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A METHOD FOR MEASUREMENTS OF HYDRAULIC CONDUCTIVITY OF BIOLOGICAL MEMBRANES *IN VITRO*

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The results of measurements of water flow across four isolated epithelial membranes (skin of frogs, colon of rats, parietal peritoneum and mesentery of rabbits) with hydrostatic pressure gradient as volume driving force have been presented herein. The membranes were mounted between two semichambers filled with Ringer or Hanks solution; the volume on both sides of the membrane was strictly controlled by photogauges coupled with microdosimeters. Transmembrane volume flow (J_v; $nL \times sec^{-1} \times cm^{-2}$) induced by the hydrostatic pressure gradient (ΔP; atm) was measured with the use of an electronic device connected with the photogauges, and with a computer which monitored values throughout the experiment. Hydraulic conductivity index (L_p) was calculated from the monitored values ($L_p = J_v/\Delta P$; in $cm \times sec^{-1} \times atm^{-1}$). The index proved to be a characteristic property of each studied membrane. Effects of some factors thought to influence the permeability of the membranes were checked: Hypertonicity of medium bathing the frog skin and parietal peritoneum induced an increase of permeability, whereas addition of mercuric chloride diminished it in the frog skin, the mesentery and the colon. The elaborated method can be useful in further studies on water metabolism and on participation of various barriers in water epithelial transport processes.

Key words: *biomembranes, water, hydraulic permeability.*

INTRODUCTION

Membranes are the most important elements in regulation mechanisms of body water metabolism, as well as in the aspect of its absorption or excretion, and redistribution. Irrespective of whichever of the four known driving forces — that cause water to move across membranes (osmotic and hydrostatic gradient, electric potential and temperature difference [1, 2]) — acts on a given membrane, effectivenes of such an action depends principally on permeability of the membrane for water (2, 3). This intrinsic property of biological membranes acts as an essential regulatory factor in water flow through all barries in the body, thus it determines organism's water homeostasis. The

results of recent studies on water transport in membranes have shown that permeability depends mostly, if not exclusively, on presence of water channels located in their structure (3—5).

The particular abundance of channels has been shown in boundary cells, which form biological barriers (epithelium, endothelium) and which are engaged in the regulation of absorption and redistribution of water in the body. They were found in salivary glands, stomach, colon, lung, skin, conjunctiva, cornea and ciliary body of the eye, trachea, gall and urinary bladders, and especially in nephrons (4, 5). Some of these structures can be studied after isolation as living memebranes *in vitro*, and this makes researches on membrane processes easier. However, as far as hydraulic conductivity index and net fluid transport across these membranes are concerned, available methods on living membranes are not precise enough to comply nowaday's requirements. It is, among others, because of technical difficulties and the lack of automation of such measurements.

In the following paper a method has been described [1] that makes determination of hydraulic conductivity possible, i. e. permeability for water of isolated biological membranes, which possess flat anatomical structure, and which participate in water metabolism within their physiological (skin, colon) or pathophysiological (peritoneum during dialysis) function. In order to show the application of the method for assessment of various factors' effects which could be pressupposed to influence permeability of these membranes for water, some actions towards a change of permeability have been included in this study. The results of the measurements after these actions have been evaluated in connection with the evaluation of the method itself.

MATERIALS AND METHODS

The experiments were carried out *in vitro* on four kinds of isolated membranes of laboratory animals: skin of frogs, colon of rats, parietal peritoneum and mesentery of rabbits. Frogs (*Rana temporaria* or *Rana esculenta*) collected in autumn, were kept in cuvettes with cooled (+4°C) tap water, changed every 3—4 days, rats (*Wistar*) and rabbits (*New Zeland, white*) came from commercial, professionally held, sources. The animals were killed with a blow on the head, with additional destruction of the spinal cord in the frogs. The membranes were taken immediately afterwards. All manipulations during the procedures of preparation of these membranes were performed as gently as possible to prevent any damage to the epithelium or the mesothelium; preparation of the mammalian tissues was carried out with additional protection against their chilling. Preparation of the skin of frogs (abdominal aspect; $2-3 \times 2-3$ cm) was in accord with the ordinary technique elaborated in Ussing's laboratory and described in related papers (6). In rats the abdomen was opened, and the colon exposed; after selection of a segment to be isolated, displacement of resting chyme with cautious palpation towards the proximal and distal direction

