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The action of antidiuretic hormone on cell membranes. Voltage transient studies

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1. The instantaneous impedance method has been used to study the effects of antidiuretic hormone (ADH) on frog skin.

2. The resting skin may be represented by a parallel RC network with a single time constant.

3. Antidiuretic hormone causes an increase in conductance and capacitance and in some cases the appearance of a polarization angle.

4. The structures in the skin responsible for the transients are located in the outermost membranes.

5. The effects of ADH have been interpreted in terms of the formation of water-filled sodium-permselective pores in the outer facing membranes which occupy, at most, 0.3% of the skin surface. These pores constitute a parallel, and hence additive, capacitance with that of the normally ion impermeable parts of the cell surface, and in addition are responsible for the increase in conductance. The polarization angle is due to the polydisperse nature of the skin after hormone treatment.

Drug action at cell membranes is usually studied by measuring an end effect several stages removed from the causative membrane change. Muscular contraction, glandular secretion and transport processes are commonly measured end effects. In this paper an attempt has been made to understand the change occurring in the cell membranes of frog skin when acted on by antidiuretic hormone (ADH). In the Koefoed-Johnsen & Ussing (1958) model of active sodium transport across frog skin the outer facing membranes are considered to be the target for ADH action. Whether the hormone acts directly (Cuthbert & Painter, 1968a) or indirectly as suggested by Orloff & Handler (1967) is not yet clear, but it is generally agreed that increased permeability of the outer facing membranes to sodium ions results.

The method that has been used in this work is the " instantaneous impedance " method (Teorell, 1946) in which step voltage functions are applied across the skin and the resulting transients recorded and analysed. The method is elaborated in the theoretical section.

Methods

Experimental procedure

Pieces of frog (*Rana temporaria* or *Rana pipiens*) abdominal skin were clamped between two Perspex chambers and the skin potential was monitored through electrodes filled with Ringer-agar and inserted through the sides of the chambers. The

closed end of each half chamber contained a platinum electrode equal in area to the skin. These electrodes were covered with platinum black by electroplating with a solution of platinum chloride (3% in 0.025 N hydrochloric acid and containing 0.025% lead acetate). The plating current density was controlled at 30 mA/cm^2 using a platinum electrode as the anode. In all 6.2×10^{-4} equiv/cm^2 of platinum was deposited, because this produces electrodes with minimal polarization impedance (Jones & Bollinger, 1935).

The experimental circuit used is shown in Fig. 1. A square pulse, from a Grass S4 stimulator unit was passed through a large (0.5 mΩ) accurately known resistor in series with the skin and electrodes. The large resistor ensured that constant current pulses were applied to the skin. The voltage transients appearing across the resistor and across the skin plus electrodes were recorded from a Tektronix type 502 dual beam oscilloscope. Because of the potential existing across the skin, current flows through the platinum electrodes to the d.c. coupled amplifier of the oscilloscope and the output stage of the square wave generator, resulting in electrode polarization. This was corrected by applying current from the potentiometer circuit connected in series with the electrodes. The adjustment was correct when no change in position of the oscilloscope trace was seen when the switch was closed. This procedure only affected the potential drop across the skin by about 1 mV.

The polarization impedance of platinum black electrodes is independent of the current passed up to densities of 1 mA/cm^2 at a frequency of 1 kHz (Schwan, 1963). The limiting density, however, decreases towards zero as the frequency falls. In the work described here, current densities never exceeded tens of microamperes/cm^2, well within the limit for frequencies above 1 kHz. Thus when square current pulses were passed across the electrode-skin system the polarization impedance of the electrodes increased with time as the frequency components of the square wave fell to zero. Correspondingly, allowance was made by the substitution method (Schwan, 1963). Transients were recorded across the electrode system in the presence of and then in the absence of skin. The transient obtained in the absence of skin was essentially a square wave the amplitude of which gradually increased by

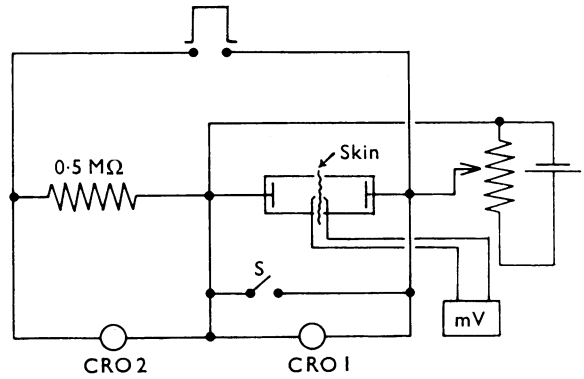

FIG. 1. Diagram illustrating the experimental arrangement. Electrode polarization resulting from the steady skin potential was corrected by the potentiometer circuit shown at the right of the diagram. Correction had been achieved when no deflection of the beam on CRO 1 occurred when the switch, S, was closed. The skin potential was monitored on a Beckman Model 76 expanded scale pH meter.

about 0.1 mV during the pulse as the electrode impedance increased. This " error " was subtracted graphically from the transient obtained in the presence of skin. The interelectrode distance was large (20 cm), so that removal of skin added a negligible amount of bathing solution, and furthermore the tissue was so far removed from the electrodes that it did not affect the electrode impedance.

Frog Ringer solution of the following composition was used throughout and was bubbled with air ; NaCl, 111 mM ; KCl, 2 mM ; $CaCl_2$, 1 mM ; NaH_2PO_4, 0.08 mM ; $NaHCO_3$, 2.4 mM and glucose 11.1 mM. All experiments were carried out at room temperature. In some experiments Ringer solution was replaced with either iso-osmotic sodium isethionate or choline chloride. In all cases 4.5 cm^2 of skin was used and set up in the apparatus for at least 2 hr before any measurements were made. Arginine vasopressin (Pitressin, Parke Davis and Co.) was added only once to the inside solution bathing each skin in a concentration (300 m-u./ml.) capable of producing a maximal effect on sodium transport. This high concentration was necessary because the commercial hormone contains a mixture of arginine and lysine vasopressin. Frog skin is six times less sensitive to the lysine analogue than the arginine-containing hormone, and many times less sensitive to both compared with the natural frog hormone, arginine vasotocin (Walter, Rudinger & Schwartz, 1967). Pitressin (Parke Davis and Co.) also contains chlorbutol as a bacteriostat. Addition of ADH 300 m-u./ml. entailed the simultaneous addition of 5×10^{-4}M chlorbutol. In some experiments pure ADH was used. This was prepared from Pitressin by vacuum distillation at room temperature over a mixture of calcium chloride and wax shavings.

Theoretical

When a current step is applied to a biological specimen the voltage appearing across the specimen is a function of time (Fig. 2a). If the curve shown in Fig. 2a represents a single exponential then the equivalent circuit, Fig. 2b, may be used to represent the biological tissue. The values $R\infty$ and Rp can be calculated from

$$R_\infty = \frac{e_\infty R}{E} \tag{1}$$

$$\text{and } Rp = \frac{e_o R}{E} - R_\infty \tag{2}$$

where R is the accurately known series resistor, and E is the voltage drop across the series resistor. The d.c. capacitance of the tissues can be calculated from the time constant, τ, of the transient from

$$C = \frac{\tau (R_\infty + R + Rp)}{(R_\infty + R)\, Rp} \tag{3}$$

In this scheme $R\infty$ represents the resistance of the electrodes, Ringer solution and cell cytoplasm, while Rp represents the cell membrane resistance, and is an indication of cell permeability. The ion-impermeable, polarizable part of the cell membrane is represented by the capacitance, C (Teorell & Wersäll, 1945). Meaningful values of C are obtained only if the transient is a single exponential. Further information about the polarization element can be obtained from measuring skin impedance at various frequencies. In general, biological tissues show dispersion ; that is, their impedances change from one constant value to another as frequency

changes. Because the steady state of response of a dispersion system as a function of frequency is formally related to the transient response to an applied force as a function of time by a Fourier integral (Cole & Cole, 1941), the dispersion can be extracted from the transient response. This analysis was performed graphically by the method of Teorell (1946), and readers should consult this paper for a full description of the method. The analysis entailed the construction of voltage vector diagrams for various frequencies from the voltage transient. In other words the voltage transient was broken down into its sine wave components. The curved part of the transient was divided vertically into say 1.0 msec intervals (starting with 0.5 msec) as shown in Fig. 3a. The values of y1, y2 – y1, y3 – y2, etc., in mV were

