

# Action of Antidiuretic Hormone on the Equivalent Pore Radius at both Surfaces of the Epithelium of the Isolated Toad Skin

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**ABSTRACT** A previously described method (1) allows the observation of swelling and shrinking of the epithelial cells of the isolated toad skin, when the solution bathing either the outer or inner side of the skin is modified. Thus, the concentration of probing molecules of graded size, isotonic to the epithelial cells, across each face of the isolated toad skin can be determined. These concentrations have been used for the estimation of the equivalent pore radius at the outer and inner face of the skin epithelium, following the approach of Goldstein and Solomon for red cells (3). An equivalent pore radius of 4.5 Å for the outer surface, and one of 7 Å for the inner surface have been obtained. Antidiuretic hormone had an effect only when added to the inner side. This effect was only at the outer surface and is interpreted as widening of the 4.5 Å pores to about 6.5 Å. A model membrane, formed by narrow and wide pores in series, may explain some of the apparent inconsistencies previously observed.

The method of MacRobbie and Ussing (1, 2) has been combined with that of Goldstein and Solomon (3) to determine the concentrations of probing molecules of graded sizes that are effectively isotonic to the epithelial cells across each face of the isolated toad skin at times close to zero. These concentrations have been used for the calculation of the equivalent radius of the pores at the outer and inner face of the toad skin epithelium. The action of antidiuretic hormone on those equivalent pore radii has been studied.

## EXPERIMENTAL PROCEDURE

*Bufo spinulosus* from Peru was used. The experiments were carried out at room temperature (16–18°C). The abdominal skin was removed from pithed animals and cleaned

of muscle and adherences, stretched out tightly, and tied onto a lucite cylinder. The cup, formed by the cylinder and the skin, was placed over a fiber glass mat on top of a lucite dish. The whole assembly was placed on a microscope stand. Therefore, there are two compartments, one (inside of the cup) facing the microscope tube and another facing the fiber glass mat. Each compartment was connected *via* polyethylene tubing to a reservoir and a syringe, so that changing the solutions in either compartment could be performed within 2 to 3 seconds. A mixture of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub> was bubbled into each compartment and provided adequate mixing. The skin was observed at a magnification of 500 times using a microscope equipped with a Leitz water immersion objective ( $\times 50$ ). The microscope was focused on a suitably chosen melanin granule and the reading on the micrometer (fine focusing screw) of the microscope recorded. An area with a sharp line on the epithelial surface was then brought into focus and the micrometer reading recorded. The vertical distance between the two reference points was thus obtained by difference. Since changes in volume of the epithelium are manifested as changes in its thickness, under the conditions of the experiments (2), changes in thickness may be followed by changes in difference of the micrometer readings. The standard deviation of ten micrometer readings when focusing one point was  $\pm 1 \mu$ . Detailed and critical descriptions of the method have been published (1, 2).

Various solutions were used in this study. All of them contained basically 25.0 mM NaHCO<sub>3</sub>, 2.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.6 mM KH<sub>2</sub>PO<sub>4</sub>, 12.0 mM KCl, 1.2 mM MgSO<sub>4</sub>, 0.6 mM Na<sub>2</sub>SO<sub>4</sub>, 1.9 mM calcium gluconate, and 26.0 mM glucose, making a total of 110 mOsm/liter. A standard solution similar to the extracellular fluid of the animal was prepared by adding NaCl to this basic medium, to give a total osmolarity of 225 mOsm/liter, isosmotic with the extracellular fluid of the animal. The experimental solutions were prepared by adding to the basic medium, instead of NaCl, one of the following non-lipid-soluble non-electrolytes: raffinose, sucrose, erythritol, glycerol, or urea. For example, three solutions were prepared with 100, 120, and 150 mOsm raffinose/liter and total osmolarities of 210, 230, and 260 mOsm/liter respectively. Similarly, various solutions of the other probing molecules were prepared with total osmolar concentrations ranging from 160 to 460 mOsm/liter. The osmolarities were determined by measuring freezing point depression.