[1] the method has been entirely elaborated and constructed in this laboratory

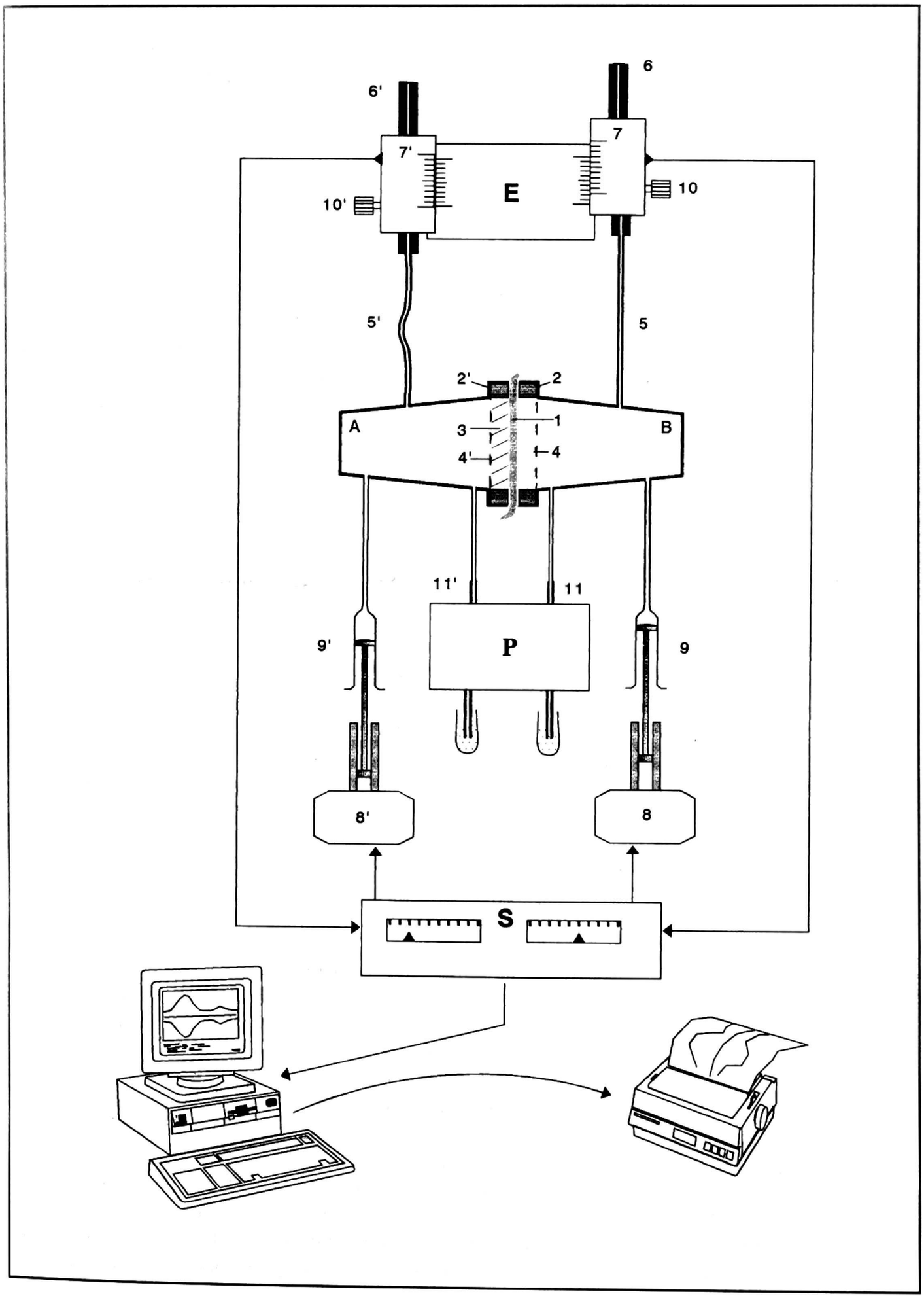

Fig. 1. Schematic representation of the set-up for measurements of the volume flux through the isolated membranes. Detailed description in the text.

was done, the segment excised, and the wall cut along the insertion of the mesentery. After careful rinsing of the mucous side of the segment with warmed Hanks solution a flat membrane with the size of 1.5×1.5 cm was created. In rabbits, after the abdomen was opened, fragments of parietal peritoneum ($2—3 \times 2—3$ cm) from the anterior wall of the abdomen were carefully excised according to the previously described technique (7, 8). In another series of experiments fragments of the mesentery of the same size were isolated from the ileal region.

Each membrane (1 *in Fig. 1*) was placed between two symmetric half-cells (A&B in *Fig. 1*) of the chamber made of perspex, possessing soft rings (2&2' in *Fig. 1*) made of slightly cured Sylgard (Dow Corning, Chicago) which prevented the membrane from edge damage. The free area of the membrane was 1.0 cm^2 (colon) or 2.0 cm^2 (all others). The ring in the left half-cell was filled with multi-perforated plate of hard-set agar (Serva; 5% in Hanks solution) which secured immobility of the membrane in that direction (3 in *Fig. 1*). The interstitial sides of the membranes were usually placed on the agar plate whereas the epithelial/mesothelial side remained free; one part of the experiments, however, was carried out with contast placement of the sides.

