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# THE EFFECT OF ALDOSTERONE ON THE ENERGETICS OF SODIUM TRANSPORT IN THE FROG SKIN

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## SUMMARY

Uncertainty persists as to whether the stimulation of active sodium transport by aldosterone is attributable to effects on permeability or energetic factors. This question has been examined with the aid of a thermodynamic formulation in which the rate of both active sodium transport $J_{Na}$ and $O_2$ consumption $J_r$ are assumed to be linear functions of the electrical potential difference $\Delta\psi$ and the affinity $A$ (negative free energy) of metabolic reaction. Previous studies have indicated constancy of a characteristic affinity on perturbation of $\Delta\psi$, suggesting the possibility of its evaluation. In studies of paired frog skins the administration of aldosterone led to a significant increase in the short-circuit current $I_0$, a suggestive increase in the associated rate of $O_2$ consumption $J_{ro}$, and a significant increase in the ratio $-I_0/\{dJ_r/d(\Delta\psi)\}$. If linearity obtains, this ratio is equal to $A$. Depression of active sodium transport and the associated metabolism with amiloride, which depresses permeability, also results in an increase in the apparent affinity $-I_0/\{dJ_r/d(\Delta\psi)\}$. The results indicate that aldosterone does not act simply by increasing the permeability or the number of transport units operating in parallel, but suggests that energetic factors are implicated as well.

## INTRODUCTION

Despite intensive investigation, uncertainty persists concerning the mechanism of stimulation of active sodium transport by aldosterone. According to the currently accepted model of the sodium transport system in epithelial cells sodium enters the cells passively at the apical membrane and is extruded by a ($Na^+$–$K^+$)-activated ATPase at the basal-lateral membrane. On the basis of this model, as discussed recently[1], it has been suggested that an aldosterone-induced protein may "exert its effect at three possible sites: (1) at the apical entry step (permease theory), (2) directly on the ($Na^+$–$K^+$) ATPase (pump theory) or (3) on the oxidative pathway generating high energy intermediates to fuel the pump (metabolic theory)". Sharp and Leaf[2] and

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others[3–7] have favored the first of these alternatives. The second alternative has been questioned on various grounds[1]. Edelman's group has urged the significance of energetic factors[8,9], and recently Lipton and Edelman[10] suggested that aldosterone might act to facilitate both passive $Na^+$ entry and active $Na^+$ extrusion.

We have presented previously a formalism for analysis of the energetics of active sodium transport by means of nonequilibrium thermodynamics[11]. Recent experimental results support the validity of this formulation, suggesting the possibility of calculating the affinity (negative free energy) of the metabolic reaction driving active sodium transport[12–14]. It should be emphasized that the affinity represents a "driving force" for reaction independent of the levels of sodium and potassium or of the rate of metabolism.

The present experiments were undertaken in order to examine the effect of aldosterone on energetics. Because of the additional possibility that aldosterone might act at the site of sodium entry, studies were also made of the effect of amiloride, an agent which is believed to act exclusively at the outer permeability barrier[5,15].

## MATERIALS AND METHODS

Frogs (*Rana pipiens*) of extra large size, 10–12 cm in length, were obtained from the Carolina Biological Supply Co., Burlington, North Carolina. They were kept at 4 °C without feeding, and were used within 2 weeks. Prior to experiments they were kept at room temperature and half immersed in 0.6% saline for at least 48 h in order to suppress the endogenous secretion of aldosterone. The skins were treated as aseptically as was practical. All glassware was autoclaved at 250 °C for 3 h. Metal instruments were immersed in boiling water for 20 min. The lucite chambers were wiped with cotton swabs soaked in 70% alcohol and rinsed several times in sterile Ringer solution. After pithing the animals the abdomen was cleansed by gentle rubbing under running water (cleansing agents have been found to depress the short-circuit current). The abdominal skin was cut vertically along the midline and resected, providing two paired skins. These were rinsed twice in sterile Ringer solution prior to use. The standard glucose Ringer solution consisted of 110.0 mM NaCl, 2.4 mM $KHCO_3$, 1.0 mM $CaCl_2$ and 10.0 mM glucose (222 mosM/kg $H_2O$; pH 8.2). Solution was freshly prepared from concentrated stock solutions immediately prior to each experiment, and was filtered with a Nalgene filtering unit (0.2-$\mu$m membrane, The Nalge Co., Inc., Rochester, New York) to remove bacteria. In early experiments (I–III), in order to inhibit bacterial growth 1 mg/ml of streptomycin sulfate (Pfizer Laboratories) and 0.5 mg/ml of sodium penicillin G (Upjohn) were added to the Ringer solution. Because some other preparations showed a progressive increase in $O_2$ consumption even in the presence of these antibiotics, we employed instead 80 $\mu$g/ml of gentamycin sulfate (Schering) in later experiments (IV–VIII and all subsequent experiments). This appeared more effective than penicillin and streptomycin, as judged by the stability of the control rate of $O_2$ consumption.

