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GLUCOSE PERMEABILITY OF LIPID BILAYER MEMBRANES

R. E. WOOD*, F. P. WIRTH, JR.** AND H. E. MORGAN***

Department of Physiology, Vanderbilt University Medical School, Nashville, Tenn. 37201 (U.S.A.)

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SUMMARY

Bimolecular lipid membranes have been prepared from total phospholipid extracts of human red cells. The electrical properties and water permeability of these membranes were similar to values reported earlier for other bilayer membranes. The urea permeability coefficient was $5.7 \cdot 10^{-7}$ cm/sec. The upper limit of the sorbitol permeability coefficient was $3 \cdot 10^{-8}$ cm/sec. The upper limit of the glucose permeability coefficient was estimated to be 10^{-10} cm/sec, some 10^6-fold lower than that of the human red cell. These observations suggest that sugar transport involves membrane constituents other than lipids. The lipid bilayer model may prove to be a useful tool for the isolation and characterization of membrane proteins that may function as carriers.

INTRODUCTION

Solute permeability is one of the most significant properties of biological membranes. Solute transfer may occur by simple diffusion or may involve participation of specific carriers. Since passive carrier transport systems have not been demonstrated to form stable intermediates, the study of transport mechanisms in broken cell preparations has been difficult.

Development of a method for reconstituting stable bimolecular lipid membranes separating two aqueous chambers by MUELLER *et al.*[1] suggested a new approach to the study of solute permeability. Thickness of the reconstituted membranes has been estimated to be between 60 and 90 Å by electron microscopy[2,3], intensity of reflected light[4,5], and membrane capacitance[6]. Electrical resistance was very high, ranging from 10^6 to 10^9 $\Omega \cdot$cm (refs. 6, 7). Since the diffusion and osmotic permeability coefficients for water were found to be identical, the membrane was suggested to be a continuous lipid film without pores[8–10].

In the present study water, urea, glucose and sorbitol permeability of bilayer membranes reconstituted from human erythrocyte membrane phospholipid will be compared to the permeability of these substances reported for the intact red cell.

* Present address: Department of Molecular Biology, Vanderbilt University, Nashville, Tenn., U.S.A.

** Present address: Department of Surgery, Johns Hopkins University, Baltimore, Md., U.S.A.

*** Present address: Department of Physiology, The Pennsylvania State University, College of Medicine, Hershey, Pa., U.S.A.

Bilayer permeability to water and urea was about 1/100 of the natural membrane, while glucose permeability was smaller by a factor of at least 10^6. The results suggest that the bilayer as presently reconstituted from red cell phospholipid does not have permeability characteristics of the natural membrane.

EXPERIMENTAL PROCEDURE

Preparation of phospholipids

Hemoglobin-free ghosts were prepared from fresh human blood by the method of DODGE, MITCHELL AND HANAHAN[11] and extracted immediately in a nitrogen atmosphere with 5 vol. of chloroform–methanol (2:1, v/v). α-Tocopherol (20 μM) was added as an antioxidant. The extract was evaporated at reduced pressure and lyophilized. The residue was taken up in chloroform, filtered, washed with 0.1 vol. of water, and again taken to dryness. The residue was dissolved in chloroform, filtered, and washed onto a column of silicic acid. The column was washed with 5 bed vol. of chloroform, followed by 5 bed vol. of methanol. The methanol eluate was evaporated and taken up in chloroform for storage in a nitrogen atmosphere at −20°. Phospholipid concentration was estimated by lipid phosphorus determination[12].

Apparatus and procedures

Membranes were formed on an aperture in a polyethylene partition separating two aqueous chambers (Fig. 1). Apertures were formed by heat pressing the partition to a thickness of 0.2 mm and punching a hole through the thinned area. The edges of the hole were smoothed by careful heating. Areas of the apertures ranged from 0.3 to 1.5 mm^2. Areas of the holes were calculated from the dimensions as measured with a calibrated micrometer eyepiece of a low-power microscope.

The partition was clamped between two lucite chamber blocks, and the chambers filled with 0.1 M NaCl buffered with 0.005 M Na_2HPO_4 at pH 7.0. Membranes were prepared by brushing a solution of 2.5% phospholipid, 2% cholesterol in chloroform–methanol–α-tocopherol (2.2:2.8:1, v/v/v) across the aperture.