After the skin and chamber were set up under the microscope, both compartments were filled with the standard solution, and allowed to equilibrate for about 1 hour. The microscope was then focused and readings of the micrometer thereon were recorded. The preparation was considered to be in a steady state when the readings in the micrometer did not vary for more than  $2 \mu$  over a period of at least 20 minutes. The standard solution was then replaced on the outer side of the skin by one of the experimental solutions containing a probing molecule, while the inner side was bathed during the entire experimental period by the standard solution. Changing of the solution was accomplished within 2 to 3 seconds. A new micrometer reading was then recorded after 15 seconds of changing the solution; subsequent readings were recorded at 15 to 30 second intervals. Errors in time recording as measured with a chronometer were  $\pm 1$  second. If the solution with the probing molecule was hyper-tonic a steady decrease in thickness of the skin could be followed as a function of time

by the change in readings of the micrometer. The change in thickness was a linear function of time usually for about 5 to 7 minutes, thereafter a plateau was reached. The experimental solution with the probing molecule was then replaced by the standard solution. This was followed by swelling (increase in thickness) until the skin recovered its original thickness and a new steady state was reached. After a period of 20 minutes, a hypotonic solution containing the same probing molecule was tried, and so forth. Experiments with one skin lasted for about 3 hours. During this period, two to three measurements were made of the osmotic effect on the outer surface of the epithelium of three solutions of the same probing molecule, one that would produce swelling, one that would produce shrinking, and one of intermediate composition. Similar experiments were performed in six to ten skins for each probing molecule.

Comparable groups of experiments were performed testing the various probing molecules at the inner side of the skin, while the outer side was bathed by the standard solution.

#### *Experiments in Which Pitressin Was Added*

Experiments were performed, in an entirely comparable manner, to study the action of antidiuretic hormone. Pitressin (Parke, Davis and Co.) was added to the various solutions to obtain a concentration of 1.5 pressor units/ml of solution. (a) Pitressin was added to the inner bathing solution and left there for the whole experimental period; after 90 minutes, solutions containing the probing molecules were tested on the outer side; (b) similarly, solutions with the probing molecules were tested on the inner side 90 minutes after pitressin was added to the outer bathing solution; (c) the outer side was bathed in standard solution with pitressin for 90 minutes prior to the addition of pitressin containing solutions of probing molecules on the same side of the membrane; (d) Pitressin was finally added to the inner side 90 minutes prior to the addition of pitressin containing solutions of probing molecules on the same side of the membrane.

#### *Adequacy of Mixing*

Observations of phenol red dispersion during the change of the solution bathing one side of the skin, indicated that mixing occurred while the new solution was still being injected and was completed within 3 to 4 seconds.

The diffusion time for the solution to the osmotic barrier may be estimated by calculating for a medium size molecule like glycerol, the time delay to reach in the extracellular space 90 per cent of the bathing fluid concentration. The following equation for diffusion in a plane sheet may be used (4, 5):

$$1 - (C_e/C_b) = (8/\pi^2)e^{-D\pi^2 t/x^2} \quad (1)$$

in which  $(C_e/C_b)$  is the ratio of the probing molecule concentration in the extracellular spaces to that in the bathing fluid.  $D$  is the diffusion coefficient,  $0.75 \times 10^{-6}$  cm<sup>2</sup>/sec. for glycerol at 17°C (6);  $x$  is the thickness of the diffusion layer; and  $t$  is the time.

At the inner side, molecules have to diffuse through the 80  $\mu$  thick corium before they reach the epithelial cells. If we assume, as an upper limit, a 100  $\mu$  non-stirred fluid layer interposed between the corium and the bathing fluid (7), it may be calcu-

lated that the solution at the epithelial cell surface will reach 90 per cent saturation in 9 seconds. At the outer side, molecules would have to diffuse through a non-stirred fluid layer  $100 \mu$  thick, taken as an upper limit, and through the stratum corneum, estimated to be  $20 \mu$  thick. Time delay for 90 per cent saturation would be 4 seconds. Therefore, only the first observation taken 15 seconds after changing the solution may have been affected by diffusion delay. However, since change in thickness was a

