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Aldosterone-Induced Moulting in Amphibian Skin and its Effect on Electrical Capacitance

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Summary. The resistance and capacitance of the isolated amphibian skin have been determined from measurements of the response of the voltage across the skin to small steps of current. Previous work indicates that the electrical impedance of frog skin, when the skin is bathed with Ringer's solution on both sides, is largely determined by the properties of the functional outward-facing membrane of the skin, the outer membrane of the stratum granulosum (P. G. Smith, 1971. *Acta Physiol. Scand.* **81**:355). This membrane can be represented by a resistance and capacitance in parallel. Aldosterone, which induces conversion of the s. granulosum into a cornified cell layer and transformation of the cell layer below into a new s. granulosum, also causes a transient rise in resistance and a short-lived decrease in capacitance to about one-half its initial value. It is suggested that these electrical changes are caused by the transitory presence of two functional outward-facing membranes in series. The method of determining resistance and capacitance from the voltage responses is discussed in the Appendix.

Application of the hormone aldosterone to the isolated frog skin induces a series of changes in the layers of cells which make up the epithelium. The stratum corneum, the outermost, dead, cell layer, is shed; the underlying layer of cells, the stratum granulosum, cornifies and becomes the new s. corneum; and the layer of cells below that takes over the functions of the s. granulosum [19, 27, 28]. Transport properties of the skin are also affected. About 2 hr after addition of the hormone the electrical potential difference (p.d.), short-circuit current (SCC) and sodium net flux begin to fall and resistance increases. The changes continue during this "inhibition period" until at about 5 hr after addition of aldosterone there are sharp rises in p.d., SCC and sodium net flux and the resistance falls; shedding of the s. corneum occurs at this time. During the period after shedding p.d. and SCC are higher than in control skins, and this has been termed the activation period. Aldosterone has similar, but not identical, effects on the isolated toad skin [12–14].

The experiments reported here were carried out with a view to elucidating the cause of the increase in resistance after application of aldosterone

to the isolated amphibian skin. With Ringer's solution bathing both sides of the skin the resistance and capacitance are largely determined by the properties of the functional outward-facing membrane of the skin, the outer cell membrane of the s. granulosum [18, 22]. This membrane behaves essentially as a resistance and capacitance in parallel. During the action of aldosterone, the identity of the functional outward-facing membrane changes from the outer membrane of the old s. granulosum to the outer membrane of the new s. granulosum. If, for a short time, the outer membranes of both old and new s. granulosa were intact there would in effect be two functional outward-facing membranes in series; the increase in skin resistance would then be accounted for, the total resistance of two resistors in series being the sum of the two. Such an arrangement would also imply the existence of two membrane capacitances in series; a transitory series arrangement of two similar capacitances would cause a short-lived decrease in total capacitance to one-half its initial value. An approximate halving of skin capacitance, occurring at the same time as the resistance increase, is therefore predicted.

The measurements of capacitance reported here show that such a decrease does indeed occur during the action of aldosterone.

Materials and Methods

Physiological

Except in one experiment noted below, frogs (*Rana temporaria*) or toads (*Bufo bufo*) were kept in the dark at 4 °C for several days before the experiment. The animals were in shallow artificial pond water (Na 1.0, Ca 1.0, K 0.1, Cl 3.1 mM); this was found to give skins with higher and more stable p.d.'s than Liverpool tap water, which contains lower salt concentrations. Belly skins were mounted in a double-chamber apparatus similar to that described previously [22] giving an exposed area of 7.1 cm^2. Ringer's solution (Na 113.6, K 2.0, Ca 1.0, HCO_3 2.4, Cl 115.2 mM) bathed both sides of the skin and was circulated by air bubbling. An equilibration period of at least 45 min was allowed before capacitance measurements were made. In some experiments the skin was short-circuited throughout, current being passed between two Ringer's-agar salt bridges. After one or two initial measurements of capacitance, aldosterone (Aldocorten, CIBA) was added to the solution on both sides of the skin to give a concentration of 6×10^{-6} M in the frog-skin experiments and 6×10^{-7} or 6×10^{-8} M in the toad-skin experiments. Further measurements of capacitance were made at intervals of $1\frac{1}{2}-2$ hr, or at shorter intervals when the p.d. or SCC was close to its minimum.