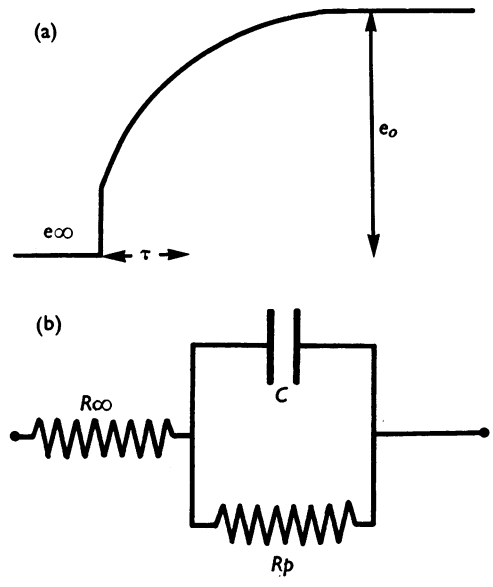

FIG. 2. (a), Voltage transient developed across a biological specimen in response to a current step. (b), Equivalent circuit representing the biological specimen.

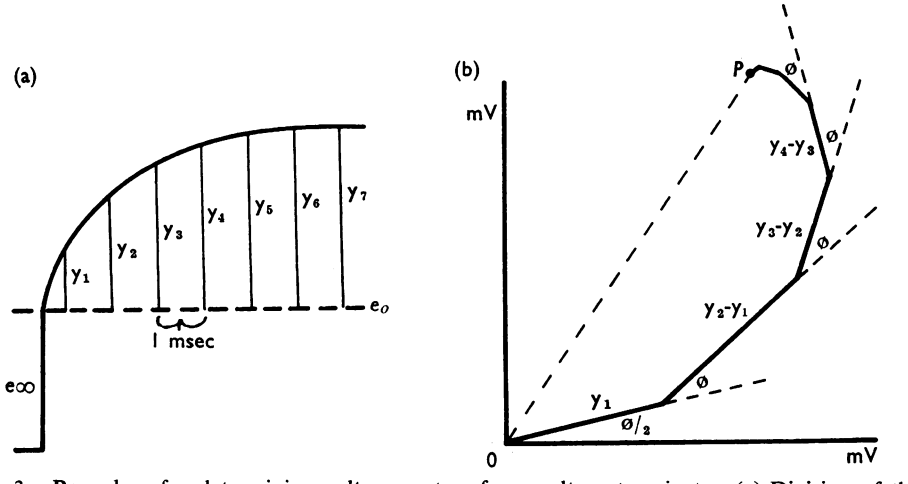

FIG. 3. Procedure for determining voltage vectors from voltage transients. (a) Division of the curved part of the transient into time intervals, frequency t_f=1,000. (b) Vectorial addition to give the voltage vector for a sine wave frequency of f where $f = \frac{\phi \times 1{,}000}{360}$.

tabulated, and then added consecutively end to end, each advancing at an angle ϕ (except the first) in comparison with the preceding value (Fig. 3b). The value ϕ determines the frequency under consideration according to

$$\phi = f/tf \cdot 360^\circ \tag{4}$$

where f is the sine wave frequency and t_f the timing frequency (in this case 1,000). The sum vector *OP* represents the sinusoidal voltage vector for a particular frequency which was then converted to an impedance vector, Z. Other impedance vectors were determined for other sine wave frequencies. These impedances are identical with those which would have been determined by conventional a.c. bridge techniques. The resulting impedance (Z) vectors were separated into their real (R) or resistive and imaginary ($-X$) or reactive components, where

$$Z = R + jX \tag{5}$$

Resistance was plotted versus reactance in the complex plane on an Argand diagram to give the impedance locus.

When the transient response is a single exponential then the impedance locus is described by

$$Z = R_\infty + \frac{(R_o - R_\infty)}{1 + j\omega\tau} \tag{6}$$

This equation describes a relaxation process with a time constant τ, and ω is the angular frequency (Debye, 1929 ; Cole & Cole, 1941). In such cases the impedance locus is a perfect semicircle with its centre on the resistance axis (Fig. 4a). The polarization angle (Teorell & Wersäll, 1945) is in this case zero, that is the phase angle is 90°, and the current–voltage relationship is in perfect quadrature. The polarizable element in such circumstances is described by a simple condenser. When the transient response is not a single exponential the impedance locus is a semicircle with its centre raised above the resistance axis (Fig. 4b). Equation 6 is now required to be of the form

$$Z = R_\infty + \frac{(R_o - R_\infty)}{1 + (j\omega\tau)^{1-\alpha}} \tag{7}$$

where α is the polarization angle and is defined by $^2/_\pi \sin^{-1} (^d/_r)$, where r is the radius of the circle and d is the distance from the real axis. The time constant, τ,

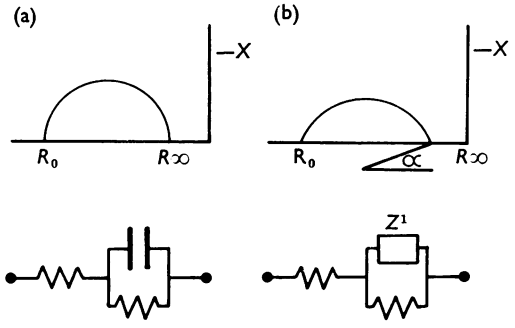

FIG. 4. (a), Impedance locus plotted in the complex plane for a parallel RC network with a single time constant. (b), Impedance locus plotted in the complex plane for a parallel RC network with several time constants. Z^1 represents a frequency dependent impedance of constant phase angle.

now represents an average time constant and the polarizable element can be represented by a frequency dependent impedance (Z^1) of constant phase angle (Schwan, 1957). Theoretical resistance and reactance values can be calculated from the impedance locus constructed from experimental data, thus giving a check on the derived locus. The relevant equations are

$$R = R_\infty + \frac{(R_o - R_\infty)\,[1 + (\omega\tau)^\beta \sin(\alpha\pi/2)]}{1 + 2(\omega\tau)^\beta \sin(\alpha\pi/2) + (\omega\tau)^{2\beta}} \tag{8}$$

$$X = -\frac{(R_o - R_\infty)(\omega\tau)^\beta \cos(\alpha\pi/2)}{1 + 2(\omega\tau)^\beta \sin(\alpha\pi/2) + (\omega\tau)^{2\beta}} \tag{9}$$

where $\beta = (1 - \alpha)$ and τ represents the time constant or average time constant.

Equation 7 can be rewritten

$$Z = R_\infty + (R_o - R_\infty) \int_0^\infty \frac{f(\tau)\,d\tau}{1 + j\omega\tau} \tag{10}$$

if it is assumed that relaxation is the result of a continuous distribution of time constants, where $f(\tau)$ is the distribution function. The time constant distribution function may be derived from equation 11, due to Cole & Cole (1941) and based on an analytical method developed by Fuoss & Kirkwood (1941).

$$f(s) = \frac{\sin(\alpha\pi)}{\cosh[(1 - \alpha)s] - \cos(\alpha\pi)} \tag{11}$$

An impedance the frequency dependence of which is described by equation 7 gives a circle when R is plotted against $-X$. The equivalent series circuit values R and $-X$ can be converted to the equivalent parallel circuit values G (conductance) and C (capacitance) by the formulae

$$G = \frac{R}{R^2 + X^2} \tag{12}$$

$$\omega C = \frac{-X}{R^2 + X^2} \tag{13}$$

Plots of G versus ωC also give semicircles but have not been used here because all the values are clustered at one extreme. Equations (12) and (13) have been used to calculate conductance and capacitance values over various frequency ranges. The average values for conductance and a.c. capacitance are given in Table 1, together with the frequency range over which they were calculated.