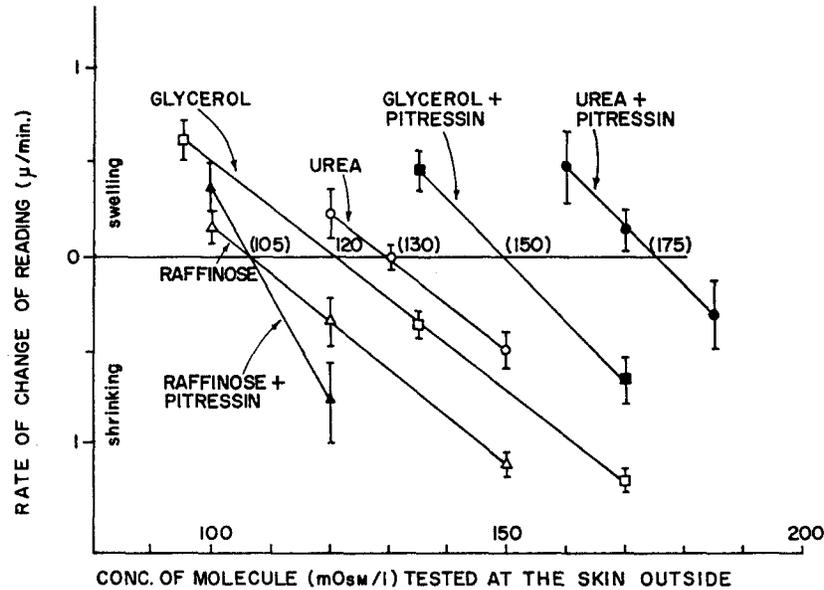


FIGURE 1. Initial rate of change in micrometer reading (rate of cell volume change) plotted as a function of the concentration of probing molecule present in the solution bathing the outer side of the skin. The inner side is bathed with standard physiological solution. The height of the vertical lines denotes the standard error of the mean obtained in experiments with six to ten skins. The intercept of the line drawn for one probing molecule at the point where rate of change of reading is zero is called  $C_{180}$ . At the outer side,  $C_{180}$  for glycerol changes from 120 to 150 (mOsm/liter) and  $C_{180}$  for urea from 130 to 175 (mOsm/liter) when pitressin is added to the inner solution.  $C_{180}$  equals 105 (mOsm/liter) for raffinose.

linear function of time during the first ten to fifteen observations recorded in 5 to 7 minutes, and they were all used in obtaining one experimental figure, it may be concluded that diffusion delay did not affect the experimental data for the outer and inner sides of the skin, and it is safe to use in the calculations the concentration of probing molecules in the bathing fluids.

## RESULTS

### *Determination of the Isotonic Concentration of Probing Molecules*

The initial rate of volume change was plotted as a function of the concentration of probing molecules, and a concentration in which there is no initial volume

change was obtained by interpolation. This concentration may therefore be called concentration effectively isotonic at times close to zero, and denoted  $C_{iso}$ . Fig. 1 shows how the value of  $C_{iso}$  is obtained from experiments performed using solutions with probing molecules at the outer side of the skin, while the inner side was bathed with standard solution. It may be seen that swelling occurs with raffinose at a concentration of 100 mOsm/liter, shrinking at 120 mOsm/liter, and further shrinking at 150 mOsm/liter. Interpolation gives 105 mOsm/liter as the concentration at which no volume change occurs

TABLE I  
EFFECT OF PITRESSIN ON  $C_{iso}$  FOR  
NON-LIPID-SOLUBLE NON-ELECTROLYTES TESTED AT THE OUTER  
SIDE OF THE ISOLATED TOAD SKIN

	No pitressin		Pitressin*		Pitressin-induced change in $C_{iso}$ ‡	<i>P</i> of difference in $C_{iso}$	
	<i>a</i>	$C_{iso}†$	$\frac{C_{iso}^i}{C_{iso}}$	$C_{iso}†$			$\frac{C_{iso}^i}{C_{iso}}$
Sulfate		105 ± 5		105 ± 4			
Raffinose	6.0	105 ± 4	1.00	105 ± 5	1.00	0 ± 5	>0.7
Sucrose	4.5	106 ± 7	0.99	103 ± 3	1.02	-3 ± 8	>0.7
Erythritol	3.2	115 ± 5	0.91	130 ± 2	0.81	15 ± 5	0.015
Glycerol	2.9	120 ± 1	0.88	150 ± 3	0.70	30 ± 3	<0.001
Urea	2.3	130 ± 4	0.81	175 ± 8	0.60	45 ± 9	0.001

*a* is the molecular radius in Angstroms, determined on Stuart and Briegleb models, except the radius for raffinose, which is taken from viscosity measurements (28).

\* Added to the solution bathing the inner side.

† Mean ± standard error of the mean. Units are mOsm/liter.