*Experimental protocols*

*(1) Standard experiments (I–VIII).* Each piece of skin was mounted in a modified Ussing–Zerahn lucite chamber of 7.1 $cm^2$ cross-sectional area, one piece (a)

to be used for a control, the other (b) for aldosterone treatment. The chambers were alternated in successive experiments. Each skin surface was bathed by Ringer solution. Electrical parameters were measured by standard means and the current was recorded continuously. The electrical potential difference $\Delta\psi$ was regulated with a voltage clamp, which permitted $\Delta\psi$ to be set at values ranging from 100 to $-100$ mV with a compensating circuit providing automatic correction for the potential drop between the voltage-sensing agar brigde tips and the membrane (A. Pandiscio, unpublished).

After an equilibration period of 2–5 h in the standard arrangement the air bubbler was detached, an oxygen electrode–micropump system was connected, and the $O_2$ consumption was determined by essentially the same procedure as reported previously[16]. The solution volume on each side was 16.7 ml. The $O_2$ concentration in each bathing solution was monitored continuously by means of Clark oxygen electrodes connected to polarographic circuits whose output voltages were proportional to $pO_2$. The total rate of $O_2$ consumption $J_r$ was evaluated from the slopes of the plots of the output voltages against time. The media were re-aerated at 6-min intervals. Following control observations the electrical potential was systematically varied and the steady-state values of $J_r$ were determined. Each perturbation of $\Delta\psi$ was maintained for 6 min and $J_r$ was evaluated during the final 3 min[13]. The sequence of potential perturbations was: $+50$, $-50$, 0, $+50$, $-50$ and 0 mV for Experiments I–III; $+50$, $-50$, 0, $+25$, $-25$, and 0 mV for Experiment IV; and 0, $+40$, $-40$, 0, $+20$, $-20$, 0, $+60$, and $-60$ mV for Experiments V–VIII. After completion of this procedure in both tissues, 10 $\mu$l of a methanol solution of d-21-aldosterone-acetate was added to the solution bathing the inner surface of skin (b), to give a concentration of $5 \cdot 10^{-7}$ M. The same volume of methanol was added to the control tissues (a). Both systems were then attached to air bubblers and the skins were maintained on open circuit for 14–18 h. Following this incubation period the bathing media were replaced with fresh Ringer solution; the inner solution of skin (b) again contained $5 \cdot 10^{-7}$ M aldosterone. Both chambers were then reconnected to the oxygen-electrode–micropump system and the short-cicuit current and the dependence of $J_r$ on $\Delta\psi$ were determined as on the previous day. The above measurements were used to calculate the apparent affinity "$A$" for each period (see analysis below).

(*2*) *Anaerobiosis experiments.* Skins were prepared as above except for the omission of the sterile technique. The paired abdominal skins were resected, mounted, and bathed in glucose Ringer solutions containing the standard concentration of gentamycin. Aldosterone to a concentration of $5 \cdot 10^{-7}$ M was added to the inside solution of one of the skins. The skins were maintained at open circuit with air bubbling for 14–16 h. The following day the bathing media were replaced with fresh glucose Ringer solutions, again with $5 \cdot 10^{-7}$ M aldosterone bathing the inner surface of the hormone-treated skin. The potential was then clamped at 0 mV and the short-circuit current was recorded continuously. About 1 h later the air supply was replaced with $N_2$ (Medical–Technical Gases, Inc., Medford, Mass., nominally 99.997% $N_2$; the findings on analysis were $N_2$, 99.875%; $O_2$, 0.125%; $CO_2$, 0.000%). After about an hour of exposure to $N_2$, bubbling with air was reinstituted.

(*3*) *Amiloride experiments.* Paired skins were prepared and mounted with the standard aseptic techniques. The inner solution of each chamber contained $5 \cdot 10^{-7}$ M aldosterone. The next day the standard glucose Ringer solutions were replaced

with fresh solutions, again containing $5 \cdot 10^{-7}$ M aldosterone in the inner baths. 1 or 2 h later, "$A$" was determined in the two skins as in the standard experiments by setting $\Delta\psi$ sequentially at 0, +30, −30, 0, +60, −60, 0 mV. $10^{-7}$–$10^{-5}$ M amiloride (Merck Sharp and Dohme) was then administered to the outside solution of skin (b), so as to decrease the level of short-circuit current to between 100 and 200 $\mu$A. If the short-circuit current later increased to a level higher than 200 $\mu$A, additional amiloride was administered as needed. 1 and 4 h after the administration of amiloride "$A$" was again determined in skins (a) and (b).