Measurement of Capacitance

The equivalent circuit of a preparation such as the amphibian skin is shown in Fig. 1 [4]. The electrical properties can be characterized by the series resistance r_1, the parallel

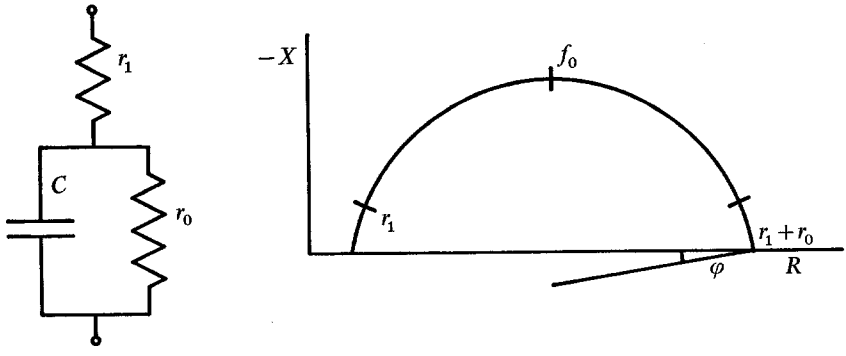

Fig. 1. Equivalent electrical circuit of the frog skin, when bathed with Ringer's solution on both sides; and the impedance locus, a graph of negative reactive component $-X$ against resistive component R. If φ is measured in degrees, $\alpha = \varphi/90$. Bars on the locus are at the frequency f_0 at which $-X$ is a maximum, at $f_0/10$ (right-hand side) and at $10 f_0$ (left-hand side)

resistance r_0 and the (nonideal) capacitance C. A further parameter, α, describes the departure of the capacitance form ideality; $\alpha = 0$ for an ideal capacitance. For the amphibian skin r_0 and C appear to be characteristics of the outward-facing membrane; r_1 is not in parallel with a capacitance and is therefore unlikely to be associated with cell membranes, but is composed of the resistances of the corium, s. corneum, and cytoplasm of the epithelial cells.

The capacitance of a biological membrane, being nonideal, is not so easy to measure as the capacitance of an electrical component. In some previous studies on frog skin [2, 22, 23] the capacitance was determined from the impedance locus, constructed from measurements of skin impedance over a wide range of frequencies (Fig. 1). To obtain a single value of capacitance using that technique it is necessary to measure impedance at several frequencies, taking about 15 min for the range of frequencies of interest here. The method cannot therefore be used when the values of the circuit parameters may be changing over this length of time, as is the case after addition of aldosterone. In these experiments, therefore, the capacitance was determined from the response of the p.d. across the skin to the passage of a step of current [5, 6, 24] which can be measured much more quickly.

A pulse of current of accurate square-wave shape was passed across the skin. The current electrodes were discs of platinum coated with platinum black. The resulting change in p.d. as a function of time was measured via silver-silver chloride electrodes in the solutions on the two sides of the skin. The use of such electrodes, which had a low impedance (no more than 1 kΩ) and therefore generated little electrical noise, allowed accurate measurements to be made of small voltage responses. Current strength was adjusted to give a final p.d. change of 3–4 mV and was of the order of 1 μA/cm^2; responses to both inward and outward current were always recorded. The p.d. was displayed directly on one channel of a dual-beam storage oscilloscope (Tektronix 5103N) and current, measured as the p.d. across a 100-Ω resistance, was displayed on the other oscilloscope channel. The traces were photographed so that the variation of p.d. with time could be determined at leisure; such a photograph is shown in Fig. 2.

For the circuit shown in Fig. 1 with $\alpha = 0$ the response of voltage V_t to a step of current I_0 is given by $V_t = I_0 r_1 + I_0 r_0 [1 - \exp(-t/r_0 C)]$, where t is time; if the capacitance is nonideal, the response is modified (Fig. 5). The nonideality of the capacitance means

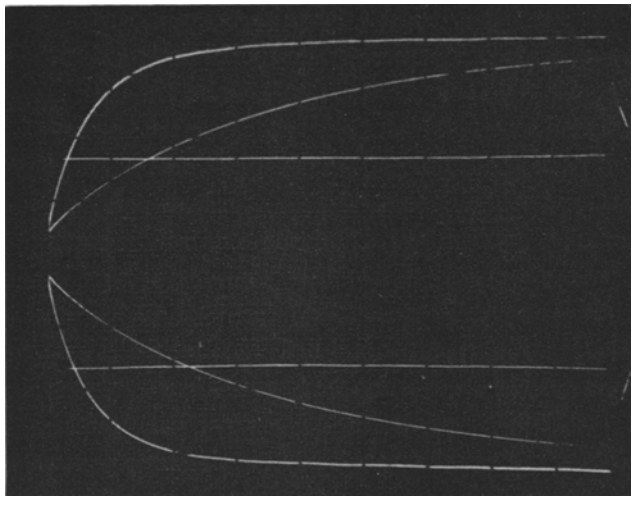

Fig. 2. Response of voltage across the isolated frog skin (curved traces) to passage of a step of current (horizontal traces). Responses to both inward current (lower traces) and outward current (upper traces) are shown. Vertical calibration: 5 μA, 1 mV. Horizontal calibration: 1 msec, 5 msec (responses were recorded at two sweep rates). The initial part of the current trace is not apparent on this photograph but was seen to begin at zero time on others taken under the same experimental conditions

that a lengthy analysis is required to determine r_0 and C. Further details of the analysis are given in the Appendix.

