocytes (sucrose-washed) in glycerin solutions and in sucrose solutions.
A: relative extinction versus glycerin or sucrose concentration. o experimental values, ● calculated values. O curve in sucrose, 1, 2, 3 and 4 curves in glycerin after 60, 120, 180 and 240 sec.
B: ratio of permeation constant and erythrocyte radius at the moment of hemolysis versus relative extinction calculated from A, using Formula (1).

physico-chemical interpretation. We therefore tried to set up as exact experiments as possible and to approach the problem from different angles.
Accordingly, this paper reports three types of activity:

1. Experiments on permeability of erythrocytes to glycerin, including work that shows the influence of some experimental conditions on the penetration rate;
2. Investigations to correlate membrane permeability with membrane structure: special attention is paid to structural membrane proteins;
3. Introduction of a hypothesis concerning the penetration of various non electrolytes (e.g. sugars) through cell-membranes.

II Permeation experiments

II 1. *Methods*

Already some years ago, methods were developed to study the penetration rate of substances through erythrocyte membranes. As none of these methods is as yet fully adequate, merits and demerits will have to be weighed carefully.
In our opinion, the hemolysis method of *Jacobs* (1950) has the best balance in terms of pros and cons, and therefore, we have adopted this method, with some modifications.
It is known that erythrocytes behave normally like perfect osmometers. As the exchange of water takes place rapidly, the osmotic equilibrium of the erythrocyte with the environment is established immediately. The experiments can be divided into two groups. In the first, the test solutions contain different concentrations of a non-permeable substance, sucrose; at a distinct low concentration of non-permeant outside the cell, the amount of water entering the erythrocytes will surpass the maximum and the erythrocyte membranes will rupture. In this way the osmotic resistance of the erythrocytes can be determined and, as water exchange takes place rapidly, this parameter is almost time-independent. In the second group of experiments, the test solutions contain different concentrations of a permeant: glycerin.
As glycerin, and therefore also water, diffuses gradually into the cell, an ever-increasing number of cells will hemolyse. So, in these experiments, the hemolysis is time dependent and to determine the permeation rate the hemolysis is stopped after different times by adding an excess of non-permeant.
To test tubes containing 10 ml glycerin or sucrose ($0 \rightarrow 0.3$ M in phosphate buffer 0.01 M, pH 7.4 and 37 °C) 0.1 ml sucrose-washed erythrocytes, haematocrite 0.3, was added. At distinct moments, the hemolysis was stopped by adding an excess of non-permeant (1 ml 2,5 M sucrose). The non-hemolysed erythrocytes were discarded by centrifugation (10 min, 3000 × g), and the extinction of the supernatant was measured at 580 nm, path length 1 cm, using a Zeiss spectrophotometer.

II 2. *Results*

The results of our hemolysis experiments are given in the form of graphs. In these graphs the relative extinction of the hemoglobin solution is plotted versus the concentration of the test solution. The extinction is given in a normalized form; we divide the extinction observed by the maximum extinction obtained at complete hemolysis (E/E max).
From these data one can calculate the ratio of the permeability coefficient, P, over the radius, R_h, of