The electrical resistance was determined routinely prior to each measurement of "$A$" by alternately setting the electrical potential difference at +10 and −10 mV for a period of 15 s.

*Analysis of the data*

Active sodium transport in amphibian epithelial tissues has been analyzed previously in terms of a linear thermodynamic formulation[11,13]. In this view, for membranes exposed to identical solutions at each surface,

$$J_{Na} = L_{Na}(-F\Delta\psi) + L_{Na,r}A, \tag{1}$$

$$J_r = L_{Na,r}(-F\Delta\psi) + L_rA. \tag{2}$$

Here $J_{Na}$ and $J_r$ are the rates of net sodium transport and metabolism ($O_2$ consumption) respectively, the L's are phenomenological coefficients, F is the Faraday constant, $\Delta\psi$ is the electrical potential difference across the membrane, and $A$ is the affinity of the metabolic reaction. The previous demonstration of linear relationships between $J_{Na}$ and $\Delta\psi$[12] and between $J_r$ and $\Delta\psi$[13] supports the validity of Eqns 1 and 2 and the constancy of the L's and $A$ on brief perturbations of $\Delta\psi$. This suggests that

$$A = -I_0 / \{dJ_r/d(\Delta\psi)\}, \tag{3}$$

where $I_0 = F(J_{Na})_{\Delta\psi=0}$ is the short-circuit current. Accordingly, we have systematically studied the behavior of the quantity on the right-hand side of Eqn 3 in this paper, and have referred to it as the apparent affinity "$A$". (In the present experiments, where pressure, temperature, and chemical potentials are presumed approximately constant, the affinity represents the negative Gibbs free energy change $-\Delta G$ of the metabolic reaction driving active sodium transport.)*

The significance of the observations was evaluated by standard statistical means[17]. Results are expressed as the mean value ± the standard error of the mean (S.E.). Values of $P > 0.05$ were considered not significant and are not stated.

* Since the affinity appears to be constant on brief perturbation of $\Delta\psi$, we cannot rule out the necessity for incorporating higher-order terms in the affinity in Eqns 1 and 2. (The observed linearities rule out significant higher-order terms in $\Delta\psi$ or, more generally, the electrochemical potential difference of $Na^+$.) In a previous paper[13] it was stated that this consideration is immaterial for the calculation of $A$ according to Eqn 3. However, this assumes the validity of the Onsager reciprocal relation. To the extent that higher-order terms or mixed terms are significant, this assumption becomes questionable.

## RESULTS

### *1. Effects of aldosterone*

(*a*) *Short-circuit current.* Observations of the short-circuit current $I_0$ are shown in Fig. 1. On Day I there was a correlation between the paired skins, and no significant difference between them. This indicates the close similarity of paired tissues obtained from a single animal and validates the use of the paired *t* test. In the control skins no significant difference in short-circuit current was observed between Day I and Day II.

As reported previously[18], aldosterone enhances $I_0$ in the frog skin. The mean value in treated skins on Day II was higher than before treatment and higher than the simultaneous value in paired untreated control tissues.