Both half-cells of the chamber were filled (2×2.5 ml) with Ringer (experiments on frog skins) or Hanks (all others) solutions. They contained (in mmol/L): 112 NaCl, 2.0 KCl, 2.5 $NaHCO_3$, 1.0 $CaCl_2$ (Ringer) or 137 NaCl, 5.4 KCl, 4.1 $NaHCO_3$, 1.3 $CaCl_2$, 0.4 $Na_2HPO_4 \times 2\,H_2O$, 0.5 $KH_2PO_4 \times 12\,H_2O$, 0.5 $MgCl_2 \times 6\,H_2O$, 0.4 $MgSO_4 \times 7\,H_2O + 5.5$ g/L of glucose; pH 7.4; osmolality adjusted to 300 mOsm/L by proper amount of mannitol (Hanks). The fluids were periodically oxygenated and mixed throughout the experiment by whirling two magnetic bars (one in each half-cell) moved by a rotating stirrer (80—100 rev./min) placed underneath the chamber. The membrane as well as the agar plate were prevented from coming into contact with the bars by means of silver rods located close to both surfaces (4&4' in *Fig. 1*).The chamber was plunged in a bath with constant temperature 20°C (± 0.1°C) in experiments carried out with the frog skin, and 37°C (± 0.1°C) in the rest of experiments. Each half-cell of the chamber was connected through an elastic polyethylene tube with a thick-walled capillary micropipette (6 in *Fig. 1*) with an infra-red photogauge complex mounted on it (7 in *Fig. 1*); the gauge controlled movement of the fluid level in the pipette [2]. Depending on the direction of the movement (up or down) the gauge had signalled "−" or "+" to an electronic device (S in *Fig. 1*) which transformed them into electric impulses ("−" or "+", respectively). They were sent to a highly precise stepper-motor (8 in *Fig. 1*) coupled with asmall, leak-proof syringe (9 in *Fig. 1*) connected with the pertinent half-cell. An identical system of connections, fully independent of the first one, was applied to the other half-cell of the chamber (5'—9'). The gauges were sensitive enough to react to any movement of the fluid levels in the micropipettes within a range of several μm (equivalent to any change in volumes). After assorting proper syringes, stepper-motors, and micropipettes it was established empirically that single step-movement of one motor gave 46 nL (added to or withdrawn from the pertinent half-cell of the chamber). Each micropipette was mounted into a set (E in *Fig. 1*) that made it possible to elevate or lower the pipette together with its photogauge but separately from the other pipette, whereas the chamber remained inert. Owing to a screw with nonius scale mounted on the set (10 in *Fig. 1*), the displacement of the pipettes could be performed with an accuracy of 0.1 mm. The electronic device was coupled with a computer (IBM PC-486) which monitored all signals received from the gauges and noted the electric impulses sent to the stepper-motors. Using an original computer programme it was possible to observe, simultaneously but separately, changes in volumes in both half-cells and to follow the whole course or chosen fragments ("zooming") of the experiment during the measurements as well as afterwards (*Fig. 2—4*).

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[2] the gauge is being turned off by the fluid optically more dense than the air

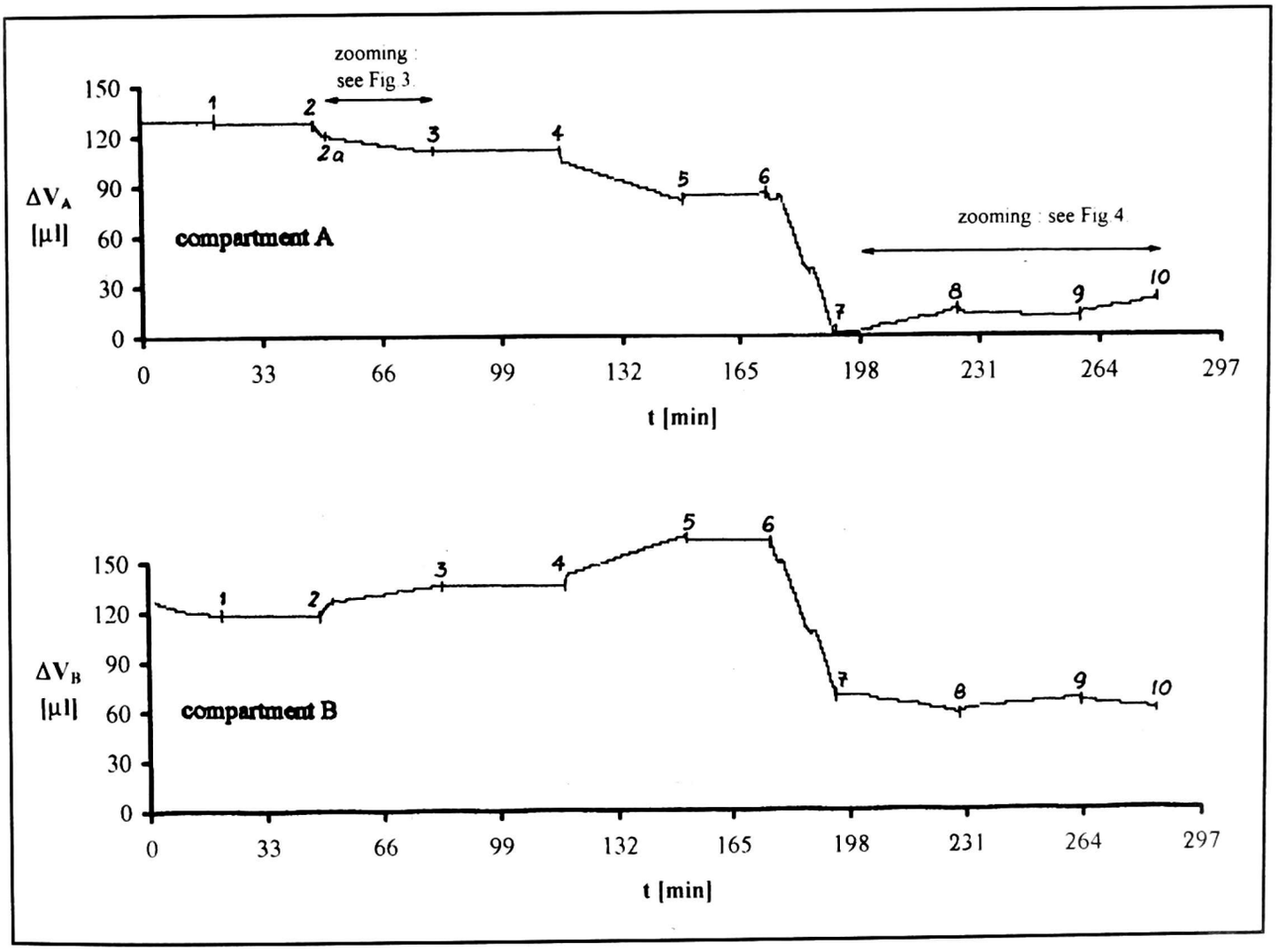


Fig. 2. A computer print-out of the whole course of the experiment carried out with parietal peritoneum, in which volume changes (ΔV) of both compartments (A&B) of the chamber were measured using the set-up shown schematically in *Fig. 1.* Between three periods of control measurements (segments 1—2, 3—4 and 5—6) hydrostatic pressure was applied two times (1.0 mm H_2O and 2.5 mm H_2O; segments 2—3 and 4—5, respectively) to the right half-cell of the chamber. During the next period lasting 17 min. (segment 6—7) a solution of 30% glucose was instilled into the right compartment and Hanks solution into the other. Then further measuremnts were carried out before (segment 7—8) and after (segment 9—10) reiterated application of hydrostatic pressure gradient across the membrane (segment 8—9).