For the estimation of permeability, radioactive solutes were introduced into the donor chamber with gentle stirring. Infusion and withdrawal of perfusate from the recipient chamber were accomplished with a syringe-type pump (Harvard Apparatus Co., Model 600-910, modified) using carefully matched 20-ml syringes. The volume of each chamber was 0.5 ml and the perfusion rate was 0.1 ml/min. The effluent was withdrawn into a coil of tubing several meters long. At the end of the experiment the tubing was clamped and samples collected by cutting the tubing into segments equivalent to 10 min of perfusion. The contents of each segment were transferred to a planchet or liquid scintillation bottle.

Permeability coefficients were calculated from the steady-state rate of appearance of counts in the recipient chamber, the area of the membrane, and the concentration of counts in the donor chamber:

$$k = \text{counts/min (recipient)}/60 \times \text{area} \times \text{counts/min (donor)}$$

where k is the permeability coefficient in cm/sec, area is in cm^2, counts/min (recipient) is the counts/min appearing in the perfusate per min, and counts/min (donor) is the counts/min per ml of donor chamber fluid. In all cases counts/min (recipient) was less

than 0.01 % counts/min (donor) so that flux was essentially unidirectional. The areas of the membranes formed on these apertures were assumed to be equal to the area of the aperture. If care was taken not to spread a large amount of lipid with the brush, this assumption appeared to be justified.

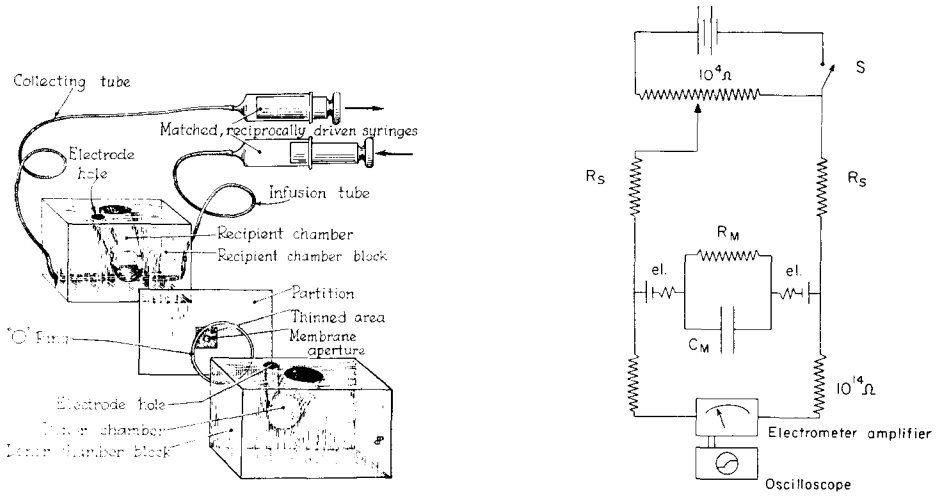


Fig. 1. Artificial membrane apparatus. The chambers were constructed of lucite blocks, 6 cm × 4.5 cm × 3.5 cm. 13-mm diameter holes were drilled at a 45° angle to form the chambers. Smaller holes were drilled to support calomel or platinum wire electrodes. 2-mm diameter holes were drilled horizontally into the recipient chamber to infuse and withdraw fluid. These holes were fitted with stainless steel cannulae connected to plastic tubing. The clamp holding the chambers together is not shown. The chambers were mounted in a metal box thermostated at 35°. The box was shock mounted on fiberglass supports and was efficiently grounded to provide electrical shielding.

Fig. 2. Electrical circuit for membrane studies. For measurement of membrane electrical properties a potential was applied to the membrane through calomel or platinum wire electrodes (el.) by closing switch S. The potential drop across the membrane resistance (R_M) was monitored by the electrometer amplifier (input impedance $10^{14}\,\Omega$). Following release of the potential by opening S, the decay of the voltage across the membrane capicatance (C_M) was monitored with the oscilloscope. The series resistors (R_S) were usually $10^8\,\Omega$.

For the measurement of electrical properties, small fiber junction calomel electrodes were used. These were not suitable for use in permeability studies, because of radioactive contamination. For such studies platinum wire electrodes were employed. The perfusion tubing leading into and out of the box was electrically shielded with grounded wire mesh. The syringes were insulated from the pump by nylon inserts.

Membrane electrical properties were measured by applying a potential across the electrodes (Fig. 2). The potential drop across the membrane was monitored with a Keithly Model 603 electrometer amplifier. The electrometer output was coupled to the vertical amplifier of a Hewlett-Packard Model 130 C oscilloscope. Resistance of the membranes was calculated from the potential drop across the membrane. Capacitance was calculated from the time constant of the decay of the potential across the membrane following release of the applied potential. The half time of a typical membrane with 10^8-Ω series resistors was 1 sec.