‡ Difference ( $C_{iso}$  pitressin -  $C_{iso}$  no pitressin) ± standard error of the difference.

|| Osmolarity given for  $\text{Na}_2\text{SO}_4$ .

at very short periods of time. Therefore, the value of  $C_{iso}$  for raffinose is 105 mOsm/liter, for glycerol 120 mOsm/liter, and for urea 130 mOsm/liter. Each value of  $C_{iso}$  was obtained from data for experiments performed in six to ten skins. Since the experiments were repeated similarly for each side of the skin, one value of  $C_{iso}$  for each side for each substance was obtained.

Table I gives values of  $C_{iso}$  obtained for the probing molecules tested at the outer side, and Table II for the probing molecules tested at the inner side. It may be seen that there is no difference in the values of  $C_{iso}$  for sucrose and raffinose, even though their molecular radii are quite different (1.5 Å difference). However, for the smaller molecules the values of  $C_{iso}$  increase while the molecular radii decrease although their molecular radii do not differ by more than 0.9 Å. It should also be pointed out that while values of  $C_{iso}$  for sucrose and raffinose are the same at both sides of the skin, values of  $C_{iso}$  for the smaller molecules are significantly larger at the inside as compared to the outside.

*Action of Antidiuretic Hormone on the Values of  $C_{iso}$* 

Pitressin was effective only when added to the inner bathing standard solution and when probing molecules were tested at the outer side of the skin. Fig 1 shows significant shifting of the lines that relate the initial rate of volume change with the concentration of glycerol and urea tested at the outer side, after the addition of pitressin to the inner side solution. Table I shows that pitressin induced changes, with high statistical significance, in the values of  $C_{iso}$ , for erythritol from 115 to 130 mOsm/liter, for glycerol from 120 mOsm/liter,

TABLE II  
EFFECT OF PITRESSIN ON  $C_{iso}$  FOR  
NON-LIPID-SOLUBLE NON-ELECTROLYTES TESTED AT THE INNER  
SIDE OF THE ISOLATED TOAD SKIN

	No Pitressin		Pitressin*		Pitressin-induced change in $C_{iso}$ ‡	P of difference in $C_{iso}$
	$C_{iso}$ †	$\frac{C_{iso}^i}{C_{iso}}$	$C_{iso}$ †	$\frac{C_{iso}^i}{C_{iso}}$		
Sulfate	105 ± 6					
Raffinose	104 ± 8	1.01	106 ± 13	0.99	2 ± 15	>0.7
Sucrose	108 ± 12	0.96	106 ± 14	0.99	-2 ± 18	>0.7
Erythritol	132 ± 10	0.80	140 ± 20	0.75	8 ± 22	>0.7
Glycerol	304 ± 30	0.35	290 ± 20	0.36	-14 ± 36	>0.7
Urea	220 ± 15	0.48	223 ± 8	0.47	3 ± 17	>0.7

\* Added to the solution bathing the inner side.

† Mean ± standard error of the mean. Units are mOsm/liter.

‡ Difference ( $C_{iso}$  pitressin -  $C_{iso}$  no pitressin) ± standard error of the difference.

|| Osmolarity given for  $\text{Na}_2\text{SO}_4$ .

to 150 mOsm/liter, and for urea from 130 mOsm/liter to 175 mOsm/liter. These changes in the values of  $C_{iso}$  were larger for the smaller molecules. No change was observed, after addition of pitressin, in the values of  $C_{iso}$  for sucrose and raffinose. Pitressin added either to the outer or the inner side solutions produced no change in the values of  $C_{iso}$  at the inner side of the skin (Table II).

## INTERPRETATION

The method of Goldstein and Solomon (3) for the determination of the equivalent pore radius of a cell membrane, depends on the determination of the external concentration of permeant solute required to prevent water movement into or out of the cell at the instant of immersion in the medium. These isotonic concentrations,  $C_{iso}$ , may be related to the equivalent pore radius and to the radius of the probing molecules (3, 9, 10). The determination of the ratio of values of  $C_{iso}$  or permeant and impermeant solutes is equivalent to

the determination of Staverman's reflection coefficient  $\sigma$  (11);  $\sigma$  describes the ability of the membrane to discriminate between the particular probing molecule, used as solute, and the solvent. Therefore,  $\sigma = 1$  if the solute does not penetrate the membrane (ideally selective membrane) and  $\sigma = 0$  if solute and solvent pass across the membrane with equal velocities.

