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CHARACTERISTICS OF ALDOSTERONE STIMULATED TRANSPORT IN ISOLATED SKIN OF THE TOAD, *BUFO BUFO* (L.)

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SUMMARY

Aldosterone not only stimulates active sodium transport through isolated toad skin but also initiates slough formation. This, in turn, affects both the active sodium transport and the passive transport of ions through the skin in a very nearly reversible manner. This overall response to aldosterone is studied by means of the short-circuit technique in combination with tracers. The analysis is based on the equivalent circuit analogue developed by Ussing, which allows the determination of the following parameters: the rate of active transport, the resistance to active transport, the electromotive force (EMF) of the active transport system, and the resistance as well as the sodium/chloride selectivity of the shunt path. The time course of the aldosterone response is tentatively divided into three more or less overlapping periods:

(1) *Initial stimulatory period*: registered shortly after addition of aldosterone and characterized by an increased active sodium transport, a decreased resistance to active transport, and a decreased EMF of the active transport system.

(2) *Refractory period*: developing as slough formation takes place and starting about three hours after addition of aldosterone. During this period, the hormonal stimulation of the active transport disappears together with an increase in the resistance to active transport and an increase in the EMF of the active transport system. In addition, the shunt resistance decreases and its selectivity is lost.

(3) *Final stimulatory period*: starting about the time slough formation is concluded, i.e. 5–7 h after addition of aldosterone. Stimulation of the active sodium transport reappears, while the resistance to active transport and the EMF of the active transport system decrease. Also, the selectivity of the shunt path reappears.

The results support the idea that aldosterone stimulates the entry of sodium into the cellular compartment from the outer solution, and are compatible with the view that slough formation and shedding of the cornified layer are accompanied by a shift of the functional stratification of the epithelium, i.e. the functionally outward-facing membrane moves to an underlying cell layer, and the same holds for the functioning tight junctions confined to the outermost part of the skin.

UNTIL a few years ago, it was assumed that the sole effect of aldosterone on sodium chloride transporting epithelial tissues was the stimulation of active sodium transport. Recent evidence suggests that this steroid, besides its direct action on active transport, also affects the passive transport of ions through the amphibian skin[1–4]. It is the primary aim of the present communication to give experimental support to this idea.

THE BIOELECTRIC RESPONSE TO ALDOSTERONE

Addition of aldosterone to the isolated skin of *Bufo bufo* initiates slough formation and shedding of the cornified layer within five to seven hours after application of the hormone. This is always observed in skin of *pars distalis* extirpated toads, and frequently observed in skin of normal toads[5]. From Fig. 1 it is seen that the active sodium flux, expressed as the short circuit current I_{scc}[6], starts to increase following addition of aldosterone. Three hours after aldosterone treat-

ment, a significant stimulation of the active sodium flux could be detected (ΔI_{scc} = 7·5 ± 2·2 μA/cm^2 between matched skins, 0·1% < P < 0·5%, n = 14)[5]. Shortly afterwards, I_{scc} decreased again and to a level below that of the paired control. As slough formation ends, stimulation of the active sodium transport reappears. This temporary elimination of typical aldosterone effect is due to loosening of *stratum corneum*[5]. Also, stimulation of the active sodium transport by oxytocin is completely blocked during this period[5]. Accordingly, the aldosterone response can be divided as follows: (1) *Initial stimulatory period*, (2) *Refractory period*, and (3) *Final stimulatory period.*

Figure 1 reveals that the response is also characterized by a temporary decrease in the spontaneous skin potential (E_t) and in the output resistance of the skin (R_T).