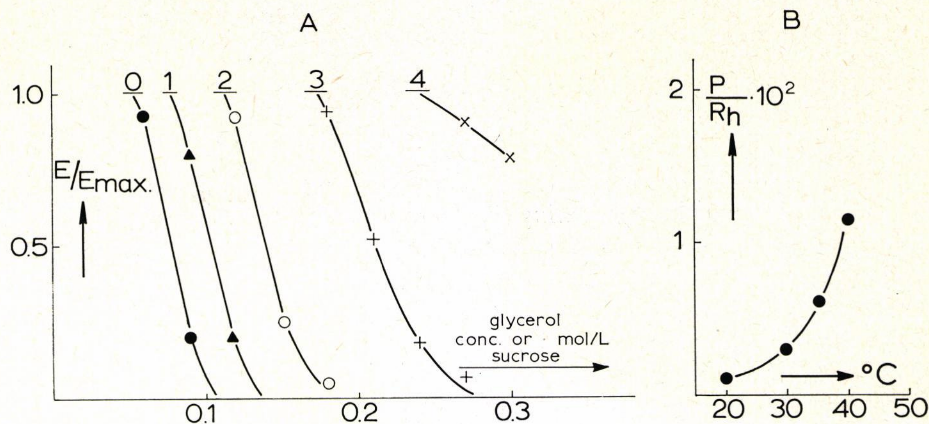

Fig. 2. Hemolysis of pig erythrocytes (sucrose-washed) in glycerin solutions and in sucrose solutions
A: relative extinction versus glycerin or sucrose concentration. ● curve in sucrose at 20, 30, 35 and 40 °C, so non temperature dependent ▲, o, + and x curvis in glycerin after 180 sec at 20, 30, 35 and 40 °C.
B: $\frac{P}{R_h}$ ratio versus temperature calculated from A, using Formula (1).

the erythrocytes at the moment of hemolysis. This ratio, $\frac{P}{R_h}$, is calculated from:

$$\frac{m_p}{m_N^h} - \frac{m_N^h}{m_p} = 2\,\frac{P}{R_h}\,.\,t_h \qquad (1)$$

where:

m_p concentration of permeant outside the cells that causes a distinct hemolysis in a distinct period.

m_N^h concentration of non-permeant outside the cells that causes, almost momentaneously, a distinct hemolysis.

P permeability coefficient.

R_h radius of the cells at the moment of hemolysis.

t_h time of hemolysis.

The results of such an experiment are shown in Fig. 1.
In the same way, we estimated the ratios for erythrocytes of other mammals; these ratios are collected in Table 1.
According to these figures, erythrocytes may, with respect to their permeability to glycerin, be classified in two groups; this suggests two types of membranes. It will furthermore be noted that the variation in each group is relatively small.
The effect of variations in temperature, too, suggests the existence of two types of membranes. Some results of such experiment are shown in Fig. 2.
The hemolysis of pig erythrocytes in sucrose is not dependent on temperature. This temperature-independence means, perhaps, that no change in elasticity of the membranes occurs. On the other hand, the velocity of hemolysis in glycerin is considerably increased by increasing the temperature, resulting into an increase of the $\frac{P}{R_h}$ ratio.
The hemolysis of human erythrocytes in sucrose is, on the contrary, considerably affected by the temperature. Increasing the temperature results into an increase of the osmotic resistance, whereas the velocity of hemolysis of these erythrocytes in glycerin is hardly influenced. Here, too, an increase of the $\frac{P}{R_h}$ ratio will be noted.
Effects of adding ionic substances, or surfactants, as well as those due to variations in pH, are still being studied.
To calculate P, the radius of the erythrocytes at the moment of hemolyse, should be known. This latter parameter we determined by means of a tracerdilution method and the results will be available soon.

Table 1.
The $\frac{P}{R_h}$ ratios for erythrocytes of some mammals in glycerin solutions.

Species	$\frac{P}{R_h}$ *Glycerin*
Ox	4.9×10^{-3} sec^{-1}
Pig	4.9×10^{-3} sec^{-1}
Sheep	3.1×10^{-3} sec^{-1}
Horse	8.5×10^{-3} sec^{-1}
Man	1.8×10^{-1} sec^{-1}
Rat	2.6×10^{-1} sec^{-1}

III Experiments to correlate membrane structure and permeability

The erythrocyte membranes were isolated according to the hemolysis procedure of *Dodge* (1963), and lyophilized.
In the literature some methods are described to solubilize cell membranes, using surfactants. In our experiments α-monolecithin, Triton-x-100 and sodiumdesoxycholate, have been used.
By means of agargelelectrophoresis according to *Wieme* (1959), these membrane solutions were studied. Whereas with α-monolecithin and Triton-x-100 the lipid-protein complex remained intact, with sodiumdesoxycholate a separation in some fractions was obtained.
Only some results with sodiumdesoxycholate will be mentioned here. The electropherograms of different mammalian erythrocyte membranes can be divided into two groups:

a. that of the membranes with a low permeability to glycerin (ox, pig, sheep and horse) showing at least two anodic protein fractions;
b. that of the membranes of erythrocytes with a high permeability to glycerin (man and rat), showing one anodic protein fraction.

In the electropherograms of both types of erythrocyte membranes, furthermore, an anodic fraction with a high electrophoretic mobility was found to contain all the lipids and a small amount of protein.
The separation into fractions is not an artefact; upon re-electrophoresis, the differences in electrophoretic mobility still existed.
The fractions will be analysed, and the results published elsewhere.