Results

Twelve experiments in which the effects of ADH on frog skin were fully analysed are reported here. The relevant data are presented in Table 1.

The results are presented under various headings.

Effects of ADH on the parallel resistance component of the skin

From the theory section it is concluded that the parallel resistance component of frog skin represents the cell membrane resistance, and is therefore an indication of cell membrane permeability. The value of the parallel resistance, *Rp*, has been

worked out from all experiments using equation 2. The changes in *Rp* with ADH for one experiment (303) are shown in Fig. 5.

The skin was allowed to equilibrate for 2 hr after setting up and then transients and potential were recorded at intervals for a further hour. ADH (300 m-u./ml.) was added to the solution bathing the inside of the skin and recording was continued for a further 2 hr. It is shown that ADH reduced the parallel resistance *Rp*, and at the same time caused the skin potential to increase.

Examination of Table 1 will reveal that the hormone always caused a fall in *Rp* with both Pitressin and pure ADH. In all experiments the skin potential increased on treatment with ADH.

Effects of ADH on the series resistance component of frog skin

The series resistance component, R_∞, of frog skin represents the resistance of the cell cytoplasm. This value was determined together with the resistance of the electrodes and Ringer solution, using equation 1. By subtraction of the resistance of the electrodes and bathing solution the value of R_∞ was found to be approximately 10 Ω for 4.5 cm² skin. ADH was without effect on R_∞ (Fig. 5).

Effects of ADH on the d.c. capacitance of the skin

The d.c. capacitance of the skin was calculated from the time constant, τ, of the voltage transient using equation 3. Figure 5 illustrates that ADH caused an increase in the skin d.c. capacitance. Examination of Table 1 will show that the hormone

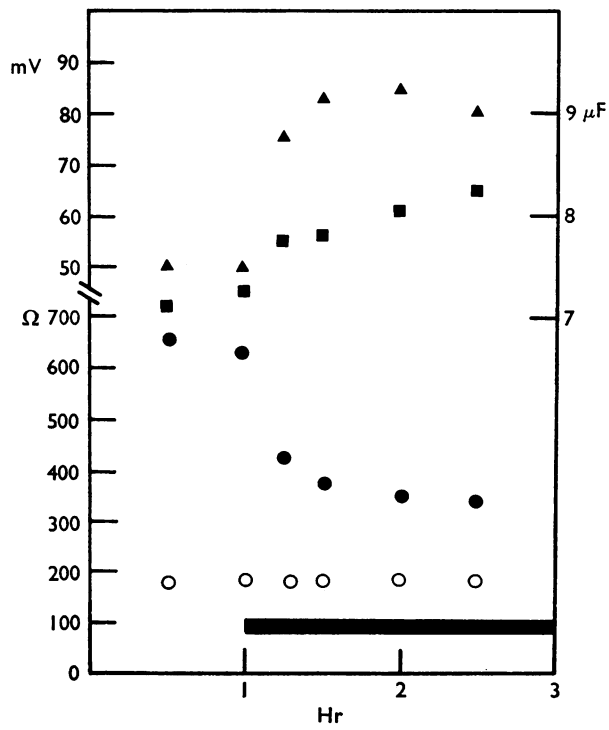

FIG. 5. Changes in *Rp* (●), R_∞ (○), d.c. capacitance (■) and skin potential (▲) for 4.5 cm² frog skin in the presence of ADH (300 m-u./ml.). The presence of the hormone is indicated by the horizontal bar. R_∞ represents the resistance of the electrodes, Ringer solution and cell cytoplasm. The latter had a resistance of 10 Ω.

TABLE 1. *Effects of antidiuretic hormone on the resistance, polarization angle, d.c. capacitance, fundamental frequency, average a.c. conductance and average a.c. capacitance of frog skins*

Date and expt. no.	Condition	*Rp* (Ω cm^2)	% fall in *Rp*	α (degrees)	d.c. capacitance (μF/cm^2)	*fo* (Hz)	*G* (mmho/cm^2)	a.c. capacitance (μF/cm^2)	% increase in a.c. capacitance	Frequency range (Hz)
May '67	Normal	2814		4	1·49	48·2	0·381	0·66		35–276
203	ADH	2278	19·0	12	1·57	54·9	0·653	1·46	121·0	
June '67	Normal	2781		0	1·61	35·6	0·344	1·76		7–55
303	ADH	1746	37·2	0	1·73	54·5	0·555	2·05	16·5	
June '67	Normal	1485		0	3·09	30·2	0·69	3·04		3–112
304	ADH	675	54·5	9	2·53	113·1	1·54	2·82	−7·2	
July '67	Normal	1541		0	1·33	88·6	0·647	1·54		7–139
309	ADH	1294	16·0	9	1·44	95·6	0·789	1·67	8·8	
Jan. '68	Normal	3240		0	1·92	27·6	0·29	1·97		7–55
365	ADH	1776	45·1	8	1·53	64·7	0·58	2·30	17·2	
Jan '68	Normal	2025		0	1·69	49·4	0·48	1·81		7–55
366	ADH	1868	7·8	9	1·74	55·1	0·56	1·95	7·9	
Jan '68	Normal	707		0	1·51	138·0	1·27	1·47		17–139
375	ADH	626	13·4	5·5	1·90	152·0	1·51	1·55	5·4	
Feb '68	Normal	1598		0	1·65	63·9	0·65	1·56		
389	5×10^{-4}M CB	1350	15·5	0	1·56	79·6	0·75	1·55	−0·6	17–139
	ADH	698	56·3	0	1·69	137·7	1·35	1·97	26·3	
Feb '68	Normal	2453		0	2·20	33·2	0·395	2·51		
390	5×10^{-4}M CB	2655	−8·2	0	2·11	31·8	0·382	2·47	−1·6	7–55
	2×10^{-3}M CB	2250	8·2	0	2·00	38·8	0·441	2·28	−9·2	
	ADH	1215	50·4	0	2·48	57·9	0·835	2·77	10·4	
Feb '68	Normal	1361		0	1·72	72·3	0·72	2·12		17–139
394	ADH	878	35·5	0	2·10	88·0	1·13	2·33	9·9	
Feb '68	Normal	855		0	2·36	80·9	1·140	2·46		17–139
396	Pure ADH	563	34·2	0	2·33	120·3	1·676	2·49	1·2	
April '68	Normal	2790		0	1·94	32·0	0·379	1·94		7–55
414	Pure ADH	2160	22·6	0	2·08	38·5	0·474	2·18	12·4	
					Mean value—Before ADH		0·609	1·80		
						After ADH	0·919	2·06		

ADH indicates addition of Pitressin 300 m-u./ml. to inner bathing solution. Pure ADH indicates addition of pure ADH 300 m-u./ml. to inner bathing solution. CB indicates chlorbutol. In calculating averages and for statistical analysis experiment 304 was omitted. Conductance and a.c. capacitance values represent averaged values calculated from equations 12 and 13 for experimental values over the frequency range given in the last column.

caused an increase in the d.c. capacitance of the skin in eleven of the twelve experiments, experiment 304 being an exception. The increase was seen with both Pitressin and pure ADH.