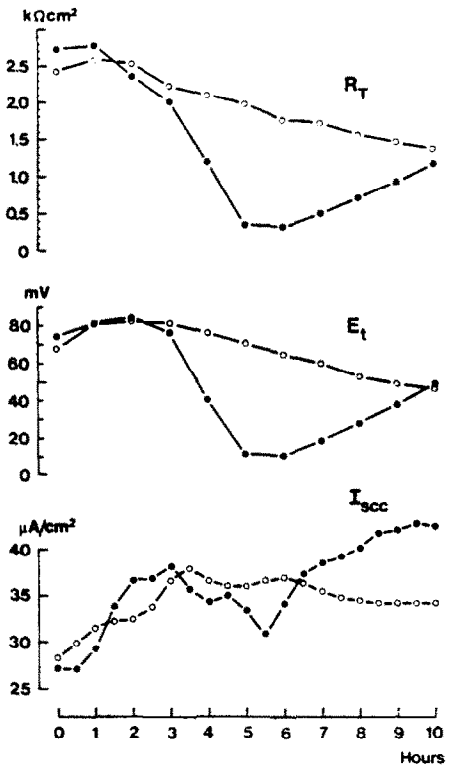

Fig. 1. Active sodium flux (I_{scc}), spontaneous skin potential (E_t) and output resistance (R_T) of paired skins from a *pars distalis* extirpated toad. ●—●: Aldosterone (0·1 μg/ml) added at zero time; slough formation completed 5–6 h later. ○—○: Control without aldosterone.

THEORY

In order to propose a causal relationship between these events it is advantageous to treat the skin as an electrical analogue in accordance with the equivalent circuit diagram successfully applied in other studies[6, 7]. The diagram (Fig. 2) consists of an active transport path represented by the "transport battery" with the electromotive force E_0 and the internal (serial) resistance R_{ser}. The passive transport path is represented by the parallel resistance R_{sh}.

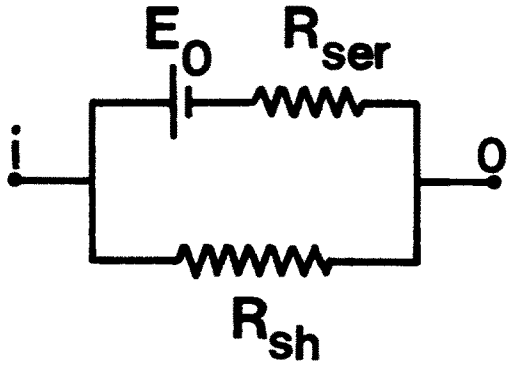

Fig. 2. Electrical analogue of amphibian skin. *i* and *o* represent points of contact between skin and inside or outside bathing solution, respectively. See text for further explanation. (From [7]).

According to the two-membrane hypothesis [8], E_o is defined as the sum of the potassium diffusion potential across the inward facing membrane and the sodium diffusion potential across the outward facing membrane:

$$E_o = \frac{RT}{zF}\left(\ln \frac{K_c}{K_i} + \ln \frac{Na_o}{Na_c}\right) \tag{1}$$

where K and Na are the chemical activities of potassium and sodium respectively in the internal (i), cellular (c), and external (o) compartments. R, T, z and F have their usual meaning.

R_{ser} is the sum of the diffusion resistance to potassium ion movement across the inward facing membrane and to sodium ion movement across the outward facing membrane.

$1/R_{sh}$ is the sum of the conductances for the ions tending to short-circuit E_o.

Applying Kirchhoff's laws to the circuit, it is easily shown that the relation between the current I through the circuit and the potential difference E_{io} between i and o is:

$$E_{io} = -IR_T + E_t \tag{2}$$

where R_T is the output resistance of the circuit:

$$1/R_T = 1/R_{ser} + 1/R_{sh} \tag{3}$$

and E_t equals the potential difference between i and o when $I = 0$, i.e. E_t corresponds to the spontaneous skin potential. Within the physiological range of E_{io}, Eq. (2) was shown to hold for the isolated toad skin bathed with solutions of identical composition and hydrostatic pressure on both sides [9]. Under these conditions, I_{scc} is given by:

$$I_{scc} = E_t/R_T = E_o/R_{ser} \tag{4}$$

the first part of which was used for the determination of R_T. The advantage of this formal treatment of the bioelectric parameters of the skin is obvious: a quantita-

tive determination of the circuit components makes it possible to differentiate a direct effect on the active transport system from a direct effect on the resistance against active transport. Furthermore, changes in the output resistance of the skin can be interpreted in terms of changes in the ion permeability of the active and passive transport pathways.

RESULTS

Two independent methods were used. The first [7] is based on the assumption that the current flowing through the active transport path is carried by sodium ions across the outward facing membrane. Thus, the output resistance of the skin approaches R_{sh} if the outside bathing solution is made sodium free.

R_T was measured bathing the skin with sodium Ringer's on both sides. Subsequently, R_{sh} was measured with potassium Ringer's as outside bathing solution and applying an out-going current to the preparation.