Radioisotopes used in these experiments were purchased from Nuclear Chicago or from New England Nuclear. All chemicals were reagent grade. Organic solvents were redistilled before use, and water was glass distilled.

RESULTS

Electrical properties. The electrical properties of membranes prepared from human red cell phospholipid were similar to those reported by other investigators using lipids from different sources[1,6,7,13,14]. The conductance was low and varied somewhat with different lipid preparations (Table I). The capacitance averaged 0.55 ± 0.01 μF/cm^2. From these values of conductance and capacitance it appeared that membranes prepared from red cell phospholipid were qualitatively similar to those prepared from different types of phospholipid.

TABLE I

ELECTRICAL PROPERTIES OF BILAYER MEMBRANES FORMED FROM HUMAN RED CELL PHOSPHOLIPIDS

10 to 20 membranes were studied with each lipid preparation. Figures are given ± S.E.

	Lipid Prep. I	*Lipid Prep. II*
Conductance, nΩ^{-1}/cm^2	160 ± 30	79 ± 6
Capacitance, μF/cm^2	0.54 ± 0.02	0.56 ± 0.01

If variation in conductance were due to leakage around the edge of the membrane, correlation of conductance with circumference of the membrane might be better than with the area. To test this, two membrane apertures were prepared with the same area but different circumference (Table II). One was round with an area of 1.48 mm^2 and a circumference of 4.34 mm. The other was rectangular, with an area of 1.54 mm^2 and a circumference of 8.35 mm. The average conductance of the rectangular membranes was not significantly higher than that of the round membranes, when expressed per unit of area. Expressed per unit of circumference, however, the

TABLE II

RELATIONSHIP OF ELECTRICAL PROPERTIES OF ARTIFICIAL BILAYER MEMBRANES TO AREA AND CIRCUMFERENCE OF MEMBRANE

12 to 15 membranes were studied in each group. Figures are given ± S.E.

Shape: *Area, mm^2:* *Circumference, mm:*	*Rectangular* *1.54* *8.35*	*Round* *1.48* *4.34*	*Significance, P*
Conductance, nΩ^{-1}	1.37 ± 0.09	1.16 ± 0.08	
per area, nΩ^{-1}/cm^2	89.2 ± 5.8	78.6 ± 5.6	< 0.30
per circumference, nΩ^{-1}/cm	1.6 ± 0.1	2.7 ± 0.2	< 0.001
Capacitance, nF	8.0 ± 0.3	8.4 ± 0.3	
per area, μF/cm^2	0.52 ± 0.02	0.57 ± 0.02	< 0.10
per circumference, nF/cm	9.5 ± 0.4	19.5 ± 0.7	< 0.001

difference in conductance between the two groups was highly significant, suggesting that for these membranes, conductance was a function of area of the film. The capacitance was also a function of area. Similar results have been recently obtained by MACDONALD[15].

Permeability

Methods for the determination of permeability coefficients (k) of radioactive solutes were developed with the use of ^{3}HHO as the penetrating species. The permeability coefficient of ^{3}HHO ($k_{^3HHO}$) was found to be $3.2 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4}$ cm/sec (14 membranes), in close agreement with the values reported by others[8,10,16]. In these experiments there was no provision for stirring the fluid in the chambers beyond initial mixing and the currents generated in the recipient chamber by the flow of perfusate. Donor chamber concentrations of ^{3}HHO were usually about $2 \cdot 10^6$ counts/min per ml.

Urea permeability of 10 membranes was estimated with [^{14}C]urea (50 mC/mmole). The low permeability required the use of high concentrations in the donor chamber, usually about $5 \cdot 10^7$ counts/min per ml. k_{urea} averaged $5.9 \cdot 10^{-7} \pm 0.3 \cdot 10^{-7}$ cm/sec. Corresponding values reported for other bilayer membranes are $3 \cdot 10^{-6}$ cm/sec (ref. 17) and $4.2 \cdot 10^{-6}$ cm/sec (ref. 9). In the human red cell k_{urea} has been reported to be $1.9 \cdot 10^{-4}$ cm/sec (ref. 18).