Effects of ADH on the a.c. capacitance and polarization angle of the skin

Calculation of the d.c. capacitance using equation 3 assumes that the transient has a single time constant. Analysis of individual transients for experiment 303 (the experiment illustrated in Fig. 5), obtained both before and after application of ADH, gave impedance loci which were perfect semicircles and suggests that the skin capacitance does behave as a simple condenser. This was not always the case, however, as for example in experiment 309. In this case ADH caused a rise in the d.c. capacitance and skin potential and a fall in skin resistance as before. Two of the transient responses (those recorded immediately before and 30 min after addition of ADH) were analysed to give the impedance loci shown in Fig. 6.

These impedance loci may be characterized by four parameters. These are the resistance at zero and infinite frequency, the polarization angle, α, and the fundamental frequency, f_o. From equation 9 the reactance is minimal when $\omega\tau = 1$, where $\omega = 2\pi f_o$. The difference between the resistance at zero and infinite frequency is Rp,

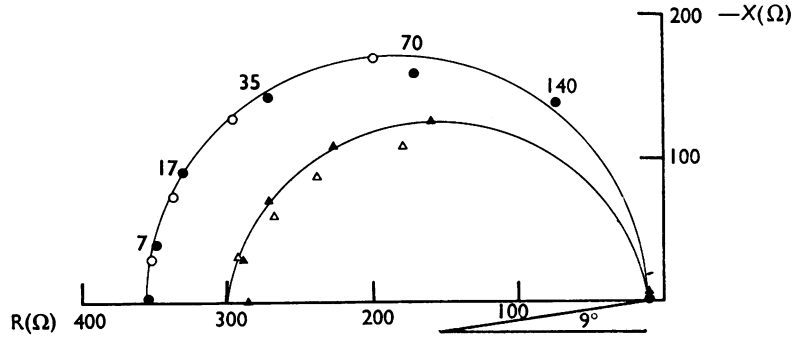

FIG. 6. Impedance loci for experiment 309. The larger semicircle represents 4.5 cm² frog skin. The smaller semicircle represents the same skin after treatment with ADH (300 m-u./ml.). Filled symbols show experimental points, open symbols indicate calculated values (equations 8 and 9). The figures by each point represent frequency (Hz). Before ADH $\tau = 2.07$ msec and after ADH $\tau = 1.76$ msec.

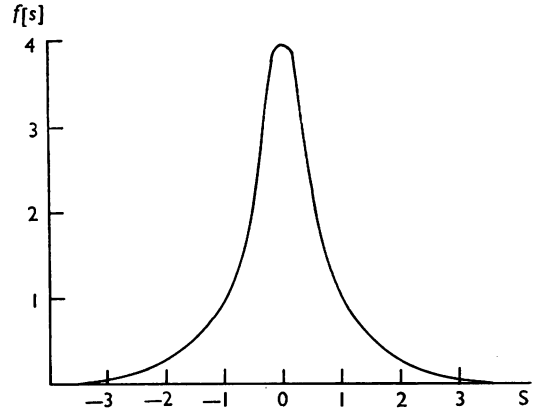

FIG. 7. Diagram showing the distribution of time constants for $\alpha = 9°$. On the abscissa, $s = (\ln \tau - \ln \tau_o)$ is plotted against the function of s calculated from equation 11. It is seen that the incidence of time constants with values differing from τ_o decreases as the difference increases.

the membrane resistance. From the loci the value of Rp fell from 343 Ω to 288 Ω, representing a fall in resistance of 16%. Before ADH the polarization angle was zero, while after ADH it increased to 9°. The fundamental frequency was 88.6 Hz before, and 95.6 Hz after ADH. Thus the skin behaved as if it had a single relaxation time of 2.07 msec before ADH was added. After treatment with hormone the most probable time constant was reduced to 1.76 msec. The distribution of time constants in this latter condition was calculated from equation 11 assuming a Cole–Cole distribution function. The time constant distribution is shown in Fig. 7. Using the determined values for Rp, $R\infty$ and α, and by applying equations 8 and 9 further values of R and $-X$ were calculated, and are shown on Fig. 6. It is seen that, although the calculated values lie fairly well on the experimental loci, their coincidence with experimental points is not good. The calculated points had higher resistance and lower reactance values than anticipated by the experimental results.

It is worth while at this point to consider these discrepancies. Schwan (1957) points out that a semicircle, either without or with a depressed centre, cannot be construed to indicate the validity of equations 6 and 7. Because the theoretical values calculated from equations 8 and 9 depend on α, then the accuracy of the theoretical values will depend on the accuracy with which α is determined. For these calculations α was derived from the transient responses and a small error in measurement of the dielectric displacement gave rise to an error in the estimation of α. There is, however, another reason for supposing that the differences between the experimental and calculated points are real and one which does not involve a consideration of the time constant. This is by considering the distribution of the frequency parameter along the impedance locus. The locus for a simple condenser is a perfect semicircle. The capacity of a simple condenser is constant, so from equation 13 we have

$$2\pi fC = \frac{-X}{R^2 + X^2} \tag{14}$$

Because $R\max/2 = -X$, it follows that

$$2\pi fC = \frac{R\,(100 - R)}{100\,R} \tag{15}$$

where R is the percentage change in resistance between R_o and $R\infty$. A curve relating the dependence of f upon R for a simple condenser is shown in Fig. 8a. The experimental values deviate considerably from the theoretical curve, indicating that equations 6 and 7 only approximately describe the behaviour of the skin. This means that although resting skin has a perfectly semicircular impedance locus the behaviour of the polarizable element is not exactly that of a simple condenser. Brown & Kastella (1965) point out that any function of frequency of the form $j^{1-\alpha}(f\omega)$ could be substituted for the terms $j\omega\tau$ and $(j\omega\tau)^{1-\alpha}$ in equations 6 and 7 and still result in a circular locus. The variations of conductance and capacitance with frequency calculated from equations 14 and 15 for experiment 309 are shown in Fig. 8b. ADH did not always cause the polarization angle, α, to increase. In six of the twelve experiments described in Table I the polarization angle was increased by the hormone. The reasons why α did not always change when ADH was added are discussed later.

Effects of chlorbutol

Commercially available ADH contains chlorbutol, so it was necessary to show if all or part of the effects of Pitressin were due to chlorbutol.

In connexion with other experiments it was noted that chlorbutol, 5×10^{-4}M, occasionally caused a minor increase in skin potential, while higher concentrations reduced the skin potential. These results are reminiscent of those of Skou & Zerahn (1959), who obtained an inhibition of sodium transport when local anaesthetics and butyl alcohol were applied to the inside of the frog skin. Alcohols also cause depolarization in tissues other than frog skin, for example in nerves (Armstrong & Binstock, 1964). Experiments were carried out using exactly the same procedure as before but with the application to the skin of increasing concentrations of chlorbutol instead of ADH. The results of these experiments are shown in Table 1 (experiments 389 and 390). In these experiments chlorbutol produced a minor change in *Rp* and *G* compared with the subsequent change caused by ADH, even when a chlorbutol concentration of 2.0×10^{-3}M was used. In contrast to ADH, chlorbutol caused a fall in skin capacitance, indicating that the increase in capacitance seen with ADH was a hormone effect. Impedance loci for experiment 390 are shown in Fig. 9.