The changes in R_T are due to changes in both R_{sh} and R_{ser} (Fig. 3). Characteristically, R_{sh} decreases to a value about 0·5 kΩ cm² illustrating a drastic increase in the passive ion permeability. This change of R_{sh} is reversible within the time of observation. Also, the change in R_{ser} is transient, and is characterized by a threefold increase during the period in which the active sodium flux reaches its minimum value. E_o reaches a maximum value during the refractory period.

When applying this method, the passive permeability of the skin is assumed to be independent of the ionic composition of the bathing solutions. Strictly speaking,

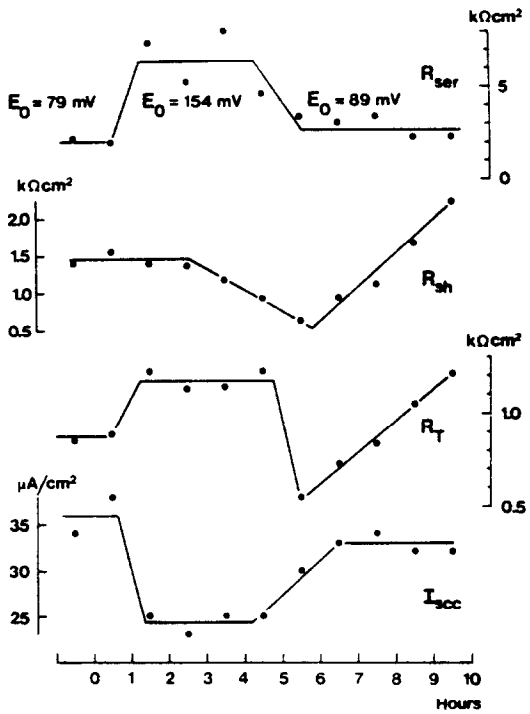

Fig. 3. Separation of the output resistance (R_T) of the skin into the resistance against active (R_{ser}) and passive (R_{sh}) transport of ions. I_{scc}, R_T, and R_{sh} were measured electrically. R_{ser} and E_o were calculated from Eqs. (3) and (4), respectively. Aldosterone (0·1 μg/ml) added at zero time, slough formation finished about 5 h later. Skin of a *pars distalis* extirpated toad.

this is not so. Actually, the passive chloride permeability tends to decrease with decreasing sodium concentration of the outside bathing solution[10], and consequently R_{sh} will be determined too high. Another objection to this method is the following: when applying an outgoing current to the preparation for the determination of R_{sh}, it cannot be ruled out that R_{ser} contributes to the output resistance of the skin. Because of this, R_{sh} would be estimated too low, though to a minor degree since the potassium concentration of the inside solution and the sodium concentration of the cellular compartment are low.

The second method was designed to overcome these objections. It is based on the fact that in the short circuited skin, the net current through R_{sh} is nil and the electrochemical activities of each ion species in the outside bathing solution are equal to those of the inside bathing solution. Under these conditions, the contribution of the single ion species j to R_{sh} is determined by[11]:

$$R_j = \frac{RT}{M_j F^2 z_j^2} \tag{5}$$

where M_j is the passive flux of j in the short circuited skin. Assuming that only R_{Na} and R_{Cl} contribute significantly to R_{sh}, and because these resistances are parallel, the following equation holds:

$$1/R_T = 1/R_{ser} + 1/R_{sh} = 1/R_{ser} + 1/R_{Na} + 1/R_{Cl}. \tag{6}$$

As R_T can be determined from Eq. (4), R_{sh} as well as R_{ser} can be calculated using the values of R_{Na} and R_{Cl} derived from Eq. (5). M_{Na} and M_{Cl} were measured simultaneously as previously described[9].