The course of the experiments was as follows: After a control period lasting 10—30 min., during which there was no driving force across the membrane, and minimal changes in the volumes of both half-cells were exactly equivalent [3], the right micropipette with its photogauge was raised by 1.0 or 2.5 mm over the other micropipette. The hydrostatic gradient across the membrane was then 0.98 or 2.44×10^{-4} atm. respectively. Only in experiments with shins taken from frogs remaining at the laboratory not longer than 4 months (carried out in autumn and winter) it was necessary to apply greater pressure (5—10 mm of water pillar, equivalent of 4.88—9.77×10^{-4} atm.) to get the estimable volume flow across these membranes. Each pressure gradient was maintained for 30—40 minutes and owing to the described servo-system it was kept at this level all the time with the accuracy of 25—35 μm (24—34×10^{-7} atm.). Then the pipette with the photogauge was lowered to exactly its previous position and the observation was continued for the next 30—40 minutes. In one part of the experiments the manoeuvre was duplicated with two different values of the gradient, all with control periods before, after and between them.

[3] a difference greater than 1.5 $nL \times sec^{-1} \times cm^{-2}$ caused the experiment to terminate

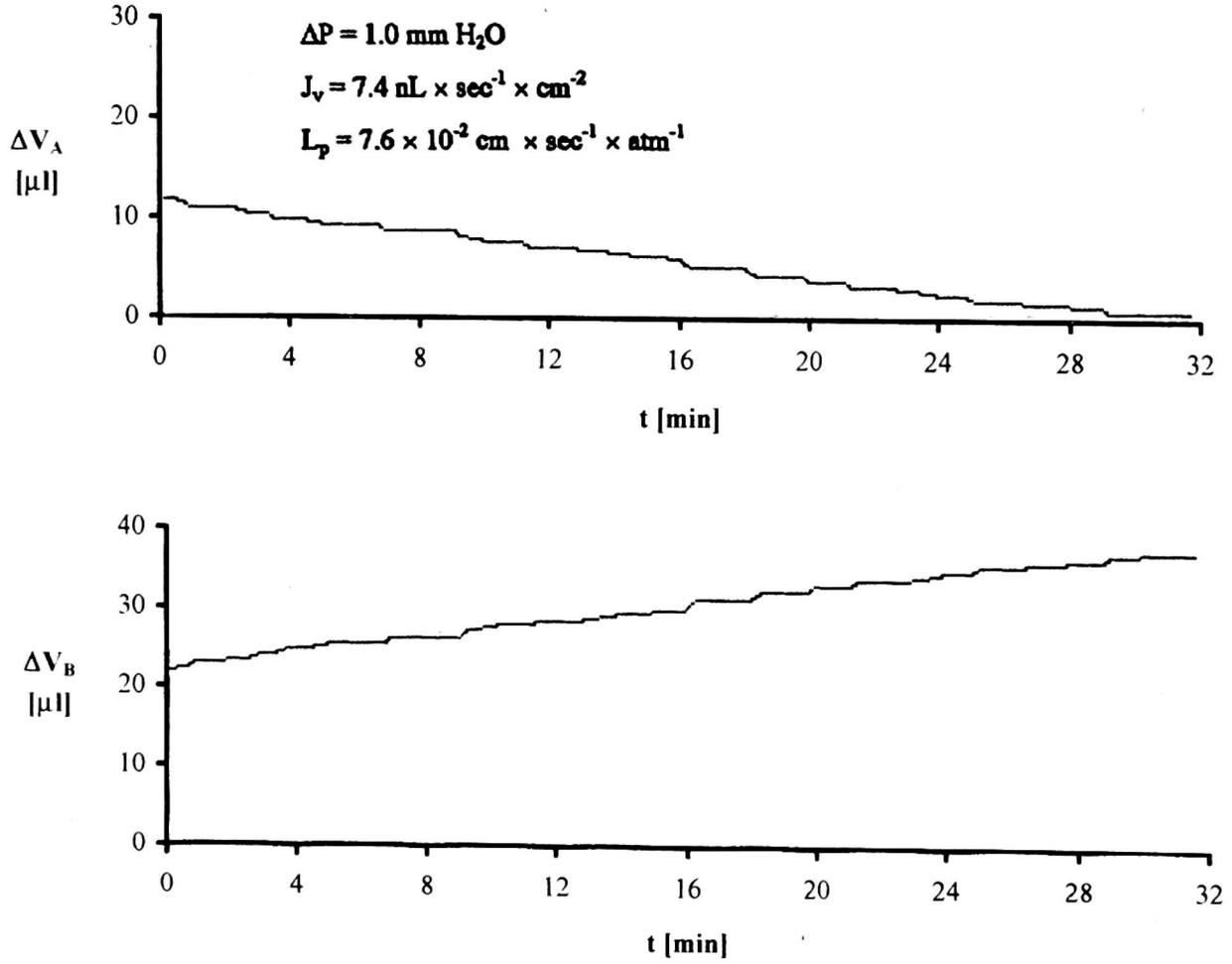


Fig. 3. Enlarged fragment (computer zooming of segment 2—3) of the experiment shown in *Fig. 2.*