Determinations of resistance and capacitance by both methods on other skins showed a close agreement between the two.

Results

Effect of Aldosterone on p.d. and SCC

In 13 out of 15 experiments on frog skin the characteristic changes in p.d. or SCC described by Nielsen [19] and Eigler [8] were observed. In one of the two anomalous experiments the animal had been kept in the light until a few hours before the experiment; aldosterone had no effect on this skin, in agreement with the finding of Nutbourne (in ref. [20]). The other anomalous experiment was carried out on the skin of a gravid female; again aldosterone had no effect. This may be related to the known antagonism between aldosterone and progesterone (for references *see* [25]), progesterone levels possibly being elevated in gravid amphibia [21]. However, the skin from another gravid female responded normally.

The results with toad skin were more variable. Lower concentrations of hormone were used in these experiments in an attempt to make the condi-

tions more physiological. Aldosterone was seen to cause shedding of the s. corneum during the experimental period in two experiments out of five at the lower concentration (6×10^{-8} M) and in two out of three at the higher concentration (6×10^{-7} M). Another skin treated with the hormone at the lower concentration showed shedding later. For only one of the four skins in which shedding occurred during the experimental period was there a well-defined minimum in p.d. or SCC. The absence of an aldosterone-induced shedding in some skins may have been caused by the small size of the hormone dose. There is also the possibility of interaction of the aldosterone effect with the natural moulting cycle of the skin; in one of the skins moulting took place just before mounting the skin in the chambers and aldosterone had no effect. Hviid Larsen [13] found similar variability in the response to an aldosterone concentration of 3×10^{-7} M in skins from nonhypophysectomized toads.

Effect of Aldosterone on Resistance and Capacitance

Responses to inward and outward current were always similar; there was no evidence for a systematic difference between the two. Short-circuited skins behaved in a manner indistinguishable from open-circuited skins and results from the two types of experiment have been combined. Upon passing a pulse of constant current across the skin the response of voltage as a function of time was always close to that expected for the circuit of Fig. 1 with $\alpha \approx 0.1$; the deviation from a single exponential was not obvious from visual inspection. In particular, evidence for the existence of two time constants was seldom found and the deviation was always small. This is an important point for it implies, as is shown in the Discussion, that the measured capacitance is little different from the capacitance of the functional outward-facing membrane.

The value of the series resistance r_1 was not the principal object of study in the experiments but its value could be determined with reasonable accuracy from the initial voltage deflection on the experimental traces. In some experiments the value of r_1 increased during the inhibition period but in no case was it greater than 6% of r_0 and it was usually much less. It can therefore be concluded that the increase in total skin resistance during the action of aldosterone must be due to an increase in the resistance of cell membranes.

Frog Skin. The parallel resistance r_0 always increased in value during the inhibition period, reaching a maximum at about the same time as the minimum in p.d. or SCC (Fig. 3). The increase was frequently superimposed