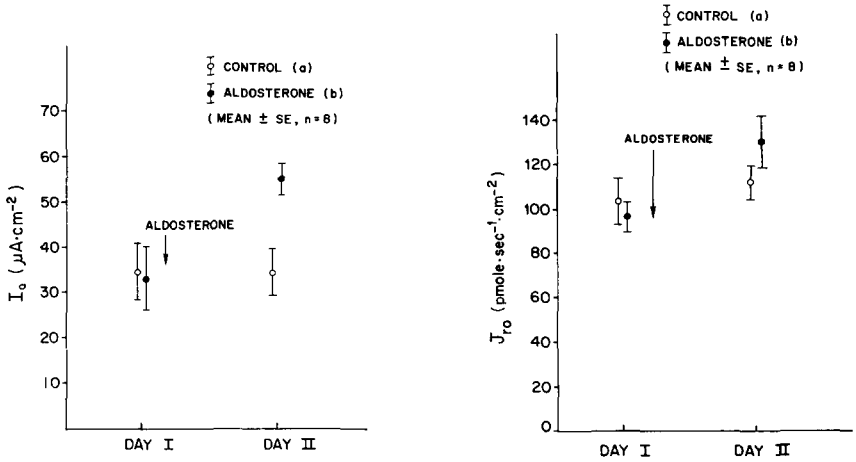



Fig. 1. Effect of aldosterone on the short-circuit current $I_0$. Each experiment was carried out on paired tissues (a) and (b). Group a were used as control tissues. Following the completion of measurements on Day 1, the tissues of Group b were exposed to $5 \cdot 10^{-7}$ M aldosterone. The skins were closely paired as indicated by an initial correlation coefficient $r = 0.99$ ($P < 0.001$). The value in treated skins on Day II was greater than before treatment ($P(b_I, b_{II}) < 0.02$) and higher than the simultaneous value in paired untreated control tissues ($P(a_{II}, b_{II}) < 0.005$).

Fig. 2. Effect of aldosterone on the rate of $O_2$ consumption at short circuit $J_{ro}$. On Day I, $r = 0.93$ ($P < 0.001$). The value in treated skins on Day II was greater than before treatment ($P(b_I, b_{II}) < 0.05$) but was not quite significantly higher than the simultaneous value in paired untreated control tissues ($0.1 > P(a_{II}, b_{II}) > 0.05$). (See legend to Fig. 1.)

(*b*) *$O_2$ consumption in the short-circuited state.* The rate of $O_2$ consumption in the short-circuited state $J_{ro}$ is shown in Fig. 2. On Day I there was a correlation between paired skins. Again, there was no significant difference between paired tissues on Day I, nor between the control skins on Day I and Day II. $J_{ro}$ increased in the presence of aldosterone. However, it is not clear that this increase could be entirely attributed to aldosterone, since there was also a slight (though non-significant) increase with time in the untreated skins. Possibly our failure to demonstrate a clear effect of aldosterone on $J_{ro}$ was because of variable bacterial metabolism following overnight incubation.

(*c*) *Dependence of $O_2$ consumption on the electrical potential difference.* As shown previously[13], in stable skins a linear relationship is observed between the rate of $O_2$ consumption and the electrical potential difference across the membrane. This is seen both in control tissues and in skins treated with aldosterone.

The slopes of the regression lines, $dJ_r/d(\Delta\psi)$, are shown in Fig. 3. Values in paired tissues on Day I were correlated. There was no significant difference between the paired tissues on Day I, nor between the control skins on Day I and Day II. A slight decrease in $-dJ_r/d(\Delta\psi)$ was observed in the presence of aldosterone, but this was not demonstrably significant.

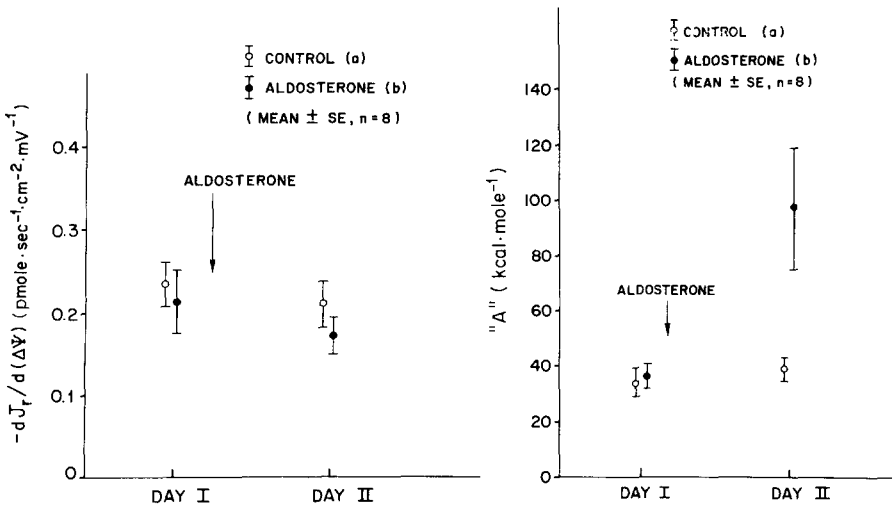



Fig. 3. Effect of aldosterone on the relation of the rate of $O_2$ consumption to the electrical potential difference $dJ_r/d(\Delta\psi)$. On Day I, $r=0.79$ ($P<0.025$). (See legend to Fig. 1.)

Fig. 4. Effect of aldosterone on the apparent affinity "$A$" (see Eqn 3). On Day I, $r=0.80$ ($P<0.025$). $P(b_I, b_{II})<0.025$; $P(a_{II}, b_{II})<0.025$. (See legend to Fig. 1.)