Comparison of Pitressin with pure ADH

Two experiments (numbers 396 and 414) were carried out using pure ADH. Qualitatively the results were the same as those obtained with either Pitressin or Pitressin in the presence of extra chlorbutol. It can be concluded that a decrease

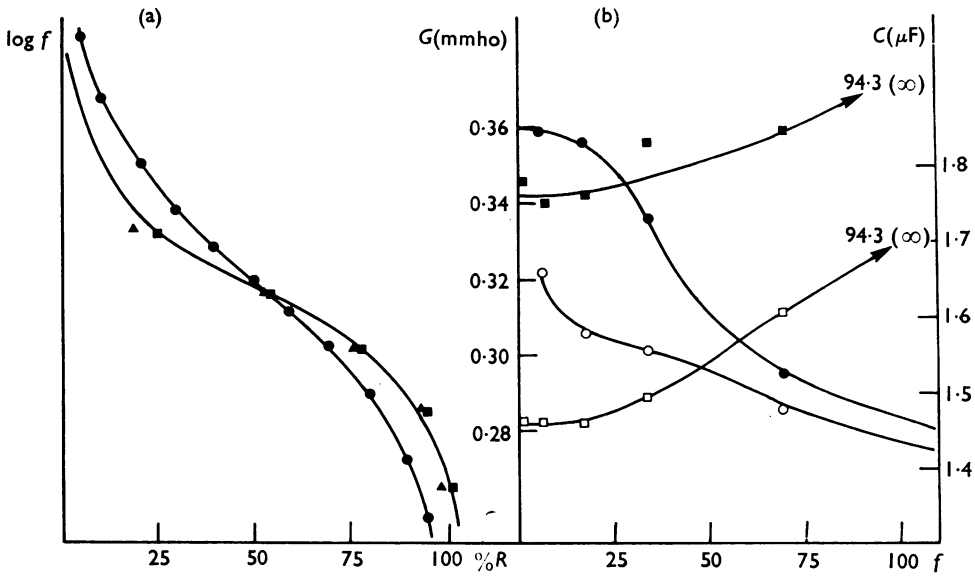

FIG. 8. (a), Curves relating the dependence of f on R. Circles (●) represent values for a simple parallel RC network. Squares (■) and triangles (▲) represent experimental values for experiment 309 obtained before and after treatment with ADH respectively. The frequency scale was normalized for each condition. (b), The variation in conductance and capacitance with frequency for experiment 309. Values for 1 cm² of skin. Circles represent capacity and squares represent conductance. Open symbols represent conditions before ADH application and closed symbols after ADH application.

in *Rp*, an increase in skin capacitance and no change in R_s are the characteristic responses due to ADH. Chlorbutol produced only minor changes in *Rp* but consistently decreased the skin capacitance. Capacitance increases due to Pitressin are therefore detected in spite of the decrease in capacitance due to the chlorbutol present. Only in one experiment (number 304) did Pitressin fail to increase skin capacitance. In this experiment the skin capacitance initially was abnormally high. The resulting fall in capacitance with Pitressin may have been due to an abnormally large chlorbutol response which outweighed the rise due to the hormone. Figure 10 shows impedance loci for an experiment in which pure ADH was used. This result should be compared with the results shown in Figs. 6 and 9.

Replacement of chloride by isethionate

As will become apparent in the discussion that follows, it was necessary to know what part, if any, of the relaxation phenomena already described for the skin might be due to the movement of ions in or around the cells. It is well known that ADH affects the permeability of frog skin to sodium ions, and for this reason frog skin was bathed in solutions in which either sodium or chloride was replaced. This section reports on the effects of bathing skins in iso-osmotic sodium isethionate.

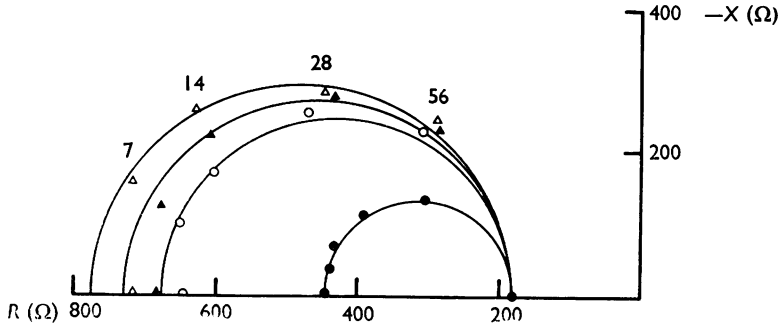

FIG. 9. Impedance loci for experiment 390. Values are for 4.5 cm² skin. Note in this and subsequent figures the resistance at infinite frequency includes the electrode and Ringer solution resistance as well as the cytoplasm resistance. The loci represent (▲) normal skin, (△) with chlorbutol 5×10^{-4}M, (○) with chlorbutol 2×10^{-3}M and (●) with ADH 300 m-u./ml. plus chlorbutol 2.5×10^{-3}M. Polarization angle constant at zero.

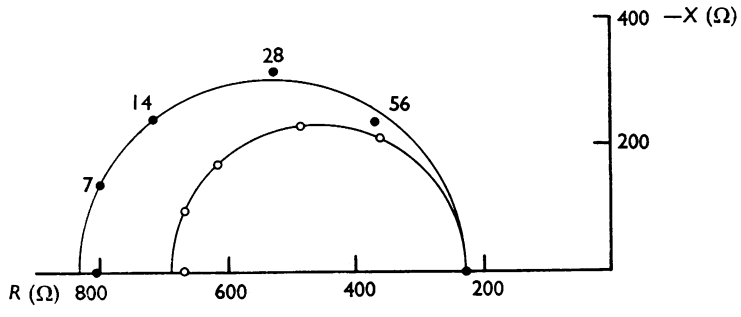

FIG. 10. Impedance loci for experiment 414. Values are for 4.5 cm² skin. The loci represent the skin before (●) and after (○) treatment with pure ADH 300 m-u./ml. Polarization angle constant at zero.

The procedure was as before, skins being mounted in the atypical solutions from the start of the experiment.

When skins were bathed in iso-osmotic sodium isethionate the membrane resistance, *Rp*, never became steady, even when 3 hr were allowed for equilibration. The value of *Rp* fell steadily throughout the experiment and if the rate of fall was great then an increased rate of fall due to ADH was difficult or impossible to detect. Thus in only those experiments in which the rate of fall of *Rp* was relatively slow could it be decided whether ADH had reduced the membrane resistance, *Rp*. Two decisive results with sodium isethionate are described here. In the first, ADH clearly increased the rate of fall of *Rp* and at the same time the polarization angle and skin capacitance increased. The fall in *Rp*, both throughout the experiment and the accelerated fall due to ADH, are shown in Fig. 11b while Fig. 11a shows the impedance loci. This result is qualitatively the same as those obtained in normal Ringer solution. In the experiment just described the skin potential fell from 80 to 10 mV in 2 hr before readings were commenced and during the experiment the potential fell further to 6 mV. Because of this another experiment in which the skin was voltage-clamped at its initial potential was tried. The potentio-