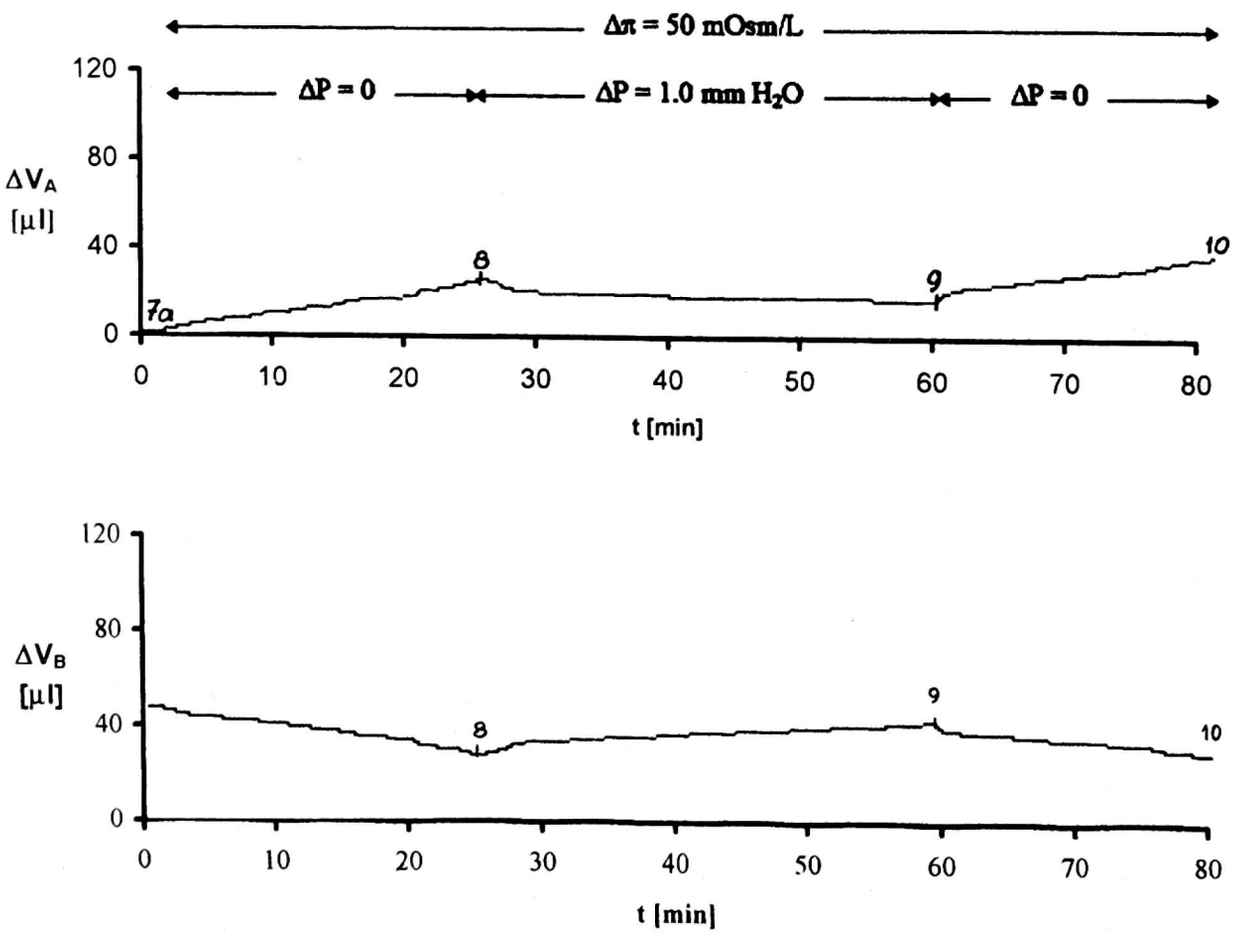


Fig. 4. Computer zooming of the further course of the same experiment (segment 7 a—10). Volume flow from the left to the right compartment was induced by the osmotic gradient; between two control periods of the measurements (segment 7 a—8 and 9—10) hydrostatic pressure gradient (1.0 mm H_2O) across the membrane was applied again.

In a separate series of several experiments the same manoeuvre was performed and measurements were carried out without any membrane but with the agar plate alone remaining at the proper place in the chamber.

Both half-cells of the chamber possessed additional inlets joined through another pair of connecting polyethylene tubes to elastic tubes made of silicon rubber (11 in *Fig. 1*) which were inserted into a precise peristaltic pump (P in *Fig. 1*). One elastic tube joined with the right half-cell (epithelial or mesothelial side of the membrane) was filled with a 30% solution of glucose whereas the remaining tubes were filled with an ordinary medium (Hanks or Ringer). After setting the pump in motion the solutions were slowly instilled into both half-cells of the chamber, while the syringes automatically withdrew the excess volume from both compartments bathing the two sides of the membrane; thus the balance of the hydrostatic pressure across the membrane was maintained throughout the time of instillation. The time was as long as it was necessary to obtain hyperosmolality of the medium bathing this side of the membrane. After a short period of stabilization, the measurements of the volume flow induced by the same ΔP were repeated. 9 such experiments were carried out with parietal peritoneum, and 7 with frog skin. In another series of experiments carried out with frog skin ($n = 8$), mesentery ($n = 3$), and colon ($n = 6$) 45 mg% solution of $HgCl_2$ was instilled instead of glucose solution with the final concentration of mercury 0.1 mmol/L (frog skin) and 1.0 mmol/L (mesentery, colon).