(*d*) *Apparent affinity of the metabolic reaction.* Values of the apparent affinity of the metabolic reaction, calculated according to Eqn 3, are shown in Fig. 4. As observed above for $I_0$, $J_{ro}$, and $dJ_r/d(\Delta\psi)$, there was a correlation between values of "$A$" in paired tissues on Day I. There was no significant difference between paired tissues on Day I, nor between the control skins on Day I and Day II.

All of the skins showed an increase in "$A$" following aldosterone, whether by comparison with the value before treatment, or with the value in the paired control skin. The mean value in the hormone-treated skins on Day II was 2.7 times that on Day I and 2.5 times the value in the control skins on Day II.

2. *Effects of amiloride in the presence of aldosterone*

The accurate calculation of "$A$" requires stable and vigorous function. In order to assure this even after depression of $I_0$ by amiloride, all tissues were exposed to aldosterone overnight prior to study.

(*a*) *Short-circuit current and conductance.* Values of the short-circuit current are

shown in Fig. 5. Prior to the administration of amiloride there is no significant difference between paired skins. Sufficient amiloride was administered to the experimental skins (b) to depress the short-circuit current to less than half of control value. These levels were maintained throughout the course of the experiment. As would be expected of an agent which acts at a permeability barrier, a fall in conductance is associated with the fall in short-circuit current (Fig. 6). Following these studies amiloride was removed by repeated washings in several skins; a transient overshoot of $I_0$ was observed as compared to the washed control skins.

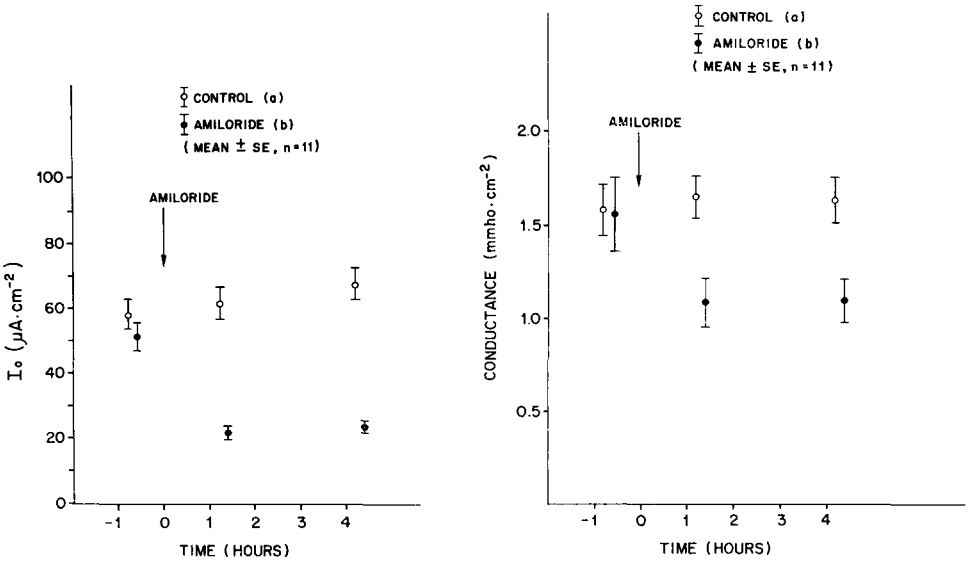



Fig. 5. Effect of amiloride on the short-circuit current $I_0$ in the presence of aldosterone. All tissues were exposed to $5 \cdot 10^{-7}$ M aldosterone. Each experiment was carried out on paired tissues (a) and (b). Group a were used as control tissues. Following initial measurements, the outer surfaces of the tissues of Group b were exposed to $10^{-7}$–$10^{-5}$ M amiloride, sufficient to depress $I_0$ to less than half of control levels. Initially there was no significant difference between paired skins, but in this series there was no significant correlation. The value of $I_0$ in amiloride-treated tissues was less than prior to treatment both 1 h and 4 h after the administration of amiloride ($P(b_{-1}, b_1) < 0.001$; $P(b_{-1}, b_4) < 0.001$). The value in treated skins was also less than the simultaneous value in untreated skins ($P(a_1,b_1) < 0.001$; $P(a_4,b_4) < 0.005$).

Fig. 6. Effect of amiloride on the conductance in the presence of aldosterone. Initially there was no significant difference (or correlation). $P(a_1, b_1) < 0.005$; $P(a_4, b_4) < 0.001$. (See legend to Fig. 5.)