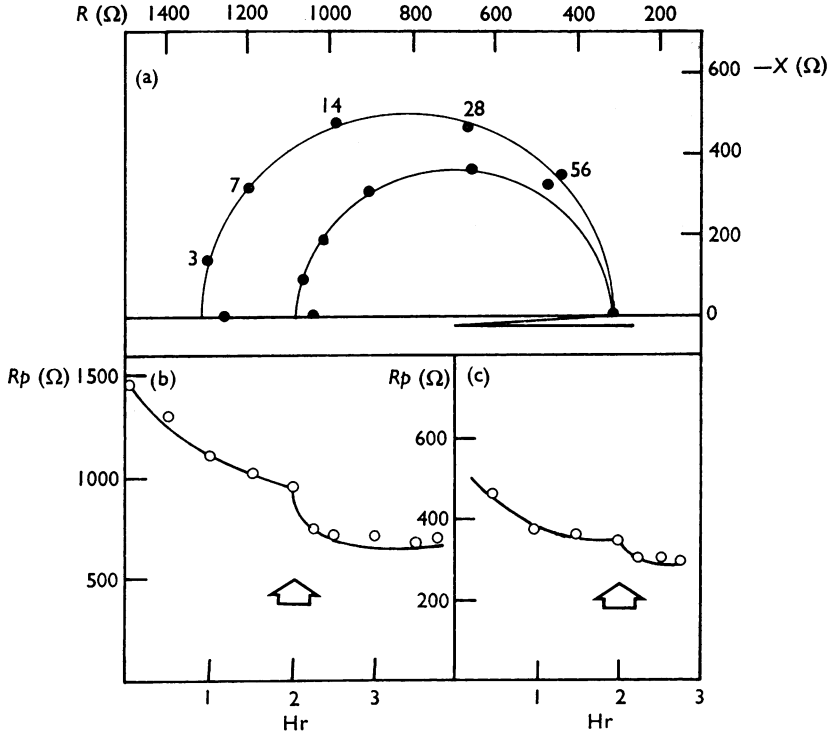

FIG. 11. (a), Impedance loci from the same experiment as (b). The larger locus represents the behaviour of the skin immediately before ADH was added. The smaller locus represents the transient obtained 30 min after addition of ADH. The value of *Rp* fell 22% on the addition of ADH, and the polarization angle increased 3.5°. The average a.c. capacitance of the skin increased from 7.24 μF to 7.91 μF over the range 3–56 Hz. (b), Change in *Rp* of 4.5 cm^2 frog skin bathed in iso-osmotic sodium isethionate. At the arrow ADH (300 m-u./ml.) was added to the inside of the skin. (c), Change in *Rp* of 4.5 cm^2 frog skin bathed in iso-osmotic sodium isethionate and voltage clamped at 41 mV (outside negative). At the arrow ADH (300 m-u./ml.) was added to the inside of the skin.

meter circuit shown in Fig. 1 was used to pass sufficient current to maintain the skin potential at the selected value. Addition of ADH again caused a fall in *Rp* as shown in Fig. 11c. In the voltage clamp experiment no attempt was made to calculate impedance loci. Because of electrode polarization the transients were recorded through an RC coupling. When corrected, these transients gave satisfactory

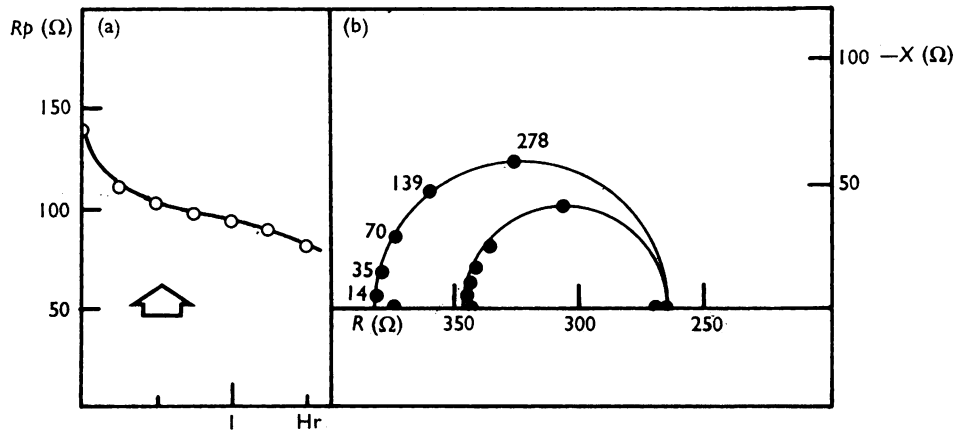

FIG. 12. (a), Change in *Rp* of 4.5 cm² frog skin bathed in iso-osmotic choline chloride. At the arrow ADH (300 m-u./ml.) was added to the inside of the skin. (b), Impedance loci from the same experiment as (a). The larger locus was determined immediately before adding ADH. The small locus was determined 30 min after adding ADH. No change in polarization angle was found. f_o remained the same, within experimental error. Calculation of the d.c. capacitance from $C=\frac{1}{2\pi f_o R}$ showed that it rose from 5 μF to 7.2 μF for 4.5 cm² skin after ADH. The average a.c. capacitance over the range 14 to 278 Hz was 4.91 μF before ADH to 6.75 μF after ADH.

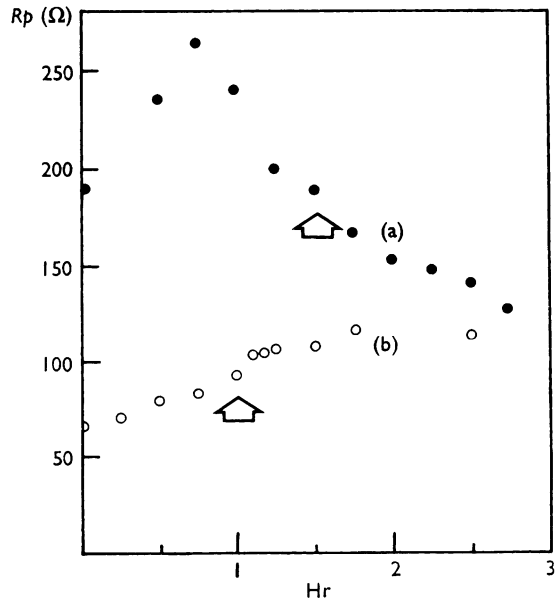

FIG. 13. (a), Change in *Rp* of 4.5 cm² frog skin bathed in iso-osmotic choline chloride. At the arrow ADH (300 m-u./ml.) was added to the inside of the skin. The skin potential was clamped at 70 mV (outside negative) throughout the experiment. (b), As in (a) but with skin bathed in choline-Ringer.

Rp values; however, the shapes of the transients were so modified that accurate impedance values for the skins could not be extracted.

Replacement of sodium by choline

When isotonic choline chloride was used as the bathing solution the situation was more complex. Not only did the membrane resistance fail to stabilize but the skin potential fell immediately to zero. ADH caused no accelerated fall in *Rp* in these conditions, as reference to Fig. 12a will show, although the skin capacitance still increased. In other experiments the skin potential was clamped at 70 mV (outside negative) immediately after setting up in the apparatus. The value of the parallel resistance, *Rp*, initially increased and then diminished in these conditions. Addition of ADH to these preparations did not alter the rate of rise or fall of *Rp* with time (Fig. 13). In further experiments the skin was mounted in choline-Ringer (sodium chloride replaced with choline chloride and other sodium salts replaced by potassium salts) because ADH is known to increase osmotic water flow across the skin in choline-Ringer (Hays & Leaf, 1962). In this case the value of *Rp* remained steady, although at a considerably lower value than for most skins in normal solution. Addition of ADH did not change this steady *Rp* value (Fig. 13).

Before the results in this paper can be discussed it is necessary to know whether or not the frog skin shows rectification, and whether the transient response is developed across the outer or inner facing membranes of the skin.

Absence of rectification

The presence or absence of rectification in the skin was tested by altering the polarity of the pulse across the skin. Examination of Fig. 14 shows that, both before and after ADH, transients obtained with either polarity were superimposable.

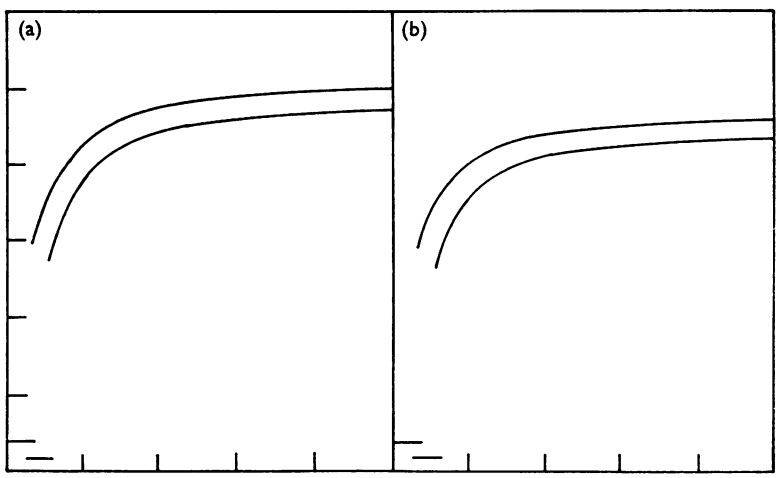

FIG. 14. (a) and (b), Uncorrected pairs of transients traced from oscilloscope records. The upper trace in each case is the transient obtained when a positive current pulse was passed from the inside to the outside of the skin while the lower trace was for a current pulse of the opposite polarity. (a), Transients recorded before ADH. (b), Transients recorded 40 min after addition of ADH (300 m-u./ml.). In this experiment ADH caused a 13% fall in *Rp*, and the polarization angle changed from 0° to 6°. Horizontal calibrations are 5 msec, vertical calibrations are 1 mV.