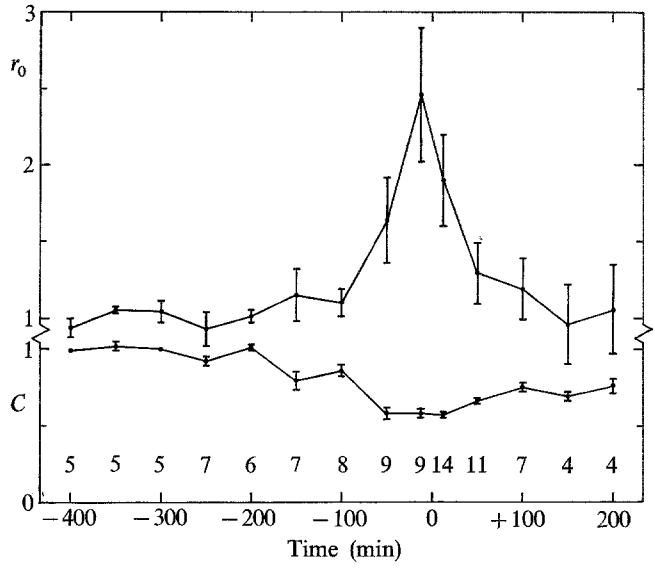

Fig. 3. Responses of resistance and capacitance of frog skin to aldosterone (6×10^{-6} M) for the 13 skins which responded to the hormone. Resistance and capacitance are shown as fractions of the values before addition of the hormone. The time between addition of the hormone and the minimum in p.d. or SCC varied from 180 to 480 min; time has therefore been plotted relative to the minimum in p.d. or SCC, rather than to the time of aldosterone addition. Readings have been averaged over 50-min periods except for two 25-min periods before and after $t=0$. Error bars are standard error of the mean, and numbers of readings are indicated below each point. For any one skin readings were made at intervals of $1\frac{1}{2}-2$ hr (except close to the minimum, where intervals were shorter); therefore not all skins are represented at each point

on small changes occurring throughout the experiment and its extent varied. The increase confirms the pattern observed by Nielsen [19] who determined resistance as the ratio of p.d. to SCC.

Variation of capacitance during the action of aldosterone on frog skin is also shown in Fig. 3. It can be seen that there was a marked decrease, the capacitance reaching its minimum value at about the same time as the minimum in p.d. or SCC. Capacitance changes in the individual experiments are shown in Table 1. The decrease was followed by a rise but in no case was recovery of the initial value complete.

Before addition of the hormone the mean values and standard deviations of the parameters for all 15 skins were as follows: r_0, 3280 ± 1670 Ωcm^2; and α, 0.066 ± 0.019.

Toad Skin. As already noted, the experiments with toad skin gave more variable results. In only two of the four experiments in which shedding occurred during the experimental period were there marked decreases in

Table 1. Effect of aldosterone (6×10^{-6} M) on the capacitance of the isolated frog skin

No.	SC or OC	Time to minimum in p.d. or SCC (min)	Initial capacitance ($\mu F/cm^2$)	Capacitance at minimum in p.d. or SCC (% initial)
1	OC	479	3.20	37
2	OC	379	2.03	58
3	SC	405	2.37	52
4	SC	no minimum	1.33	–
5	SC	428	2.36	64
6	SC	331	2.54	51
7	SC	400	1.76	58
8	SC	382	2.06	55
9	OC	no minimum	2.49	–
10	OC	258	1.96	63
11	OC	182	2.15	64
12	SC	295	2.89	60
13	SC	283	2.50	55
14	OC	333	2.64	49
15	OC	220	2.29	68
mean		337	2.30	56
SD		86	0.46	8

The second column indicates whether the skin was short-circuited (SC) or open-circuited (OC). The value of capacitance at the minimum in p.d. or SCC was determined by interpolation between the closest experimental values before and after the p.d. or SCC minimum.

capacitance – to minima of 0.52 of the initial value in both cases. In none of the other six experiments was there a clear-cut change in capacitance, and there appeared to be no pattern in the resistance changes. Mean values and standard deviations of the parameters for all eight skins before aldosterone addition were r_0, $3790 \pm 3740\ \Omega cm^2$; C, $3.37 \pm 0.82\ \mu F/cm^2$; and α, 0.067 ± 0.020.

Discussion

It is well established that aldosterone causes a shedding of the s. corneum of frog skin, and transformation of the underlying layer of cells into a new s. corneum [27]. Aldosterone also induces shedding of the s. corneum in toad skin [12] and similar changes occur during the natural moulting cycle in toad skin [3].

That the increase in skin resistance is a result of effects on the membranes of living cells, and not on the s. corneum, is supported by the negligible

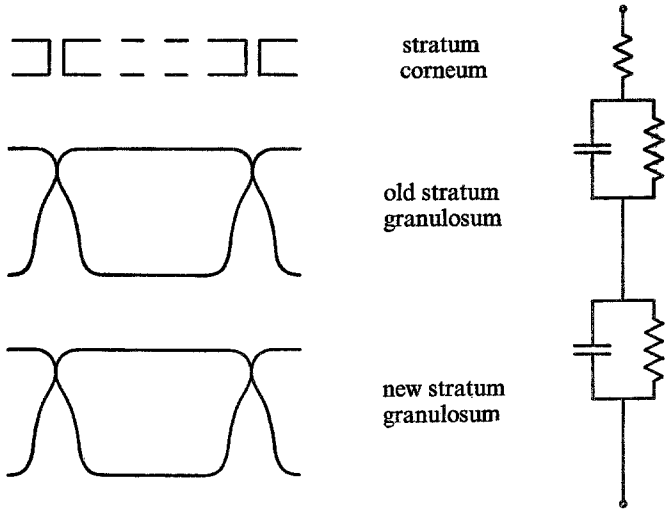

Fig. 4. Diagram of the outer cell layers in the frog skin epithelium, at the time of the minimum in p.d. or SCC, and the equivalent electrical circuit. The equivalent circuit has been simplified as described in the text

change in r_1 found here and the findings of Nielsen [20] that the isolated s. corneum has negligible resistance and that the aldosterone-induced resistance can be abolished by polyene antibiotics, which increase the permeability of sterol-containing cell membranes [11]. Ionic concentrations in the s. corneum are similar to those in the solution bathing the outer surface of the skin [7] supporting the idea that this layer consists of cells which are essentially dead.