The values of the volume flow across the membranes were read by the computer (3 samplings per second); the hydraulic conductivity index (L_p) was then calculated from these values and the actually applied pressure gradient according to the following equation (1, 2):

$$L_p = J_v/\Delta P$$

where

L_p = the hydraulic conductivity (in $cm \times sec^{-1} \times atm^{-1}$) of the membrane during the application of the application of the hydrostatic pressure gradient

J_v = the volume flow across the free area of the membrane (in $nL \times sec^{-1} \times cm^{-2}$)

ΔP = the hydrostatic pressure applied into one half-cell of the chamber (in atm.)

All results were expressed as means $\pm$ standard error of the mean (SEM). The Mann-Whittney test was used for statistical analysis.

Statistical significance of differences was established at the level of $P < 0.01$.

RESULTS

During all control periods, while the hydrostatic pressure on both sides of the membrane was in balance, no transmembrane volume flow was observed. After applying the hydrostatic gradient the flow appeared immediately (within the range 0.04—48.9 $nL \times sec^{-1} \times cm^{-2}$) always in the same direction as the gradient, i.e. from the right to the left half-cell; whenever the gradient ceased, the flow diminished to zero. Qualitatively the same effects were observed when no biological membrane but the agar plate alone was in the chamber dividing its two half-cells. However, the latter measurements showed several times higher values ($\Delta P = 1$ mm H_2O induced flow over 250 nL/sec, which was the upper limit of reliable measurements of flow through the chamber). Thus a conclusion could be

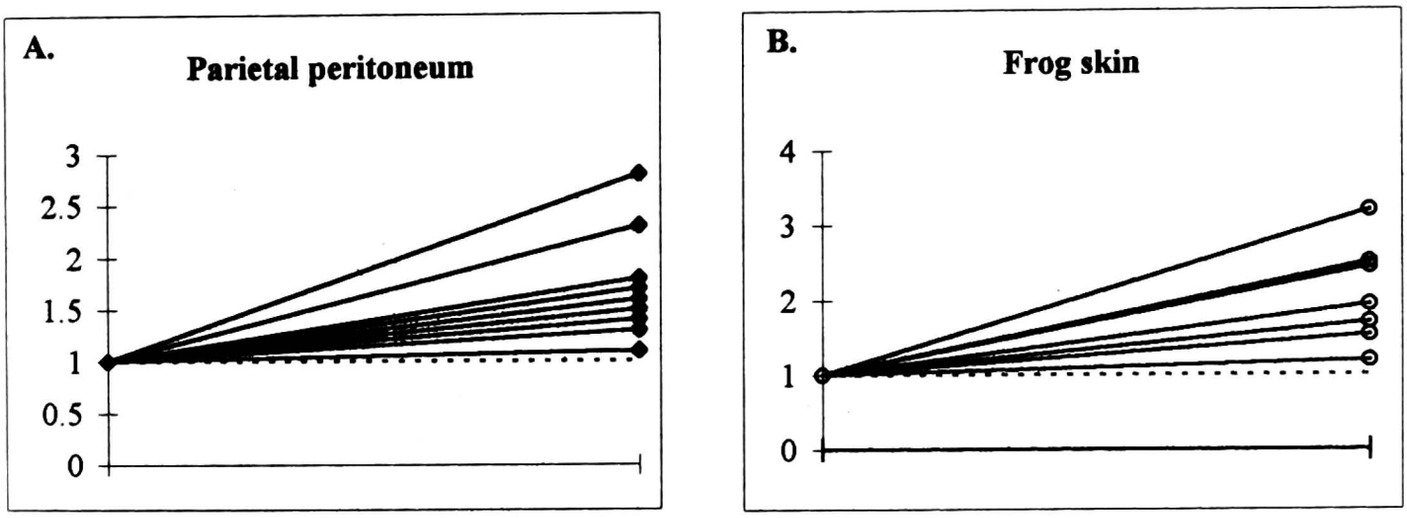
drawn that the agar plate alone made no hindrance for the volume flow through the chamber.

Fig. 2 presents an example print-out of the computer data from the whole course of the experiment carried out with the parietal peritoneum. After a period of stabilization of temperature inside the chamber and the first control period of measurements (segment 1—2) hydrostatic pressure of 0.98×10^{-4} atm. (= 1.0 mm H_2O) was established in the right half-cell of the chamber and kept at this level for 30 minutes (segment 2—3). As shown in a separate print-out of this period (*Fig. 3* computer zooming of the segment 2a—3), stable volume flow from the right to the left half-cell was 7.4 $nL \times sec^{-1} \times cm^{-2}$ and the calculated index of hydraulic conductivity was 7.6×10^{-2} $cm \times sec^{-1} \times atm^{-1}$. Then the hydrostatic gradient was extinguished and another control period with measurements followed (*Fig. 2,* segment 3—4). During both control periods the transperitoneal flow was zero and it could convince that the membrane permeability was in a steady state throughout the experiment. In further part of the experiment the maneouvre was repeated but with higher pressure gradient (2.5 mm $H_2O = 2.44 \times 10^{-4}$ atm.). The resultant transperitoneal flow was 17.5 $nL \times sec^{-1} \times cm^{-2}$ and the hydraulic conductivity index 7.2×10^{-2} $cm \times sec^{-1} \times atm^{-1}$.