Thus it is clear that no overall rectification occurs when pulses are passed across the skin. Similarly the impedance loci must of necessity be identical since they are derived from the transients.

Location of the ADH effect at the outer membranes

It has been shown that the transient response of the skin to a current pulse is affected in both size and shape by addition of ADH, and it was therefore important to discover the origin of the voltage transients. To do this a modification of the previously described technique was used. Pieces of skin larger than normal were clamped between the Perspex chambers and the excess was allowed to hang down into a vessel containing saturated potassium chloride solution. An electrode placed in this solution is in electrical connexion with the cell contents between the inner and outer facing membranes (Steinbach, 1967). Current pulses were passed in the normal manner but the transients were measured (*a*) across the whole skin, (*b*) between the outward facing membranes and the cell interiors and (*c*) between the inward facing membranes and the cell interiors. Examples of these three types of transients are shown in Fig. 15. Examination of these shows that the algebraic sum of the potentials developed across the inner and outer membranes equals the potential developed across the whole skin. More important, structures responsible for the transmembrane transient of the skin are located mainly in the outer facing membranes. The contribution of the inner membrane to the total transient response is small. From the transients shown in Fig. 15, rough calculation shows the value of Rp for the inner and outer membranes is 70 Ω and 560 Ω respectively. The time constant of both the inner and outer transients is approximately 5 msec, indicating a d.c. capacitance of approximately 70 μF and 10 μF, or 16 μF/cm^2 and 2 μF/cm^2 for the inner and outer membranes. When the transient for the whole skin is

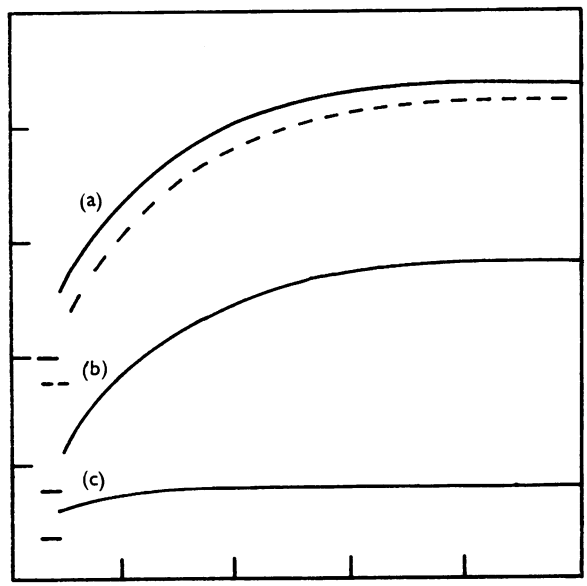

FIG. 15. Frog skin transients in response to constant current pulses passed across the skin. Transient (a) was measured across the whole skin, transient (b) across the outer facing membranes, transient (c) across the inner facing membranes. The dotted transient was constructed by adding (b) and (c). Horizontal calibrations are 5 msec and vertical calibrations are 5 mV.

considered $Rp = 630\ \Omega$, $\tau \simeq 5$ msec and the d.c. capacitance is somewhat less than 2 $\mu F/cm^2$. It is clear that when the transient across the whole skin is considered, then the properties of polarizable elements mainly in the outer facing membranes are measured. Routine recordings of transients across the inner and outer membranes were not made, neither were these subjected to detailed analysis because of various imponderables concerning the tissue flap through which contact with the intracellular regions of the cells was made.

Discussion

Impedance loci have been used extensively to characterize the frequency-dependent passive electrical properties of biological materials. The α-dispersion, that is the frequency-dependent electrical properties over the d.c. to 10 kHz range, describes the properties of biological cell complexes (Schwan, 1963). In most investigations a.c. methods, using conventional a.c. bridge techniques, have been used, as for example in the study on guinea-pig amniotic membranes (Silver, Strauss & Misrahy, 1965), and on frog skin (Brown & Kastella, 1965), and on frog gastric mucosa (Teorell & Wersäll, 1945). Rarely has the effects of drugs on dispersion phenomena in biological tissues been investigated.

It is evident, by comparison with the literature cited, that impedance loci determined by the a.c. method are superior to those obtained by the instantaneous impedance method. This latter method, however, has a unique advantage in that the time required to record a transient is very short, whereas a complete run of measurements using a.c. methods may take many minutes. Rapidly developing drug effects cannot therefore be usefully investigated other than by the method used here. The reason for the inferiority of the d.c. method is due undoubtedly to problems associated with electrode polarization. In these experiments electrode polarization has been partially corrected by the substitution method. The electrode impedance, even at low current densities, increases as the frequencies of the applied voltage tend to zero. When the skin is present the potential difference across the electrode-tissue system takes some tens of milliseconds to become constant, while in the absence of tissue the transient only lasts a few microseconds. This means that the increase in electrode impedance due to polarization is greater in the absence of tissue than in its presence. Consequently the skin transients are over-corrected for electrode polarization. This is then responsible for the deviation of the experimental points from the semicircular locus at low frequency (see Figs. 9 and 11). A second source of error in the d.c. method is due to the redistribution of ions. In the initial instantaneous phase of the transient the polarizable element of the skin is a short circuit and the potential drop occurs across R_∞. The subsequent phase can be regarded as a charging process and describes the time course of development of a potential of $e_o - e_\infty$ across the polarizable and resistive elements. When charging is complete any further current causes a redistribution of ions in and around the membrane, and consequently alters the potential across the membrane. It was easy to recognize the redistribution phenomenon on the transients because of the exceedingly long "time constants" (seconds, or even minutes). Redistribution of ions following completion of charging in membranes has been considered theoretically by Cole (1965) and practically, for gastric mucosa, by Rehm (1967).

Consideration must next be made of what part of the frog skin is responsible for the transients recorded under the conditions of these experiments. The major

part of the skin resistance must be located in the other facing membranes, for the potential drop across these membranes is approximately eight times that occurring across the inner facing membranes (Fig. 15). This does not mean, however, that the specific resistance of these membranes is necessarily so different, for it will depend on the cell geometry. If the view, developed by Ussing and Windhager (1964) of the frog skin epithelium is correct then it is clear that current flows into the skin first through the outer facing membranes of the more superficial cell layers, then into the cells and then into the intercellular spaces which communicate rather freely with the inner bathing solution. This picture of the frog skin is confirmed by electron-microscopic studies by Farquhar & Palade (1963). It is necessary that the intercellular spaces in the outer layers of the skin be obliterated because otherwise they would act as a low resistance shunt. The zonulae occludentes seen with the electron microscope seem to perform this function. In a recent study on frog skin using microelectrodes, Lindemann & Thorns (1967) also came to the conclusion that the major part of the transepithelial resting resistance was located in the outermost border of the tissue. These workers were investigating the fast spike potential of frog skin generated when inward, but not outward, current flows through the skin. The current densities required to produce this effect are far greater than those used in this work, where it is shown that skin does not have rectifying properties. It is concluded therefore that the outermost outward-facing membranes of the skin present the major resistance barrier and that this resistance can be reduced with antidiuretic hormone.