Nielsen [20] has proposed a scheme for the action of aldosterone on the isolated frog skin. He suggests that during the gradual decrease in p.d. and SCC, the inhibition period, three processes occur: the attachments between the s. corneum and the s. granulosum are broken down, the old s. granulosum begins to turn into a new cornified layer, and tight junctions begin to form in the next cell layer below. These processes continue until at the time of the minimum in p.d. or SCC there are tight junctions between the cells in two layers, so that the skin has two intact barriers in series, giving an increased electrical resistance (Fig. 4). Later, during the activation period, the old s. granulosum becomes very leaky to ions.

If the outer membranes of the old and new s. granulosa are the only important membranes so far as the impedance of the skin is concerned, and if these two membranes have identical resistances and capacitances at the time of the p.d. or SCC minimum, then the total resistance at this time is twice that of either membrane; the total capacitance, being the resultant of

two identical capacitors in series, is half the capacitance of either membrane. Thus, both the doubling of resistance and the halving of capacitance which were observed during the action of aldosterone can be accounted for. Before this explanation is accepted however it is necessary to consider the possibility of other interpretations. The fact that the voltage response did not have more than one time constant limits the possibilities.

There is good reason to believe that the capacitance of the normal skin is determined by the outward-facing membrane of the s. granulosum [22, 23]. One reason for considering this to be the case is the similarity between the value of the skin capacitance and the "typical" cell membrane capacitance of 1 $\mu F/cm^2$ [4], which indicates that the area of the membrane which determines the total capacitance is of the same order of magnitude as the area of the skin. It is however conceivable that during the action of aldosterone other cell membranes may contribute.

Let us first consider the impedance of a single layer of cells, such as the old s. granulosum in Fig. 4, joined at their apical borders by tight junctions. This layer has two functionally different membranes, one facing the external solution and one facing the internal. There is no evidence for a change in the areas of these two membranes during the action of aldosterone and it can therefore be assumed that the capacitance of the two membranes is constant. But would it be possible for a change in the relative resistances of these two membranes to give rise to a substantial change in the measured capacitance of the whole cell layer? A series combination of two parallel $r - C$ circuits does not have a single time constant unless the time constants of the two parallel elements are equal, but in some circumstances the deviation from a single time constant may be too small to be easily detectable.

It is possible to estimate the areas of the two membranes and thus their relative capacitances. The cells in the s. granulosum are roughly cubical and have five faces in contact with the inner solution as opposed to one face in contact with the outer solution. The membranes facing the inner solution are more convoluted than those facing the outer solution [9, 17, 26, 29, 30]; thus the area of membrane facing the inner solution is about 10 times greater than that of the membrane facing the outer solution and the capacitance is likely to be about 10 times greater also. The theoretical responses of such a circuit were worked out and subjected to the same analysis as the experimental results. If the resistance of the inner membrane is much smaller than that of the outer membrane then the response appears to have a single time constant, that of the outer membrane; the inner membrane therefore has a negligible effect. If the inner membrane resistance is much larger then the response again appears to have a single time constant, but it is the time

constant of the inner membrane, and the total capacitance is close to that of the inner membrane, which experiment shows to be untrue. Between the two extremes the response appears to have a single time constant only if the inner membrane resistance is one-half that of the outer membrane or less; the total capacitance calculated by the analysis is then between 91 and 100% of that of the outer membrane. In other words, if the response is not obviously to have more than one time constant, and the total capacitance is not to exceed 1 $\mu F/cm^2$ by a factor of about 10, the total capacitance determined by the analysis used here is only slightly different from that of the outer membrane. Even if the inner membrane capacitance is taken as only five times that of the outer membrane, similar considerations show that the total capacitance is determined as 84–100% of the outer membrane capacitance.

If the resistance of the tight junctions is low – comparable to the outer membrane resistance – then it is not possible to work out exactly the voltage response; however, inspection of the impedance loci indicates that if the response is to approximate to a single time constant the total capacitance is again little different from the capacitance of the outer membrane.