Stavermann (11) has pointed out that for solutes that can cross a membrane the osmotic pressure differs from the classical van't Hoff osmotic pressure. The relationship of these osmotic pressures may be expressed formally in terms of:  $\sigma = (\pi_{\text{exp}}/\pi_{\text{th}}) = (\pi_{\text{exp}}/RTC)$  where  $\pi_{\text{th}}$  is the theoretical thermodynamic (van't Hoff) osmotic pressure and therefore equal to  $RTC$ , and  $\pi_{\text{exp}}$  is the effective osmotic pressure measured with a membrane, for a penetrating solute at a concentration  $C$ ,<sup>1</sup> as the pressure difference at which volume flow stops;  $R$  is the gas constant,  $T$  the absolute temperature.

#### *Determination of $\sigma$*

In the stationary state there exists a balance of water between cells and surrounding fluid at equal total osmotic pressures (12, 13). In these conditions, an osmotic pressure gradient across the cell membrane may be established by making the extracellular fluid hypotonic. Osmotic equilibrium (isotonicity) may be restored by addition to the extracellular solution of an impermeant solute at a concentration  $C_{\text{iso}}^i$  that will therefore exert an osmotic pressure equal to  $RTC_{\text{iso}}^i$ , since  $\sigma = 1$ . Transient osmotic equilibrium may also be restored by addition of a solute that can permeate the membrane. As Staverman (11) has pointed out, the concentration,  $C_{\text{iso}}$ , required to produce transient osmotic equilibrium is higher for permeant than for impermeant solutes. The value of  $C_{\text{iso}}$  will exert an effective osmotic pressure,  $\sigma RTC_{\text{iso}} = RTC_{\text{iso}}^i$ .  $\sigma$  may therefore be calculated from the experimental determination for the ratio ( $C_{\text{iso}}^i/C_{\text{iso}}$ ).

When the radius of the probing molecule is close to the radius of the water molecule, the value of  $C_{\text{iso}}$  will be very large; as the molecular radius increases, approaching the equivalent pore radius, the value of  $C_{\text{iso}}$  will become progressively smaller, approaching the value of  $C_{\text{iso}}^i$  for the impermeant molecule. It may be seen in Tables I and II that this is the case for our experimental data for the outer and inner sides of the skin. The concentration approached is about 105 mOsm/liter. The values of  $C_{\text{iso}}^i$  for erythritol, a 3.2 Å radius molecule, are 115 and 130 mOsm/liter for the outer side, without and with pitressin, respectively, and 132 and 140 mOsm/liter for the inner side, without and with pitressin. The values of  $C_{\text{iso}}$  for sucrose, a 4.5 Å radius molecule, are 106, 103, 108, and 106 mOsm/liter for the same experimental

<sup>1</sup> The concentrations of non-penetrating,  $C^i$ , and penetrating solutes,  $C$ , are expressed in osmolar units.

conditions, respectively. The values of  $C_{iso}$  for raffinose, a 6.0 Å radius molecule, are 105, 105, 104, and 106 mOsm/liter, respectively. It seems safe therefore to consider the isotonic concentration of non-penetrating molecules that keeps isotonicity at both skin sides,  $C_{iso}^i$ , as 105 mOsm/liter. (See Appendix.)