The results of a single experiment are depicted in Figs. 4 and 5; the corresponding values of I_{scc}, E_t and R_T are represented in Fig. 1 During the observation period, R_{Na} and R_{Cl} (and thus R_{sh}) decrease in the control skin. Obviously, the time course of these parameters in the aldosterone treated skin is different and is characterized by a steep decrease, reaching a minimum value within the refractory period. As was the case for the experiment depicted in Fig. 3, R_{sh} increases during the final stimulatory period. In addition, important information concerning the nature of the shunt path can be obtained: by calculating the ratio of the passive chloride permeability to the passive sodium permeability (P_{Cl}/P_{Na}), the selectivity of the shunt path is determined (Fig. 5, lower curves). Two important conclusions may now be drawn. As a consequence of isolation, the skin loses its selective permeability to sodium and chloride ($P_{Cl}/P_{Na} = 1{\cdot}5$ corresponds to the ratio of the mobilities for these ions in water). In the presence of aldosterone this phenomenon is accelerated and becomes transient. Consequently, eight to ten hours after addition of aldosterone, the selectivity of the shunt path is the same as it was at the very start of the experiment.*

The initial increase in the active sodium flux (Fig. 1) coincides with decreases of R_{ser} and E_o (Fig. 5). Again, R_{ser} and E_o maximize during the refractory period, and, within the final stimulatory period, reach values comparable to those of the initial stimulatory period.

These results were obtained in all experiments (Table 1).

*The error introduced by ignoring the contributions of K^+, Ca^{++}, and HCO_3^- to R_{sh} can now be estimated. Within the hour of observation during which the selectivity is lost, the passive permeability of these ions and of Na^+ and Cl^- must be proportional to their mobilities in water. Taking these values from the literature, the error was calculated to be 2·7%.

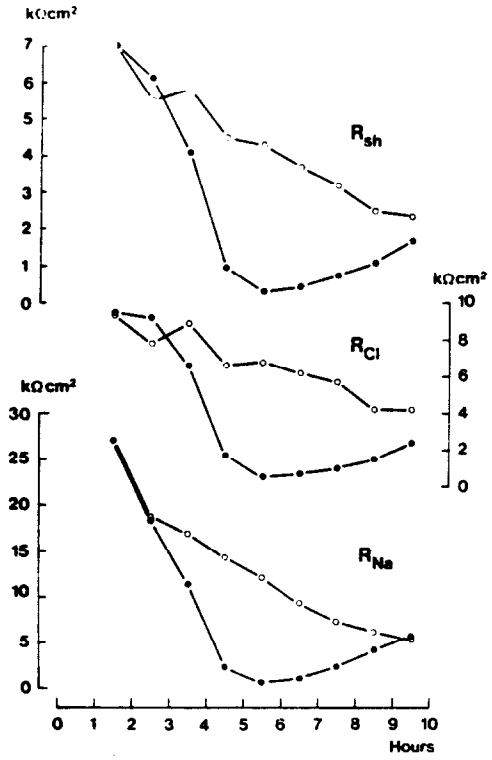

Fig. 4. Time course of shunt resistance (R_{sh}) calculated from separate determinations of sodium (R_{Na}) and chloride (R_{Cl}) resistance by means of sodium-24 and chloride-36. Continued from Fig. 1.

Table 1. Transport characteristics of the isolated skin of *Bufo bufo* in the presence of aldosterone (mean ± SEM, $n = 8$)

	Initial stimulatory period		Refractory period	Final stimulatory period
Active transport path	I*	II*	III*	IV*
R_{ser} kΩcm²	3·1 ± 0·3	2·3 ± 0·2	5·1 ± 1·0	1·9 ± 0·3
E_o mV	108 ± 8	89 ± 10	161 ± 27	91 ± 10
Passive transport path				
R_{Na} kΩcm²	16·5 ± 2·5	7·1 ± 1·1	1·4 ± 0·3	3·3 ± 0·4
R_{Cl} kΩcm²	6·1 ± 0·7	4·1 ± 0·5	0·8 ± 0·2	1·1 ± 0·2
R_{sh} kΩcm²	4·4 ± 0·5	2·6 ± 0·3	0·5 ± 0·1	0·8 ± 0·2
P_{Cl}/P_{Na}	2·7 ± 0·3	1·7 ± 0·1	1·4 ± 0·1	3·7 ± 0·7

*I 2nd
II 4th
III 6th (passive path) or 7th (active path)
IV 10th
} h after aldosterone treatment.