If the interiors of the cells in all layers of cells in the epithelium are electrically connected [22] then the capacitance of the functional inward-facing membrane is about 50 times that of the outward-facing membrane; considerations similar to those described above once more show that if the response is to appear to have no more than one time constant then the total capacitance determined by the analysis is close to the capacitance of the outward-facing membrane.

It can therefore be concluded that only a series arrangement of two similar membrane capacitances can give rise to the changes observed, and the obvious candidates are the outer-membrane capacitances of the old and new s. granulosa. This implies that tight junctions are present in both layers of cells. Again the measured total capacitance depends on the relative resistance of the two membranes; the voltage response always appears to have a single time constant. If the two membranes have equal resistances the measured capacitance is 0.50 that of either; if the resistance of one is one-half that of the other, measured capacitance is 0.53 that of either; if one-fifth, 0.68; if one-tenth, 0.81. Even if one of the membranes has a comparatively low resistance, therefore, the total capacitance is substantially lower than that of one of the membranes alone. This can account for the fact that under the action of aldosterone the capacitance decrease is initially faster than the resistance increase (Fig. 3). It may also account for the failure of the capacitance to return to its original value after shedding

of the s. corneum, if the resistance of the old s. granulosum does not decrease immediately to zero.

Nielsen [20] suggests that one of the reasons why sodium transport is low in the inhibition period is that the inner membrane of the old s. granulosum is neither actively transporting sodium nor allowing sodium to diffuse across. The present results indicate that this membrane must have a low resistance compared with the outward-facing membranes; but this does not imply that all ions can cross the membrane with equal ease and Nielsen's suggestion seems a likely explanation of the fall in sodium transport, and thus SCC. However, his proposal that the increased resistance of the skin is a reflection of a high resistance of this inner membrane is in conflict with the results reported here.

In frog skin the minimum in p.d. or SCC and the maximum in resistance occur at about the same time, whereas in toad skin Hviid Larsen [12–14] found that the resistance maximum occurred some time before the p.d. or SCC minimum. Another difference is that sodium efflux, and chloride fluxes in both directions, across the toad skin are elevated during and after the period in which p.d. and SCC are depressed, while in frog skin there is little change in these fluxes from the normal level [19]. Furthermore, the shunt resistance in toad skin drops during this period [15]. This suggested that in toad skin the tight junctions in the new s. granulosum might not form until after their breakage in the old s. granulosum – unlike the situation in frog skin, where formation of the new junctions takes place before breakage of the old. If this were so, the toad skin would never possess two functional outward-facing membranes in series and a capacitance decrease would not be observed during the action of aldosterone. The results on toad skin indicate that this hypothesis might be correct for two skins of the four which showed moulting (those where capacitance did not change) but not in the other two of the four (where a marked decrease in capacitance was seen). These latter two had the lowest p.d.'s of the eight at the beginning of the experiment and it is possible that they are atypical.

In summary, then, it seems clear that the reduction in capacitance observed during the action of aldosterone on the frog skin means that the skin possesses two functional outward-facing membranes for a short period, implying that tight junctions are formed in the new s. granulosum before they break in the old.

The storage oscilloscope was purchased with the aid of a Science Research Council grant. I thank Mr. T. Hine for construction of the pulse generator and Mr. S. Drinkwater for constructing the experimental chambers.

Appendix

Determination of Resistance and Capacitance from Step-Function Response

Theoretical Step-Function Response. It has been shown [23] that the impedance Z of the normal, isolated frog skin as a function of angular frequency ω is tolerably well described by the equation

$$Z = r_1 + r_0/[1 + (j\omega\tau)^{1-\alpha}] \quad (1)$$

where $j = \sqrt{-1}$ and τ is a time constant. If the equivalent circuit of the skin is as shown in Fig. 1 then $\tau = r_0 C$, where C is the membrane capacitance. For an ideal capacitance $\alpha = 0$.

From the relationship between impedance and frequency it is possible to work out the response of the voltage across the skin to a step of current. If the value of α is zero then the response of voltage V_t as a function of time t after the application of a current step of magnitude I_0 is given by

$$V_t = I_0 r_1 + I_0 r_0 [1 - \exp(-t/\tau)]. \quad (2)$$

For larger values of α the response is modified [10, 16] (Fig. 5). The most prominent difference is the "tail" at large t; at $t = 5\tau$, for example, V_t is well within 1% of its final value for $\alpha = 0$ but is about 5% below for $\alpha = 0.1$.

This exact response was used to determine the accuracy of the analysis of the experimental results.