The apparent capacitance of the inner facing membranes was high (10 $\mu F/cm^2$). This value too will be incorrect if the geometry discussed previously applies. In their paper on frog skin, Brown & Kastella (1965) calculated the dimensions of the capacitative element assuming a parallel plate condenser. The average value of 1.8 $\mu F/cm^2$ noted in this work gives a value of approximately $4k \times 10^{-10}$ m for the thickness of the dielectric layer, where k is the dielectric constant. The value of this latter is probably about 3 (lipids), giving a thickness of 12×10^{-10} m. The average conductance of the untreated skin was 0.61 $mmho/cm^2$.

It is interesting to compare the properties of lipid bilayers with the values obtained for frog skin. Lipid bilayers have thicknesses of 45 to 90×10^{-10} m, capacitances of 0.38–1.0 $\mu F/cm^2$ and conductances of 10^{-3} to 10^{-6} $mmho/cm^2$. In addition they have a constant capacity over a wide frequency range (for references, see Cuthbert, 1967). The thickness of the polarizable element in frog skin from these results is less than the thickness of a single cell membrane. Hanai, Haydon & Taylor (1965a), however, showed that capacitance of lecithin bilayers was determined only by the hydrocarbon chains. They (Hanai, Haydon & Taylor, 1965b) also showed that the capacitance of lecithin bilayers was not altered by adsorption of protein. It is clear that membrane thickness determined from capacitance measurements may be considerably thinner than the actual membrane thickness. Addition of cholesterol to lipid bilayers of egg lecithin caused an increase in capacitance due to thinning of the membranes (47 to 35×10^{-10} m) and an increase in the dielectric constant of the hydrocarbon chains (Hanai *et al.*, 1965b). Cholesterol is normally present in cell membranes, so it partly accounts for the high capacitance of cell membranes as compared with lipid bilayers. The actual cell membrane area in frog skin will be greater than the skin area since the cells bulge slightly. This factor would increase the estimated membrane thickness. It is concluded that the transient responses

obtained in these experiments probably represent the dielectric displacement across a single cell membrane located in the outer surface of the skin. The estimated thickness of this membrane is low for the reasons discussed.

It is relevant at this point to discuss why the frog skin should show α-dispersion before the changes in the dispersion caused by ADH are examined. Schwan (1957) points out that it is not possible to come to a definite conclusion because the nature of membrane conductance is unknown. If conductance is controlled by a gating mechanism then the frequency dependance of the impedance represents the electrical phase shift between the applied alternating potential and the current penetrating the gate. In this model the membrane capacitance proper may be frequency independent. Alternatively, if membrane conductance represents a dielectric loss conductance, dielectric dispersion could result from the series arrangement of two layers, each of which may have frequency independent characteristics. This view supports the " sandwich " type structure of cell membranes. Finally dispersion may result from counterion movement in relation to charged particles on the cell membrane, that is relaxation of the electric double layer. Fricke & Curtis (1937) have shown that the impedance of the electrical double layer is frequency dependent.

The effects of ADH on frog skin are an increase in conductance and skin capacitance, often with an increase in the polarization angle. Our early results tempted us to suggest in a preliminary communication (Cuthbert & Painter, 1968b) that the increase in polarization angle was obligatory for ADH action. Subsequent experiments have indicated that the polarization angle does not always increase (Table 1). Other workers have not consistently obtained polarization angles of zero in resting frog skin. For instance Teorell (1946) obtained values of between 0° and 5° whereas Brown and Kastella (1965) obtained values of 4°–21°. In only one experiment did we have a polarization angle greater than zero in the resting condition (4°, experiment 203). We suggest that our results are due to the considerable time allowed for equilibration of the skins before recording was commenced, so that endogenous hormone levels were very low. It seems clear that the increase in polarization angle caused on occasion by ADH is a property of the system rather than a fundamental expression of a drug effect. The presence of a polarization angle indicates that the polarizable elements in the skin do not have a single time constant, but a distribution of time constants. If the capacitor in the equivalent circuit of Fig. 2b is replaced by an infinite number of series RC elements in parallel, then this modified circuit has a frequency dependent impedance with a constant phase angle, each additional RC element representing a particular time constant. A sheet of epithelial cells, each responding differently in extent from its neighbours, may be the morphological correlate of the phase angle. Gatzy & Clarkson (1965) showed that the mucosal membranes of a small fraction of toad bladder epithelial cells were more permeable than others. The failure to observe changes in polarization angle with ADH in more recent experiments is believed due to seasonal variation. Frog skin responses to ADH over the period February–March are poor, that is during the period following awakening from hibernation. When the responses are poor then the differences between cells will be small.

Conceptually the easiest way of explaining the increase in conductance of the skin with ADH is to propose the appearance of pores, holes or gates within the normally ion impermeable structures. These sites would need to be selectively

permeable to sodium ions, for no increase in conductance was seen with ADH when skins were bathed in iso-osmotic choline chloride, whereas this did occur if iso-osmotic sodium isethionate was used. There is no apparent reason why the hormone should not induce the conformational changes in the membrane, resulting in the opening of pores, in the absence of sodium ions. If these gates have high specificity, however, then no increase in conductance should result, as will be the case when the skin is bathed in iso-osmotic choline chloride. It should be mentioned that the skin impedance in choline chloride was much less than in Ringer solution, whereas Brown & Kastella (1963) found it was increased. This is probably a result of their impedance measurements being made after changing to choline-Ringer solution from normal Ringer. In these experiments the tissues had been immersed in choline chloride solution for several hours before impedances were measured. Koblick (1959) showed that frog skin was permeable to choline, so high conductances in choline chloride are not unexpected. Table 1 gives the average a.c. capacitances over the various frequency ranges investigated. On the whole these values are similar to the d.c. capacitances, although the errors involved in estimating these latter were considerable. The only atypical result was for experiment 304 in which the capacitance was initially very high. The fall in capacitance caused by ADH in this case may be due to a large chlorbutol response. The average a.c. capacities before ADH was 1.80 μF/cm^2, rising to 2.06 μF/cm^2 after ADH. Although the initial capacitance of the skins varied widely analysis showed that the increase in capacitance due to ADH was significant, $P=0.0025$.

If the increase in capacitance was due to the appearance of water-filled polar pores in regions of the membrane where none previously existed then regions of low dielectric constant (3 to 5) are replaced with regions of high dielectric constant (80). It can be shown that 0.3% of the total skin area must be occupied by such pores, assuming the skin thickness calculated earlier, to account for the capacitance increase caused by ADH. If the conductance within these pores was of the same order as in Ringer solution then the conductance change caused by ADH was too small by several orders. This is not necessarily a problem because the effective conductance within the pores is probably several orders smaller than in Ringer solution since (*a*) the pores are sodium permselective and (*b*) the anionic groups conferring the permselectivity polarize water molecules in their vicinity. Because the dielectric constant of ice-like water (88) is similar to normal water (80), the pores formed in response to ADH would contribute fully to the increased capacitance, whereas only a small conductance increase would be seen. Also in the absence of sodium ions ADH should still increase skin capacitance without affecting skin conductance. This was found to be so with skins bathed in isotonic choline chloride. ADH also increases water flow down an osmotic gradient across frog skin. This may be due to the coalescence of small water-filled pores into larger ones (see Leaf, 1964) which then allows viscous flow. If this is so then the action of ADH on the barrier to water flow will not affect the skin capacitance. Alternatively the increase in skin capacitance due to ADH may be partly due to the formation of additional pores in the barrier to water flow.

In summary it is concluded that the resting skin behaves as a parallel RC network with a single time constant. After treatment with ADH water-filled sodium permselective pores are formed in the outermost membranes which occupy no more than 0.3% of the surface area of the skin. These pores constitute a parallel, and

therefore additive capacitance, with the normally ion impermeable parts of the membrane. In addition the pores increase the skin conductance. In some skins, after ADH, the tissue behaves as a frequency-dependent impedance with a constant phase angle paralleled by a resistor. This is considered a consequence of the polydisperse nature of the skin after hormone treatment.

One of us (E. P.) was in receipt of an M.R.C. scholarship for training in research methods.

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