Fig. 4 depicts in detail changes of volumes of both half-cells, i.e. the transmembrane volume flow, during the further course (segment 7—10) of the experiment shown as a whole in *Fig. 2*. After instillation of the concentrated solution of glucose into the right half-cell (segment 6—7 in *Fig. 2*) medium in this compartment became hypertonic and transmembrane volume flow occurred. Its direction was from the left to the right side of the membrane, in accordance with the osmotic gradient ($\Delta\pi$). After the 30-minute period (segment 7a—8), during which stable transperitoneal volume flow was 10.0 $nL \times sec^{-1} \times cm^{-2}$, hydrostatic pressure gradient of 0.98×10^{-4} atm. was applied again (segment 8—9). The transfer changed its direction (from the right to the left side of the membrane) reaching the values 2.9 $nL \times sec^{-1} \times cm^{-2}$; the calculated index L_p was then 13.2×10^{-2} $cm \times sec^{-1} \times atm^{-1}$.

As shown in *Fig. 5-A* similar results were found in the overwhelming majority of other experiments with the same schedule. The absolute values (initial & final) were fairly different so in the drawing relative values (final/initial ratio) have been shown. This effect was also observed when experiments with the same procedure were carried out on frog skin; it is depicted in *Fig. 5-B*. Contrasting effects were observed when solution of mercuric chloride was instilled into the chamber instead of glucose: Volume flows diminished as compared with the control periods in all experiments carried out with frog skin, mesentery and colon (*Fig. 5-C* and *-D*, respectively).

Effect of hypertonicity



Effect of $HgCl_2$

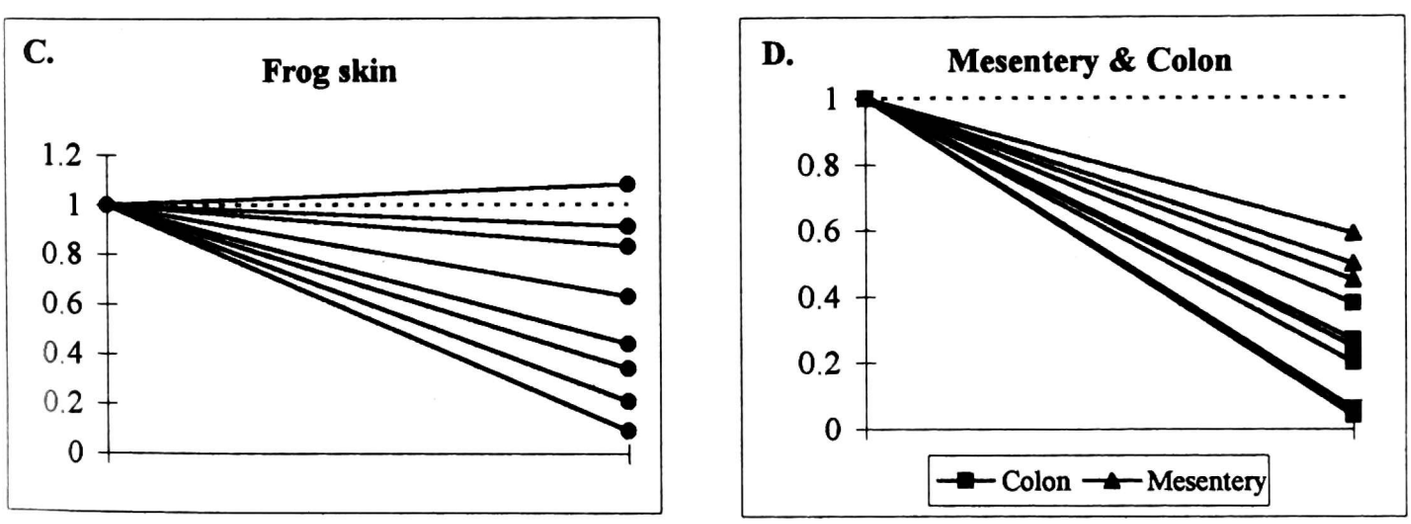


Fig. 5. Effects of hypertonicity of medium (A&B) and mercury (C&D) on water permeability of frog skin (B&C), parietal peritoneum (A), mesentery (D-Δ-) and colon (D-□-). All results expressed as ratio between the final and the initial values of L_p, i.e. measured after and before application of the factors under study.

The values of the flow were different depending on the type of the membrane used, the circumstances in which experiments were carried out, as well as on the magnitude of the applied gradient. The lowest values (0.04—0.33 nL/sec with $\Delta P = 5$—10 mm H_2O) were found in experiments carried out with the amphibians during autumn & winter, i.e. on skins taken from frogs kept out of their natural environment no longer than 4 months[4], significantly higher values in experiments with parietal peritoneum, and with frog skins carried out in spring & summer (these frogs were kept in the laboratory > 4 months) whereas the highest values were in experiments with mesentery and colon (36.8—48.9 nL per second under $\Delta P = 1$ mm H_2O).

[4] it has not been established yet which of the two possibilities (season of the year or time of maintenance in the laboratory) was the cause of difference between the two groups of experiments done with frog skins

The results of the measurements of flows with regard to the actual ΔP have been conversed in the index of hydraulic conductivity (L_p) which can be accepted as a common denominator to compare all the membranes under all circumstances. The table (*Tab. 1*) presents a comparison between the hydraulic conductivity indices of all membranes studied under basic circumstances (Ringer or Hanks solutions on both sides of these membranes).