IV Introduction of a hypothesis concerning the penetration of non-electrolytes through erythrocyte membranes

At least for slowly permeating substances, the most characteristic features of transport of non-charged substances through erythrocyte membranes are the following:

(1) In contrast to normal diffusion, the total flux of permeant is only proportional to the concentration difference across the membrane at very low concentrations. At higher concentrations, the flux tends to go to a limiting maximum value. This behaviour suggests the existence of a limited number of diffusion pathways through the membrane;
(2) Moreover, from experimental data published, it can be concluded that the inward and outward fluxes through the membrane do not interfere with each other.

Of all mechanisms of permeation of sugars proposed so far, the „carrier" mechanism of *Widdas* (1954) agrees closest with experimental findings.
In, or at, the membrane, Widdas assumes carrier-molecules (unknown membranecomponents) to be present; these carrier molecules would shuttle through the membrane, forming reversible complexes with the penetrating substance. Some rather serious discrepancies, however, can be observed when one goes into details of the experimental results published. As *Regen and Morgan* (1964) pointed out, the permeation of sugars through rabbit erythrocytes cannot be explained by such a simple carrier mechanism alone. The same holds true for human erythrocytes *(Levine et al.* 1965).
As no shuttling carriermolecules could so far be detected in the membrane, though adsorbing membrane components were observed e.g. by *Bobinski & Stein* (1966), the question whether carriers play a part in permeation, or not, is still open.

Although Widdas' carrier mechanism, except for some details, can describe the permeation of non-electrolytes, another model which, we think, enables a more natural explanation is proposed. This model has the following features:

(1) The whole membrane surface, or at least a considerable part of it, is supposed to be able to adsorb, and solve, permeant molecules.
(2) Apart from inhomogeneities in the composition of the membrane surface, not all spots on the membrane are at the same moment in the same energetic state, or have the same conformation.

    Due to thermal motion, the state of all spots is liable to changes.
(3) Only a small locally verying, but proportionally constant part of the spots will be liable to react with molecules of the permeant.
    In order to complete the model we furthermore suppose that:
(4) a permeant molecule, once it is adsorbed, has a distinct probability to reach the opposite surface of the membrane.
    As at each moment a distinct finite number of active spots will be present, a limiting maximum flux will be achieved when the concentration of the permeant is increased.

As the chance that two spots on the membrane surfaces just opposite to each other would become active at the same time is negligibly small, feature (2) likewise applies.
Therefore, fluxes in both directions do not interfere with each other. The adsorption of permeant to the active spots is described by the Langmuir equations:

$$F \sim \frac{c}{c + ø}$$

where: F is the number of excitated spots.
c is the concentration of the permeant, and
ø is the affinity coefficient of the spots.

The flux from the outside to the inside is proportional to $F \sim \frac{c}{c + ø}$ whereas the flux from the inside to the outside is proportional to

$$F^1 \sim \frac{c^1}{c^1 + ø}.$$

The net flux is equal to $K \left(\frac{c}{c + ø} - \frac{c^1}{c^1 + ø} \right)$,

where: K is a constant, including the diffusion constant and the number of activated spots.

So our model leads to the same mathematical formulation as does Widdas' carrier model.
A more detailed description of our hypothesis will be published elsewhere.

Acknowledgement

The investigations reported above are those of a research team of the Central Laboratory TNO. In this team, Miss C. van Harberden, Mrs. Th. E. van Kesteren and Miss J. A. Luyendijk took part in the actual experiments.

References

H. Bobinski and W. D. Stein: Nature *211* (1966) 1366-1368.

J. T. Dodge, C. Mitchell and D. J. Hanahan: Arch. Biochem. Biophys. *100* (1963) 119-130.

M. H. Jacobs, H. N. Glassman and A. K. Parpart: J. Exptl. Zool. *113* (1950) 277-300.

M. Levine, D. L. Oxender and W. D. Stein: Biochim. Biophys. Acta *109* (1065) 151-163.

D. M. Regen and H. E. Morgan: Biochim. Biophys. Acta *79* (1964) 151-166.

W. F. Widdas: J. Physiol. *125* (1954) 163-180.

R. J. Wieme: Clim. Chim. Acta *4* (1959) 317-321.