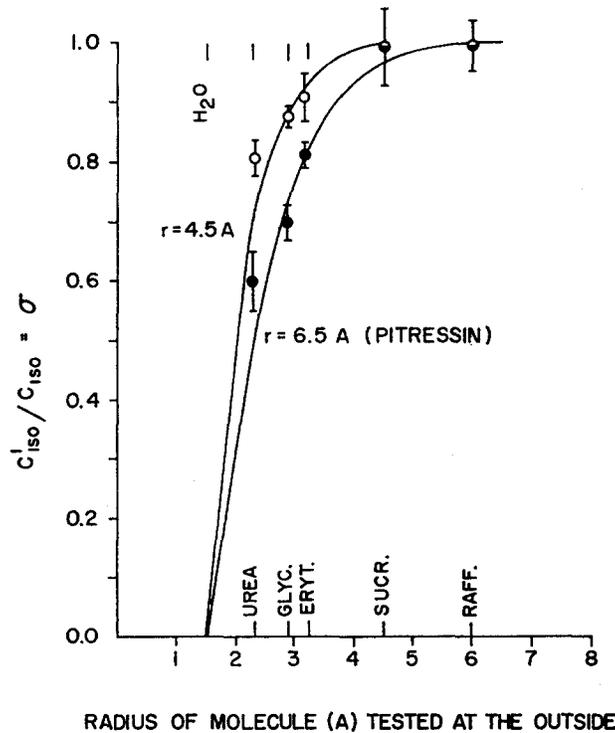


FIGURE 2. ( $C_{iso}^i / C_{iso} = \sigma$ , plotted as a function of the radius of the probing molecule tested at the outer side of the skin. The radius of the water molecule is taken as 1.5 Å (reference 19, and p. 12 of reference 14). The height of the vertical lines denotes one standard error of the mean. Values obtained without pitressin (open circles) are fitted by a theoretical curve that corresponds to an equivalent pore radius of 4.5 Å. Values obtained after pitressin was added to the inner side (filled circles) are fitted by a theoretical curve that corresponds to an equivalent pore radius of 6.5 Å.

#### *Relationship between $\sigma$ , Molecular Radius, and Equivalent Pore Radius*

Renkin (17) has related  $A_{sf}$  and  $A_{wf}$ , the effective pore areas available for filtration of the solute and water, to the molecular radius of the solute,  $a$ , of the solvent,  $a_w$ , and to the equivalent pore radius,  $r$ . It has been demonstrated (18, 10) that  $\sigma = 1 - (A_{sf}/A_{wf})^2$ . Therefore,  $\sigma$  is a function of the

<sup>2</sup> Independently, from Equation 4-11 of reference 15, a comparable expression may be obtained for the ratio of the areas available for diffusion when the net water flow is zero.

radius of water, the radius of the probing molecule, and the equivalent pore radius of the membrane (3, 9, 10) as expressed by Equation 6 in reference 3 which is as follows:—

$$\sigma = 1 - \frac{[2(1 - a/r)^2 - (1 - a/r)^4][1 - 2.104a/r + 2.09(a/r)^3 - 0.95(a/r)^5]}{[2(1 - a_w/r)^2 - (1 - a_w/r)^4][1 - 2.104a_w/r + 2.09(a_w/r)^3 - 0.95(a_w/r)^5]}$$

Using this equation, a family of theoretical curves may be constructed for

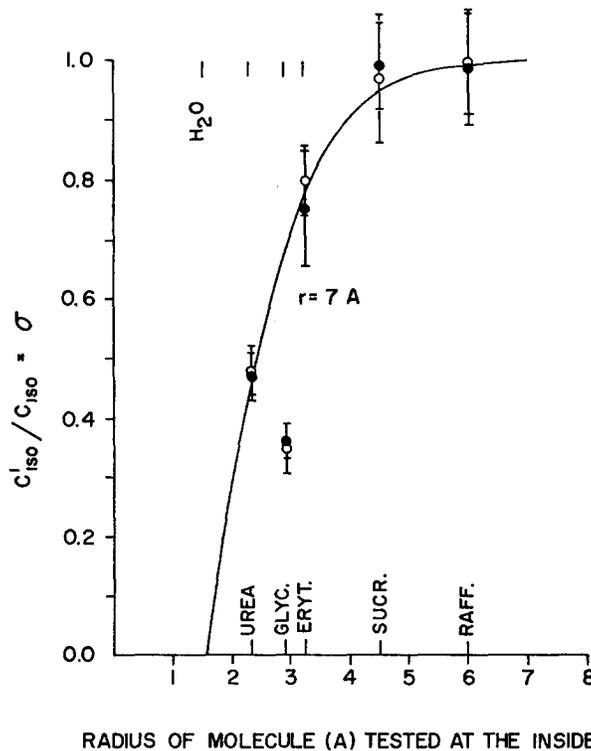


FIGURE 3.  $(C'_{iso}/C_{iso}) = \sigma$ , plotted as a function of the radius of the probing molecule tested at the inner side of the skin. The height of the vertical lines denotes one standard error of the mean. Open circles, obtained without pitressin, overlap with filled circles, obtained after pitressin was added to the inner side. The continuous line is a theoretical curve that corresponds to an equivalent pore radius of 7 A. The point corresponding to glycerol, for the inner surface, is aberrant. We have no explanation for this finding.

various equivalent pore radii in a membrane, relating values of  $\sigma$  in the ordinates, to the radii of the probing molecules in the abscissae. The equivalent pore radius of a membrane may be evaluated from the experimental determination of the values of  $\sigma$  in that membrane for various probing molecules, and from their molecular radii. The experimental values are then superposed on the best fitting theoretical curve.