Table 1: Hydraulic conductivity indices (L_p) of the membranes studied *in vitro*.

Frog skin		Peritoneum		Colon
winter season	spring season	parietal part	mesentery	
(1) 0.20 ± 0.02 (n = 11)	(2 2.50 ± 0.63 (n = 9)	(3) 6.3 ± 0.6 (n = 73)	(4) 38.9 ± 3.7 (n = 34)	(5) 37.6 ± 4.1 (n = 6)

The values are means ± SEM ($\times 10^{-2}$ cm $\times$ sec^{-1} $\times$ atm^{-1}). The differences between all values (but col. 4 and 5) are statistically significant.

DISCUSSION

The elaborated method made it possible to establish both hydrostatic and osmotic pressure gradient across the membrane. They acted as a driving force for water transmembrane flow. However, it proved to be much easier and more precise to establish the former gradient than the latter. Moreover, owing to the described arrangement (servo-mechanism) it was also quite easy to maintain the gradient at the desired level throughout the requested time. Threfore, in the majority of experiments that tested the method, hydrostatic pressure gradient was used to induce the flow and in this paper only the results obtained with this driving force have been presented.

A question can be raised how far the hydrostatic pressure corresponds with the osmotic one. Both appear in organisms, and both play a role as the driving forces for water movement through membranes (1, 2, 9—11). They are mutually denumerable, although recalculations are much more complicated in biological (physiological, and the more pathophysiological) studies than in a physico-chemical laboratory where all factors can be strictly controlled (1, 2, 12). In physiological systems the osmotic pressure depends not only on composition of fluids bathing both sides of a membrane and the sort of solute which creates the osmotic pressure, but also on kind and type of the membrane, its actual state (e. g. the age of the organism the membrane comes from), not mentioning many humoral influences on the tissue. Thus, it seemed rational to

use rather hydrostatic than osmotic pressure gradient as the standard driving force in testing the applicability of the described method for further studies on water movement through various membranes, and for investigations of influence of some factors on it.

It is worth mentioning that the measured values relate strictly not only to water but also to solutes with as small molecular sizes as they could allow them to pass the barriers. In this study, like in other investigations on water membrane transfer, one cannot know which and how much of the solutes pass the membrane, e. g. within a mechanism of "solvent drag". As it depends not only on type of the membrane but also on conditions of experiments, it has been generally accepted in investigations of that kind to replace the expression "water" with "volume" (2, 10). In practice, however, such differentiation between "volume" and "water" is superfluous, especially in elaboration of problems of medical pharmacology, clinical physiology and pathophysiology. Transmembrane flow of free water (i. e. without solutes) takes place only in very rare places & circumstances, e. g. in collecting duct of the nephron under the influence of vasopressin (4, 10).

Assessing the method in general, one should say that the results were as it could be expected: After the gradient across the membrane had been established, water flow appeared with the direction in accordance with the direction of the gradient, and with the flow as stable as the gradient. Values of the flow were roughly proportional to the values of the gradient, so the index of hydraulic conductivity could be surely calculated. The indices, however, were quite different and rather characteristic for different membranes (within the margin of error designed by $\pm$SE): they were greatest in colon and mesentery, and smallest in frog skin during winter (shortly maintained in the laboratory). The indices accord obviously with *in situ* water transfer which is big in such membranes as the large intestine and also in the peritoneum, but not in its parietal part (11). The results also show that permeability for water of frog skin can be different under different conditions.

Since the main subject of this study was description of the method itself, with the presentation of only some results that could prove its validity for further studies on water transmembrane flow, there should be no discussion about merits of the effects which have been found during the series of testing experiments. Nevertheless, it is worth stressing that hypertonicity of the bathing medium induced an increase of hydraulic conductivity not only of frog skin but also of peritoneum. This observation which confirm our earlier findings as regards peritoneal solute transport (13) can be interesting not only because of its importance from the clinical point of view (efficiency of peritoneal dialysis) but also because an opinion is still maintained that mesothelium (main component of this membrane in *in vitro* study) does not count as a barrier for peritoneal ultrafiltration, i. e. for water transfer across

peritoneal membrane (12). Based upon our previous results (7, 13, 14) as well as the ones presented herein a contrasting opinion can be upheld.

Another interesting observation should be noticed, i. e. the inhibitory effect of mercuric chloride which was shown in all (but one) of the experiments carried out on frog skin, mesentery and colon. This effect should be looked at in the context of contemporary discoveries that permeability of various membranes depends on water channels, a majority of which are mercurial-sensitive as they become blocked under the influence of this toxic metal ion (3—5).

The results presented in this paper seem to show that the elaborated method can be useful for further studies on water metabolism and on participation of various barriers in water transport processes. Presumably it will occur to be applicable for research on some specific medical problems connected with water membrane transport like peritoneal dialysis (ultrafiltration failure) or some abnormalities in the function of alimentary tract (diarrhoe, constipation).

CONCLUSIONS

1. Biological membranes with an even anatomic structure when mounted into a chamber to divide it into two compartments can permeate water under hydrostatic pressure gradient; the volume flow is proportional to magnitude of the gradient thus hydraulic conductivity index (L_p) of the membranes can be calculated.
2. Value of L_p is a characteristic property of each membrane, and may be influenced by such factors as hypertonicity of medium bathing the membrane, drugs or toxins (e. g. $HgCl_2$).
3. The method of assessment of L_p may be useful for further studies on water membrane transport across biological barriers in the body.

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