(*b*) *$O_2$ consumption in the short-circuited state.* The rate of $O_2$ consumption in the short-circuited state differed insignificantly between the paired skins untreated with amiloride. The administration of amiloride resulted in a sustained depression of $J_{ro}$ (Fig. 7).

(*c*) *Dependence of $O_2$ consumption on the electrical potential difference.* The slopes $-dJ_r/d(\Delta\psi)$ in paired skins initially differed insignificantly. The administration of amiloride resulted in a sustained depression of this quantity (Fig. 8).

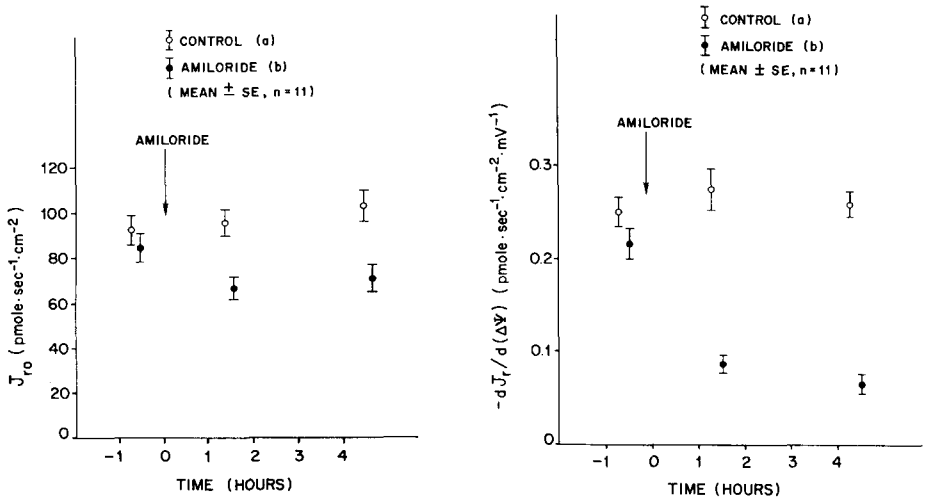



Fig. 7. Effect of amiloride on the rate of $O_2$ consumption at short circuit $J_{ro}$ in the presence of aldosterone. Initially there was no significant difference (or correlation). In this series $J_{ro}$ in the untreated tissues increased with time ($P(a_{-1}, a_4) < 0.005$). There was a difference between the simultaneous values in treated and untreated skins ($P(a_1, b_1) < 0.001$; $P(a_4, b_4) < 0.001$). (See legend to Fig. 5.)

Fig. 8. Effect of amiloride on the relation of the rate of $O_2$ consumption to the electrical potential difference $dJ_r/d(\Delta\psi)$ in the presence of aldosterone. Initially there was no significant difference (or correlation). $P(b_{-1}, b_1) < 0.001$; $P(b_{-1}, b_4) < 0.001$; $P(a_1, b_1) < 0.001$; $P(a_4, b_4) < 0.001$. (See legend to Fig. 5.)

(*d*) *Apparent affinity of the metabolic reaction.* Values of the affinity of the metabolic reaction, again calculated according to Eqn 3, are shown in Fig. 9. In this series of studies in the presence of aldosterone values of "*A*" were some 60 kcal/mole, and differed insignificantly in paired tissues untreated with amiloride.

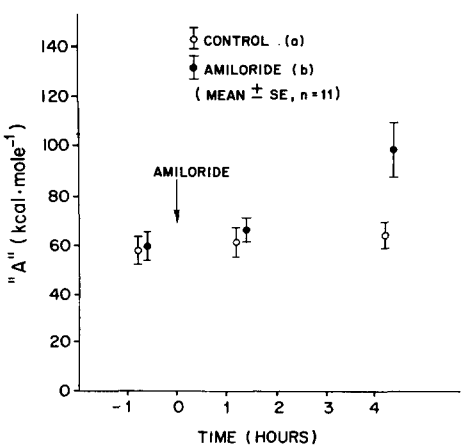



Fig. 9. Effect of amiloride on the apparent affinity "*A*" in the presence of aldosterone (see Eqn 3). Initially there was no significant difference; the initial correlation coefficient $r = 0.63$ ($P < 0.05$). 4 h following the administration of amiloride the value of "*A*" was greater than prior to treatment ($P(b_{-1}, b_4) < 0.005$) and greater than in untreated skins ($P(a_4, b_4) < 0.02$). (See legend to Fig. 5.)

An hour following the administration of amiloride no significant effect on "$A$" was demonstrable. 3 h later "$A$" had increased to about 50% above control level.