The glucose permeability of the bilayers proved difficult to measure because of its small magnitude. Donor chamber concentrations of [^{14}C]glucose of about 100 μC/ml were necessary to obtain measurable radioactivity in the recipient chamber. A typical experiment is shown in Fig. 3. The average value of $k_{glucose}$ was $5.1 \cdot 10^{-8} \pm 0.5 \cdot 10^{-8}$ cm/sec (12 membranes). In all experiments the appearance of radioactivity in the recipient chamber resulted in counting rates at least 3 times background.

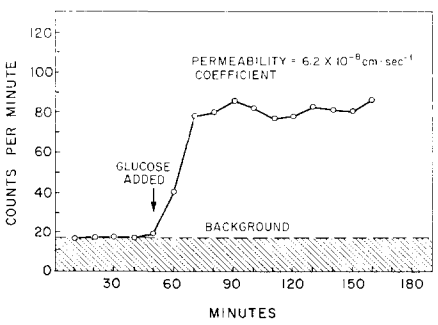
In an attempt to determine whether the bilayer glucose permeability had any characteristics of the red cell transport system, $k_{glucose}$ was measured in the presence of 0.1 mM phloretin or 25 mM unlabeled glucose. In the intact cell such additions would have greatly diminished the rate of tracer penetration. In the bilayer, $k_{glucose}$ was $4.4 \cdot 10^{-8} \pm 1.3 \cdot 10^{-8}$ cm/sec (3 membranes) in the presence of phloretin and $3.9 \cdot 10^{-8} \pm 0.8 \cdot 10^{-8}$ cm/sec (3 membranes) in the presence of 25 mM unlabeled glucose. These differences are not significant.

When the glucose concentration employed in the present experiments (0.7 mM) was used to calculate the $k_{glucose}$ for the human red cell, the data of BRITTON[19] gives a value of $1 \cdot 10^{-4}$ cm/sec. This is some 2000 times greater than the apparent $k_{glucose}$ found for the bilayer. A similar calculation for the rabbit red cell from data of REGEN AND MORGAN[20] gives a value of $3 \cdot 10^{-8}$ cm/sec. In one successful experiment $k_{glucose}$ of a bilayer prepared from rabbit red cell phospholipid was $5 \cdot 10^{-8}$ cm/sec. Although the bilayer permeability for glucose was far less than the human red cell, permeability of the bilayer was of sufficient magnitude to account for the glucose permeability of the rabbit red cell. However, the penetration of glucose into the rabbit red cell has been shown to be mediated by a mobile carrier system[21]. This consideration led to a suspicion that the material penetrating the bilayer might be a contaminant in the [^{14}C]glucose.

When a sample of the [^{14}C]glucose added to the donor chamber was fractionated on a column of Dowex-1 formate using a gradient of formic acid–ammonium formate,

the bulk of the counts came off the column with the water wash (Fig. 4). A small impurity was present, amounting to about 1 % of the total activity. This peak came off just after the start of the acid gradient. This sample of glucose, after reaction with hexokinase, and MgATP still showed a small peak at the front of the gradient, but the bulk of the counts came off the column much later. This later peak was presumed to be glucose 6-phosphate. After reaction with hexokinase, recipient chamber samples did not show any detectable activity in the portion of the chromatogram where glucose 6-phosphate was expected. Instead, all the counts were found near the start of the acid gradient. From these observations it was concluded that essentially none of the material penetrating the membrane was actually glucose. The identity of the material which did cross the membrane is unknown. These data suggest that the permeability coefficient for glucose must be revised downward by at least two orders of magnitude. The upper limit of glucose permeability would appear to be 10^{-10} cm/sec.

The permeability of the bilayer to sorbitol was measured in hope of demonstrating specificity of penetration. Most cells are virtually impermeable to sorbitol,



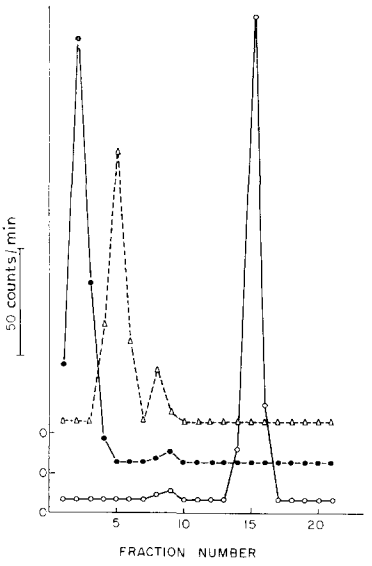


Fig. 3. Measurement of glucose permeability of a bilayer formed from human red cell phospholipid. The membrane was formed at zero time. After 50 min [^{14}C]glucose was added to the donor chamber to give a final concentration of $2.63 \cdot 10^8$ counts/min per ml. Perfusion was continued for 110 min, then stopped and samples collected as described in the text. Experiments for the measurement of water, urea and sorbitol permeability were conducted in a similar manner.