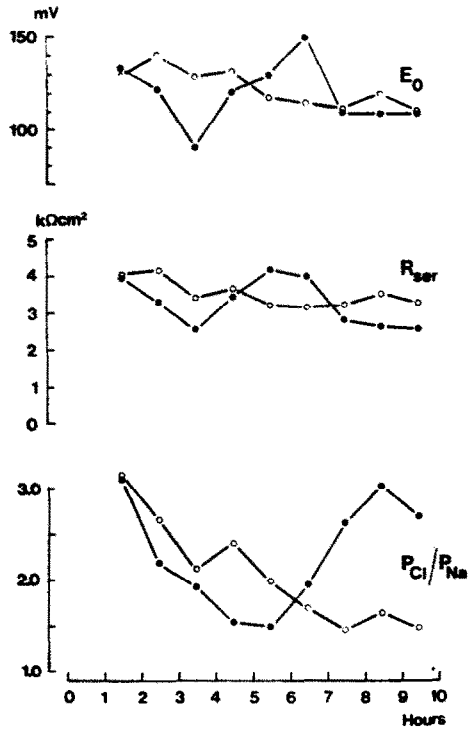

Fig. 5. *Lower diagram*: The selectivity of the shunt path expressed as the ratio of the passive chloride and sodium permeabilities (P_{Cl}/P_{Na}). *Two upper diagrams*: Time course of the resistance of the active transport path (R_{ser}) and the electromotive force of the active transport system (E_o). Continued from Figs. 1 and 4.

CONCLUSIONS

(1) *Initial stimulatory period.* Impressive evidence supports the theory that the initial aldosterone-tissue interaction triggers an early protein synthesis [12–15]. The decrease in R_{ser} within the initial stimulatory period (Fig. 5, Table 1) is compatible with the view [16] that the primary site of this *de novo* synthetized protein is the outward facing membrane of the transporting cells, the effect being an increased sodium permeability. More sodium now enters the cellular compartment, as is reflected in the decrease in E_o (compare Eq. (1)). The accumulation of sodium in the cellular compartment indicates that the active sodium flux is limited, either by a saturation of the Na^+/K^+-exchange pump or by the turnover rate of the ATP yielding metabolic processes.

Addition of aldosterone to the isolated toad skin also initiates slough formation. The effect of loosening of *stratum corneum* characterizes the subsequent period.

(2) *Refractory period.* Both aldosterone and oxytocin stimulation of the active sodium transport disappears completely within this period. The baseline activity of the active transport is affected to a minor degree.

The transient increase in R_{ser} and in E_o and the decrease in I_{scc} might be the consequence of either a decreased sodium permeability of the outward facing membrane or a decreased potassium permeability of the inward facing membrane; it is not possible to distinguish between these possibilities on the basis of the present observations. On the other hand, light microscopic studies of small pieces

of the skin incubated in aldosterone Ringer's (Aabye and Hviid Larsen, unpublished experiments) showed normal appearance of the granulosa cells during the refractory period in contrast to a distinct swelling of these cells *after* shedding of the stratum corneum (i.e. during the final stimulatory period). As, however, a decrease in the potassium permeability of the inward facing membrane would lead to swelling of the transporting cells during the refractory period, the above evidence strongly supports the hypothesis that the mechanism behind the observed increase in R_{ser} is to be found in a decreased sodium permeability of the outer barrier. The outward facing membrane of the transporting cells is situated just beneath the cornified layer[17–19]. This part of the skin is distinguished by an extracellular mucopolysaccharide material almost closing the space between the keratinized cells and the superficial granulosa cells. It was suggested[2] that disintegration of this material, along with loosening of the cornified layer liberate a factor which, in turn, plugs the "sodium channels" of the outward facing membrane. This is, of course, a highly speculative hypothesis as long as direct experimental evidence of such a factor is lacking. It cannot be ruled out that functioning "sodium channels" in contrast, depend on an interplay between the intact mucopolysaccharide material and the outward facing membrane of the granulosa cells. Also in this case, disintegration of this layer leads to loss of function of the outer part of the epithelium.

Slough formation also affects the passive transport pathway through the skin. As a most interesting consequence, the selectivity of this pathway is lost during the refractory period. This is most readily explained by assuming that slough formation causes an opening of an extracellular pathway, and is compatible with the view that extracellular ion transport normally is suppressed by tight junctions between the outermost cells of the epithelium[20].

(3) *Final stimulatory period.* From a functional point of view, the skin recovers during the final stimulatory period, starting about the time slough formation is completed.