## DISCUSSION

Previous attempts to define the mechanism of action of aldosterone have utilized a series of diverse approaches. Investigations of the permease function have depended on radioactive labelling, electrical effects, and measurements of sodium content of isolated cells. The pump theory has largely been evaluated by means of ($Na^+$–$K^+$)-ATPase studies. The metabolic theory has been studied biochemically from several standpoints using a variety of substrates, intermediates, and inhibitors[1,2].

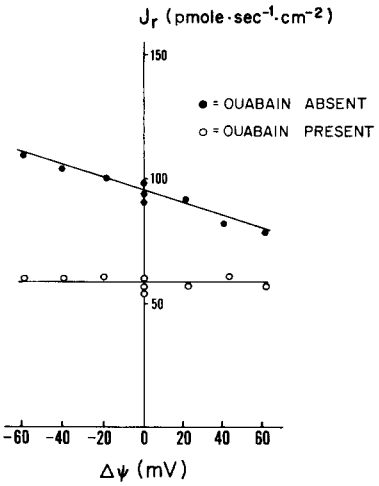
The present results provide some insight as to the mechanism of aldosterone action from another point of view. For one thing, it should be noted that aldosterone cannot act solely by increasing the number of active transport units operating in parallel, since this would cause $I_0$ and $-dJ_r/d(\Delta\psi)$ to increase proportionally. For another, aldosterone cannot act solely by having the converse effect to that of amiloride on the permeability, since both agents increase the ratio $-I_0/\{dJ_r/d(\Delta\psi)\}$ although they have opposite effects on $I_0$. Aldosterone does in fact affect electrical conductance[4] and sodium permeability in toad bladder[19], but in frog skin the above observations indicate this cannot be its only effect. Energetic factors must be involved.

Attempts to evaluate energetic factors directly have generally involved measurements of high-energy intermediates. Such measurements suffer from the shortcoming that the mean cellular concentrations determined do not necessarily reflect the effective concentrations at the site of transport. In studies of this type in toad bladder aldosterone has been observed to cause a significant decrease in the creatine phosphate: creatine ratio, but no detectable change in the ATP: ADP ratio[20]. Somewhat different findings were reported by Kirsten *et al.*[21] for rat kidney.

Energetic problems are usefully approached with the aid of the formalism of linear nonequilibrium thermodynamics. If the formalism is appropriate for this system, the determination of the short-circuit current $I_0$ and the dependence of the rate of $O_2$ consumption on the electrical potential difference $dJ_r/d(\Delta\psi)$ permits the evaluation of the affinity of an oxidative metabolic reaction "driving" sodium transport. While biochemical assays reflect total tissue content of metabolites, the affinity determined in this way should be that pertinent to the function of the sodium pump. The previous demonstration of linear relationships between the rate of active sodium transport and $J_r$ and $\Delta\psi$ support the validity of this formulation.

In attempting to apply the formalism for the present purposes, the following preliminary considerations are of significance:

(1) It is necessary that the dependence of $J_r$ on $\Delta\psi$ be specific, reflecting the function of the active transport system, rather than other aspects of tissue metabolism. This has previously been shown to be the case in the absence of aldosterone, as was indicated by the insensitivity of $J_r$ to $\Delta\psi$ when sodium transport is blocked by ouabain. This issue was re-examined here. As is shown in Fig. 10, in the presence of aldosterone as well as in its absence the dependence of $J_r$ on $\Delta\psi$ is abolished by ouabain.



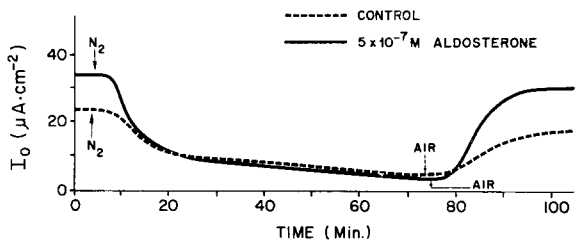




Fig. 10. Dependence of the rate of $O_2$ consumption $J_r$ on the electrical potential difference $\Delta\psi$ in the aldosterone-treated frog skin; influence of ouabain ($10^{-3}$ M) added 30 min prior to perturbation of $\Delta\psi$.

Fig. 11. Effect of anaerobiosis on short-circuit current in control and aldosterone-treated frog skins.