*The Equivalent Pore Radius for the Isolated Toad Skin*

Tables I and II show the experimental results for the ratio  $(C_{iso}^i/C_{iso}) = \sigma$  for the various probing molecules tested. A value of 105 mOsm/liter has been used for  $C_{iso}^i$ , which corresponds to values of  $C_{iso}$  obtained with raffinose and sucrose. Figs. 2 and 3 have been constructed as outlined above. Values of  $\sigma$  for the various probing molecules have been plotted as a function of their respective molecular radii. The continuous lines are theoretical curves given by the above equation with values of  $r$  that best fit the data.<sup>3</sup> Fig. 2 refers to data obtained for probing molecules tested at the outer side of the skin. A curve that corresponds to an equivalent pore radius of 4.5 Å limited by values of 4.2 and 4.8 Å, best fits the open circles which represent data obtained without pitressin. A curve that corresponds to an equivalent pore radius of 6.5 Å limited by values of 6.2 and 6.8 Å best fits the filled circles, which represent data obtained after the addition of pitressin to the inner solution. Fig. 3 shows that an equivalent pore radius of 7.0 Å best fits the data for the inner side; limiting values are 6.2 and 7.8 Å. There is no difference between the data without pitressin (open circles) and with pitressin (filled circles). Therefore, in the absence of hormonal action, the outer side of the epithelium appears to have smaller equivalent pore radius (4.5 Å) than the inner side (7.0 Å). Antidiuretic hormone widens significantly, up to 6.5 Å, the equivalent pore radius at the outer side, and appears to have no action on the equivalent pore radius at the inner side. Similar "widening" of pores by pitressin has been described for *Necturus* kidney slices (20).

#### DISCUSSION

Fig. 1 shows that, at the outer side of the skin, the slopes relating initial rate of volume change with concentration of probing molecules are steeper after pitressin was added to the inner solution. The slopes have the units of a permeability coefficient for the filtration of water ( $P_f$ , units of  $\text{cm}^3/\text{cm}^2$  second per unit osmotic pressure gradient). Therefore, after the addition of pitressin, the value for  $P_f$  increases at the outer side of the epithelium. MacRobbie and Ussing have reported similar observations (2). They calculated values of  $P_f$  at the outer side to be about 5 per cent the value of  $P_f$  at the inner side of the epithelium. Addition of antidiuretic hormone produced no alteration in the value for  $P_f$  at the inner side, while at the outer side it increased the value of  $P_f$  to about 20 per cent of the value of  $P_f$  at the inner side. This was interpreted as being produced by an increase in pore size in some layer towards the outer side of the skin. The area available for water filtration may be evalu-

<sup>3</sup> The radius of the water molecule was taken as 1.5 Å (reference 19, and p. 12 of reference 14).

ated (Renkin's Equation 19) from the equivalent pore radius, and the radius of the water molecule (17). Taking our values for equivalent pore radius, it may be calculated that the value of  $P_f$  at the outer side should be 20 per cent the value of  $P_f$  at the inner side, without hormone; and after pitressin addition, the value of  $P_f$  at the outer side should be about 70 per cent of the value of  $P_f$  at the inner side. Therefore, the present results agree qualitatively with those of MacRobbie and Ussing (2). The outer side of the epithelium appears tighter in their experiments. This may also be deduced from their data on sulfate-Ringer; the skin volume appeared to be independent of the sulfate-Ringer tonicity at the outer solution (see Appendix). Explanations for the quantitative differences are several: the experimental animals belong to different suborders, there were some differences in the solution used, and there are seasonal variations in the animals themselves.