Fig. 4. Chromatography of donor and recipient [^{14}C]glucose samples. Donor and recipient chamber samples of [^{14}C]glucose were reacted with 50 μg hexokinase, 2 mM ATP, 3 mM $MgSO_4$, 1 mM unlabeled glucose and 20 mM phosphate buffer (pH 7). The reacted samples and an unreacted donor chamber sample were applied to 1 cm × 10 cm columns of Dowex-1 (formate). The columns were eluted with water followed by a gradient of 4 M formic acid *plus* 0.2 M ammonium formate. The acid gradient began at Fraction 7. 7.5-ml fractions were collected and subsequently plated on planchets for counting in a low-background counter. The three chromatograms have been separated vertically for clarity. Approx. 500 counts/min were applied to each column. ●—●, pattern obtained using a donor chamber sample unreacted with hexokinase; ○—○, the same sample after reaction; △---△, a recipient chamber sample after reaction with hexokinase.

and it is frequently used as an extracellular marker[21,22]. The apparent k for [^{3}H]-sorbitol was $1.7 \cdot 10^{-7} \pm 0.2 \cdot 10^{-7}$ cm/sec (8 membranes). However, radioactive purity was a problem in this case as well as with glucose. The sorbitol was lyophilized prior to use to remove any ^{3}HHO, the most likely contaminant. When recipient chamber samples were lyophilized, however, at least 80 % of the counts were lost, suggesting that the actual k_{sorbitol} was much lower than $1.7 \cdot 10^{-7}$ cm/sec. Under the conditions of these experiments a contamination with 0.1 % ^{3}HHO would give an apparent k of $2 \cdot 10^{-7}$ cm/sec.

DISCUSSION

Attention has been focused on the lipid bilayer as the most likely basic structure of biological membranes since the work of GORTER AND GRENDEL[23]. This concept was extended and developed formally by DANIELLI AND DAVSON[24,25]. Transfer of glucose across the plasma membrane of many cell types is thought to involve binding of the sugar to a mobile carrier in the membrane[26].

Both the phospholipid and protein components of the membrane have been discussed as potential carriers[27]. Interest in the role of phospholipids was heightened by the demonstration of specificity of binding of sugars by these compounds[28]. If phospholipids were to serve as carriers by binding sugar, the binding would presumably reduce the hydrophilic nature of the sugar and allow it to diffuse through the membrane as the sugar–phospholipid complex. While no definite conclusions as to the role of phospholipids as sugar carriers can be drawn from the present experiments, the extremely low glucose permeability of the bilayer does not support a simple role of phospholipids in sugar transport.

Recently the involvement of a membrane protein in the penetration of sugars, amino acids and sulfate has been suggested[29–32]. These proteins could catalyze the binding of sugar and phospholipid or could bind and translocate the sugar without direct involvement of the lipid. The lipid bilayer offers an attractive model for the study of proteins thought to be components of transport systems. Since the glucose permeability of the bilayer is very low in the absence of protein, a small amount of carrier activity might be detected. If carrier could be added to the bilayer in the concentration found in the human red cell membrane, glucose permeability would increase by a factor of 10^6.

The diffusion of ^{3}HHO across the bilayer has been shown to be restricted by the presence of unstirred layers adjacent to the membrane[10,16]. Since the permeabilities of urea, glucose and sorbitol were much lower, it is unlikely that their fluxes would be significantly affected by such stagnant layers. A more significant consideration in these experiments is radioisotope purity. This has been illustrated in the present experiments with glucose and sorbitol. The commercial preparations of labeled glucose and sorbitol were stated to be greater than 99 % pure, but nearly all of the material that penetrated the membrane consisted of unidentified impurities. Such limitations of isotopic purity must be fully considered in future experiments involving isotopic fluxes and low permeabilities.

The perfusion technique offers several advantages for the measurement of bilayer permeability. First, background radioactivity of the recipient chamber can be accurately assessed. This feature is of particular importance when very high levels

of radioactivity must be added to the donor chamber. Second, replicate measurements of permeability can be made on a single membrane with a minimum of manipulation. Third, continuous renewal of recipient chamber fluid insures that the flux is essentially unidirectional. Fourth, valid measurements of permeability can often be obtained even if the membrane should break during the experiment.

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