R_{ser} decreases to a value often below that prevailing at the end of the initial stimulatory period. More sodium enters the skin and, as a consequence, the active sodium flux increases. The decrease in E_0 indicates that the rate of active sodium transport again is limited by the pump activity at the inward facing membrane. The increased sodium permeability of the active transport pathway could be due to diffusion of the "inhibitory factor" to the outside bathing solution[2]. Alternatively, it could be ascribed to re-formation of functioning mucopolysaccharide material, i.e. re-formation of a functioning outward-facing membrane in the cell-layer below. On the basis of the present observations, one cannot distinguish between these possibilities.

Likewise, passive transport of ions undergoes changes within this period. The resistance of the shunt path increases only to a minor degree, whereas its selectivity to sodium and chloride is re-established, indicating that the extracellular ion transport is now suppressed, and that chloride is transported across the skin along the opened cellular pathway. This appears to be a reasonable guess since the cellular pathway should not be available to passive sodium transport due to the very low sodium permeability of the inward facing membrane[8]. Such an interpretation makes an interesting point because it implies reappearance of functioning tight junctions between the outer cells of the epithelium.

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DISCUSSION

Voûte: You mentioned that there might be extracellular pathways due to opening of the intercellular tight seals. Don't you think that there could be an alternative explanation: the degradation of the next underlying cell layer (which supposedly forms with the cornified layer the main diffusion barrier) and the various physiological and metabolic changes accompanying this cornification process, which could explain the loss of its asymmetric permeability and transport characteristics, and which could mimic extracellular shunt increase in spite of being transcellular? (Voûte *et al.*, *Exptl. Cell Res.* **61** (1971) 133).

Hviid Larsen: During the refractory period, the resistance against active transport increases, indicating that the sodium permeability of a barrier in series with the inward facing membrane decreases. Accordingly, during this period, the sodium ions passing passively along the cellular pathway are faced with a considerable diffusion barrier at their passive entrance to, as well as at their passive exit from[8] the cellular compartment. Therefore, in order to explain the observed unrestricted diffusion of Na^+ and Cl^-, the theory of an opened extracellular pathway was suggested. Of course we cannot rule out that this pathway includes dying or keratinizing cells at the outer part of the epithelium. But even so, it seems reasonable to assume that they become excluded from contributing to this pathway during the final stimulatory period by the formation of functioning tight junctions in the cell layer below.

Voûte: The trouble is that we have never seen open extracellular pathways even in this situation of increased passive movements during aldo induced moult. That probably depends on the methods.

Hviid Larsen: Well, this seems to be an important point, and I was aware of this in using the term *functioning* tight junctions. I think we have to consider

whether the morphological appearance of *zonulae occludentes* can be used as the sole criterion for their function as tight seals.

Ussing: I just want to make some comments on some experiments Dr. Erlij performed. He has found openings of the tight seals under certain circumstances by using the lanthanium method. He only saw these leak paths when using a hypertonic solution on the outside of frog skin. Then he could see how the colloidal particles penetrated the tight seals. That would tally with the idea that during the period of moulting passive ion movements following the electrical gradient will increase. This would also go with the idea that the tight seals of the old outermost layer are leaky and the ones of the new one not yet formed and functionally tight. This would happen only during the short period we are talking of now. This hypothesis could be tested with the lanthanium method or with the barium sulfate method we have been using in our institute.

Morel: Have you tried the effect of amiloride on this preparation?

Hviid Larsen: I have not, but Nielsen and Tomlinson have studied the effect of amiloride on sodium transport in *frog* skin during aldosterone induced moulting (*Acta physiol. scand.* **79** (1970) 238).

Crabbé: What are the environmental conditions during the days preceding sacrifice of the animals? As I remember from one of your recent publications, you often use hypophysectomized *Bufo bufo*. Am I correct in assuming that you did the same for the experiments you have just discussed?

Hviid Larsen: You are correct. Precocious slough formation due to corticosteroid treatment is always observed after *pars distalis* extirpation of the toad, whether you inject the hormone *in vivo* (*Gen. Comp. Endocrinol.* **4** (1964) 389) or add the hormone to the *in vitro* preparation. Furthermore, initiation of slough formation is frequently observed in aldosterone treated skin of normal toads, but now the appearance of slough formation is dependent on the prehistory of the animal [5]. In the present work *pars distalis* extirpation of the toads was performed in order to get reproducible results.