The equivalent pore radius is proportional to the ratio of the permeability for filtration of water to the permeability for diffusion of water (21-23), so that by determining these parameters, the equivalent pore radius of a membrane can be estimated. Let us now envisage a composite membrane, formed by narrow pores in series with wide pores (10), such that the main resistance to filtration of water occurs at the narrow pores and the resistance to diffusion of water is larger at the wide pores than at the narrow ones. This may occur, (a) if the wide pores are long enough, or (b) if the total area of the narrow pores is greater than that of the wide pores. If one constructed such a model and used measurements of permeability coefficient for diffusion and filtration of water to estimate the equivalent pore radius of the whole composite membrane, one would be bound to get, from the calculation, an equivalent pore radius bigger than that of the narrow pore; the value obtained would be only an upper limit for this equivalent pore radius. The true radius would be between the radius of the water molecule and this upper limit. Villegas and co-workers proposed a similar model for the squid nerve (9, 24). Andersen and Ussing (8) calculated an equivalent pore radius of 6 to 20 A for the toad skin, using water permeability coefficients. However, these pores were too big to explain the marked resistance they found to the movement, through the skin, of thiourea and acetamide (molecules bigger than water). Consequently they suggested a narrow end in the pores. Later, MacRobbie and Ussing (2) deduced that this change in pore size (narrow end) occurred at the outer border of the epithelium, because water permeability at the outer side of the epithelium was found to be smaller than that at the inner side. They suggested that antidiuretic hormone widens this narrow end, because of a marked increase in water permeability at the outside after addition of antidiuretic hormone. These findings are also in accord with the composite membrane suggested here which is the simplest model so far necessary to explain the experimental results. It may also explain some apparent inconsistencies of the

pore theory (see for example reference 25). The present findings are in accord with an interpretation of permeability studies on the toad bladder (26) if the mucosal and serosal surfaces of the toad bladder are compared with the outer and inner surfaces of the skin, respectively.

Solomon (23) has pointed out that the concept of an equivalent pore radius, in spite of its limitation, does provide a useful description of a simple membrane's behavior with respect to the passage of water and solutes. Because the values of molecular radii have inherent errors, and because of other possible experimental errors, the theoretical curves with which the experimental data are fitted are given with a tolerance that is assumed to cover the uncertainties mentioned above. The present experimental results are self-consistent, however, and independent of the interpretation given here.

Evidence has been given for the existence of water-filled channels going through the skin (22, 8, 27). The present experiments are concerned only with the toad skin epithelium. The results may be interpreted as showing that (a) the epithelial cell layer that responds to swelling and shrinking has towards the outer surface, an osmotic barrier with narrow pores of 4.5 Å, which are assumed to be the main resistance to water filtration; it has towards the inner surface, probably facing the basement membrane, an osmotic barrier with wide pores of 7.0 Å, which are assumed to be the main resistance to water diffusion; (b) water moves through the cells and not between them; the water-filled channels therefore perforate the cell walls; (c) narrow and wide pores are in series; (d) antidiuretic hormone acts on the narrow pores (although it is effective only when applied to the skin inside), widening them to 6.5 Å, so that resistance to water filtration markedly diminishes but resistance to water diffusion stays the same, as has been found by others (22, 8).

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#### APPENDIX

When a salt diffuses in solution, anion and cation move in the same direction at equal speeds, since otherwise separation of electrical charges in the solution would result (14). Kedem and Katchalsky (15) have shown that although  $\sigma$  is concentration-dependent, the equations for  $\sigma$  for salts in solutions are similar to the equations for  $\sigma$  for non-electrolytes, provided the charge density in the membrane is not extremely high. Whittembury, Sugino, and Solomon (16) have found in kidney slices that from

the point of view of osmotic pressure, electrolytes behave like non-electrolytes. Experiments on the osmotic behavior of the epithelial cells of frog skin support the contention based on studies of the skin electrical potential that the skin is virtually impermeable to sulfate ions (2). Therefore,  $\text{Na}_2\text{SO}_4$  was tried as probing molecule in a similar way as has been described under Methods. A figure of 105 mOsm/liter was obtained, for the values of  $C_{i_{80}}$  for  $\text{Na}_2\text{SO}_4$  for the outer and for the inner side of the skin (Tables I and II) similar to the values of  $C_{i_{80}}$  for raffinose and sucrose. These results give further support to a value of 105 mOsm/liter as  $C_{i_{80}}^i$  for the osmotically impermeable substances.

Separate experiments were performed using a reference solution containing the basic composition plus 105 mOsm/liter  $\text{Na}_2\text{SO}_4$ . This solution was placed bathing both sides of the skin. After equilibration, solutions of the probing molecules and of NaCl were tested at the outside. The following values for  $C_{i_{80}}$  were obtained: 105, 110, 117, and 123 mOsm/liter for sucrose, erythritol, glycerol, and urea, respectively, and 115 mOsm/liter for NaCl.

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