Analysis of Step-Function Response. The nature of the voltage response – in particular the long tail – suggests that a voltage record of considerable length is required for accurate analysis. For this reason the response was always recorded at two sweep rates, one four or five times slower than the other. Values of voltage were read from the oscilloscope photograph at each vertical division of the graticule, to give nine voltage values at the high sweep rate (including the value at zero time) and up to a further seven at the low rate.

The average value of α in frog skin is 0.09 [23]. This low value suggested that the experimental response might be close enough to that described by Eq. (2) for it to be possible to determine the parameters of the network by fitting an equation of this form to the experimental results. However, analysis of the exact response for $\alpha = 0.1$ in this way gave values of the parameters which were incorrect by up to 8%, indicating that the procedure was in fact unsatisfactory.

Teorell [24] and Cuthbert and Painter [5, 6] determined the parameters of the network from an approximate reconstruction of the frequency response by Fourier analysis [1], and a similar procedure was used here. Calculations were carried out with the aid of the University of Liverpool's

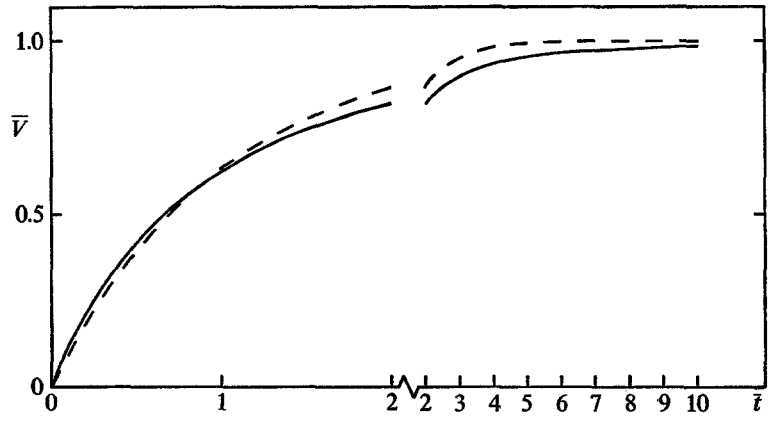

Fig. 5. Response of voltage across a membrane to the passage of a step of current. It is assumed that the membrane impedance obeys Eq. (1), and the value of r_1 has been taken as zero. $V = V_t/I_0 r_0$ and $t = t/\tau$, where the variables are as in Eq. (2). Values are taken from refs. [10] and [16]. The dashed line is for $\alpha = 0$ and the continuous line for $\alpha = 0.10$

KDF9 computer. The equations for resistive and reactive components of the impedance at frequency f, $R(f)$ and $X(f)$, respectively, determined from a series of values of voltage V_t equally spaced at intervals Δt are

$$R(f) = (1/I_0) \sum_{k=1}^{N} (V_k - V_{k-1}) \cos(2k-1)\psi/2 \tag{3}$$

and

$$X(f) = (-1/I_0) \sum_{k=1}^{N} (V_k - V_{k-1}) \sin(2k-1)\psi/2 \tag{4}$$

where $\psi = 2\pi f \Delta t$, $k = t/\Delta t$ and $N = T/\Delta t$. T is the length of the voltage record and N the number of voltage readings. The approximation improves as $\Delta t \to 0$ and $T \to \infty$.

Eqs. (3) and (4) can be written, with the aid of trigonometrical identities,

$$R(f) = (1/I_0) \left[-V_0 \cos\psi/2 + 2 \sin\psi/2 \sum_{k=1}^{N-1} V_k \sin k\psi + V_N \cos(2N-1)\psi/2 \right] \tag{5}$$

and

$$X(f) = (1/I_0) \left[V_0 \sin\psi/2 + 2 \sin\psi/2 \sum_{k=1}^{N-1} V_k \cos k\psi - V_N \sin(2N-1)\psi/2 \right] \tag{6}$$

and these were the equations used in the computer program. The value of r_1 was calculated as V_0/I_0. V_0 was then subtracted from all values of V_t so that the first term in Eqs. (5) and (6) became zero.

Fourier analysis of a series of values spaced at intervals of Δt over a total length of time T gives values in the frequency domain at frequencies spaced at intervals of $1/T$, ranging from $1/T$ to $1/2\Delta t$. These fundamental

limitations do not appear explicitly in the type of analysis used here; any value of f can be substituted into Eqs. (5) and (6) and the impedance at this frequency calculated. However, this does not necessarily produce useful results. Impedance values calculated at two frequencies closer than $1/T$, for example, are not independent. Substitution of $f=0$ can give a value for impedance which bears no relation to the direct current impedance; such a substitution tacitly implies that the voltage at very long times is constant at the final measured value.