(2) The application of Eqn 3 requires that $I_0$ represent active sodium transport attributable to oxidative metabolism. In our previous studies of frog skins, in the absence of aldosterone, "glycolytic sodium transport" (*i.e.* sodium transport dependent on glycolysis) appeared to be small[16]. However, although it is clear that the stimulation of active sodium transport by aldosterone is highly dependent on oxidative metabolism[1,2], Handler *et al.*[20] have reported that in anaerobic toad bladders the short-circuit current is appreciably higher in the presence of aldosterone than in its absence. In order to determine the behavior of anaerobic active sodium transport in the present study, the short-circuit current under anaerobic conditions was examined in five pairs of skins, with one member of each pair serving as a control and the other exposed to aldosterone. A typical result is shown in Fig. 11. As is seen, the short-circuit current was significantly enhanced by aldosterone in the presence of air, but fell to the same level in the two tissues during anaerobiosis. Thus, in this study, the enhancement of active sodium transport by aldosterone is completely dependent on aerobic metabolism.*

* Since transport persisted at a significant rate during anaerobiosis it may be asked what effect this would have on the validity of our calculations of free energies. To the extent that glycolytic metabolism supports active transport, even in the presence of $O_2$, the use of uncorrected values of $I_0$ in Eqn 3 would of course lead to overestimates of $A$ for the oxidative reaction. However, presuming glycolytic transport to be equal in the presence and absence of aldosterone as in Fig. 11, the (fractional) error would be greater in the control tissue; under these conditions glycolytic sodium transport would if anything lead to an underestimate of the difference in $A$ between control and aldosterone-treated tissues. Handler *et al.*[20] found that aldosterone induced a ouabain-sensitive increment in aerobic glycolysis in toad bladders exposed to solutions equilibrated with 97% $O_2$–3% $CO_2$. However, these findings seem not directly relevant to our results since they also found an aldosterone-induced increment with solutions equilibrated with 97% $N_2$–3% $CO_2$. This difference presumably reflects the use of different tissues, solutions, and gases.

It might have been expected that following prolonged incubation tissue function would deteriorate owing to substrate depletion. As shown in Figs 1 and 4, however, there was no significant change in either $I_0$ or "$A$" in the control skins, even after 14 to 18 h of incubation. This stability of function may have reflected the use of 10 mM glucose in the Ringer solutions, and the maintenance of an open circuit during overnight incubation. In this state, as shown above, oxidative metabolism is diminished, presumably minimizing the rate of depletion of tissue substrates.

Despite the admitted complexity of the biochemistry of the system, it is worthwhile speculating on the significance of the observed changes in the apparent affinity. From a simple point of view it might be assumed that "$A$" is determined by the substrate–product activity ratio of some reaction intimately associated with transport. In this view it would be expected that any factor acting primarily to alter the rate of sodium transport would have a predictable effect on the affinity. Thus, decreasing the rate of sodium transport would be expected to result in increased accumulation of substrates with a resultant increase in the affinity, whereas increasing the rate of sodium transport would have the opposite effect. This picture is quite consistent with our previous results in frog skin, where perturbations in the potential for 10 min or more produced a "memory effect"[13]. Accelerating the rate of sodium transport by negative clamping transiently lowered the subsequent short-circuit values of current and $O_2$ consumption relative to control levels. Positive clamping induced the opposite effects.

This picture also permits an interpretation of the amiloride studies. Since this substance is thought to act exclusively at the outer permeability barrier[5,15], its effects on metabolism should be attributable to its depression of sodium entry. It might be anticipated, therefore, that following the application of amiloride the substrate level would gradually rise. This picture is consistent with the results observed. An hour following the depression of sodium transport by amiloride the apparent affinity was not demonstrably different from its value in the control state. 4 h after amiloride "$A$" had risen appreciably. This increase in affinity would account for the overshoot of the short-circuit current observed following the removal of amiloride.

The above model may also be applied to the mechanism of action of aldosterone. If aldosterone acts either by increasing the permeability of the outer barrier, or by activation of sodium pumps, there would be an increased rate of utilization of substrates. With time, in the absence of compensating regulatory mechanisms, this might be expected to result in a decrease of the affinity. On the other hand, if aldosterone increases the local concentrations of substrates which support transport, the affinity might be expected to increase. The present results support the likelihood of the latter mechanism, but do not exclude a direct effect on sodium permeability or the pump as well.

We appreciate of course that other interpretations of our results are possible. As mentioned, our treatment requires linearity in $A$. Furthermore, the thermodynamic formulation may reflect the affinity of different metabolic pools before and after aldosterone. This might occur in either of two ways. On the one hand, different regions of the metabolic chain might show constancy of the affinity on perturbation of $\Delta\psi$. On the other hand the sloughing of superficial layers of skin following aldosterone may result in our seeing the affinity of a different cell population[18]. Never-

theless we feel that these data are suggestive, and emphasize the need for studies designed to correlate biochemical and thermodynamic concepts.

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