These limitations were borne in mind when deciding at what frequencies impedance should be calculated. First, at low frequencies: the slow approach of V_t to its final value (Fig. 5) indicates that for a record of finite length the last measured value may not be a good approximation to the value at infinite time. The direct-current impedance calculated by substituting $f=0$ into Eqs. (5) and (6) is therefore likely to be inaccurate too; its value was not calculated in the analysis. It was, however, desirable to calculate impedances over as wide a range of the impedance locus as possible so that lower and more closely-spaced frequencies than are strictly justified were used (to determine impedance at a frequency one-tenth of that at which $-X$ is a maximum a time record 60 times longer than the time constant is needed). Analysis of exact responses plus error showed that this gave useful results (*see below*). The maximum frequency at which impedance can usefully be calculated is $1/2\Delta t$; in fact, the accuracy of the method used here decreases at much lower frequencies [1]. The value of Δt for the Fourier analysis was decreased by interpolation between adjacent values of V_t read from the oscilloscope traces; interpolation was also necessary to obtain an equally-spaced series of values from readings at the two sweep rates. The sampling frequency $1/\Delta t$ was always at least 10 times greater than the highest frequency at which impedance was calculated. Impedance was calculated at nine frequencies spaced more or less evenly around the locus; it was also known that the locus passed through the origin.

From the nine values of impedance the values of r_0, C and α were calculated. The center of the locus was determined as the "best" point of intersection of the perpendicular bisectors of the lines joining the origin to each point on the locus, using a procedure in the University of Liverpool's Algol Procedure Library for the best solution of n linear equations in n unknowns ($m>n$). The radius of the circular arc was taken as the mean value of the distances of the points from the center; the standard deviation of this distance was also calculated to give some idea of the fit of the points to the circular locus. Knowledge of the position of the center of the semicircle and the value of its radius fixes the values of r_0 and α.

The value of the time constant τ was then determined by a least-squares method as described previously [23], and the capacitance from the relation $\tau = r_0 C$. The residual error after finding the best value of τ gave an estimate of the conformity of the impedance locus to Eq. (1). The fit of the points was not as good as that found previously [23], because that fit was carried out on impedance values measured directly and the preceding Fourier analysis necessary here introduced further errors. The standard deviation of the radius was nearly always less than 2% of the value of the radius and the fit of τ gave a residual error about twice as great as that found previously [23].

The method for determining r_0, C and α was rather lengthy and it therefore seemed appropriate to investigate the accuracy and reproducibility of the entire procedure.

The response of a network of the type shown in Fig. 1 made up from electrical components was investigated in the same way as the skin. Several responses were recorded. It was found possible to repeat a voltage reading with a standard deviation of about 0.01 mV in 3 mV. The standard deviations of the results of the analyses were 1.2% for r_0 and 1.6% for C; the value of α was -0.005 with a standard deviation of 0.008, the correct value presumably being zero. Most of the variability in fact resulted from the measurement of current rather than voltage. The fit to Eq. (1) was rather closer than for the experimental traces, probably because the small value of α meant that there was no "tail" at long times.

The accuracy of the computer analysis was determined by analysis of the exact response; the effect of error added to the exact response was also investigated. The error was a random normal deviate with a standard deviation 0.3% of the final voltage value; it was added to each voltage value and 10 replicates were made of the analysis. The averages of the 10 values of resistance and capacitance had an accuracy better than 1% and a standard deviation of about 1%. The value of α tended to be slightly underestimated but the value was not of great interest and the error was considered unimportant. Conformity with Eq. (1) was similar to that for the experimental traces.

The technique of analyzing the exact response with added error was valuable when developing the program; it was, for example, possible to investigate systematic and random errors at each of the points on the impedance locus, which showed that errors, and correlations between adjacent values, at low frequencies (but not at zero frequency) were less than might have been expected from the fundamental limitations of Fourier analysis.

The method of analysis finally used gave unsatisfactory results if (a) the final voltage value was at a time less than 6τ; or (b) if the spacing of the original voltage ordinates (i.e. those read from the photograph) was wider than 0.5τ. At the very least, 12 equally-spaced voltage ordinates are therefore needed; this confirms the desirability of recording the response at two sweep rates. The experimental responses did not violate the constraints and were usually well within the limits.

The main objects of study in the present work, the values of r_0 and C, appear to be correct to about 2%. Each value used in the construction of Fig. 3 was the mean of the results of analyzing two responses, recorded with inward and outward current steps, and should therefore be closer to the correct value.

Previous workers [5, 6, 24] who have used this type of analysis do not appear to have investigated possible errors in any detail and it is difficult to assess the accuracy of their results.

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