SUMMARY

Prolonged incubation with aldosterone causes an increase in the rate of sodium transport and in the rate of glycolysis in the urinary bladder of the toad (*Bufo marinus*) *in vitro*. When sodium transport was inhibited by ouabain after the effect of aldosterone was manifest, the rate of sodium transport as well as the rate of glycolysis fell to the same low value in aldosterone-treated tissue and in paired control tissue that had been incubated without hormone. When bladders were made anaerobic after the aldosterone effect was manifest, short circuit current fell in both preparations, but remained significantly higher in aldosterone-treated tissue than in paired control tissue. During anaerobiosis, the rate of lactate production was higher in aldosterone-treated tissue than in paired anaerobic control tissue. Thus the effect of aldosterone on glycolysis appears to be related to its effect on sodium transport and not secondary to an effect of the hormone on energy production.

It is generally agreed that the aldosterone-induced stimulation of sodium transport by aldosterone (or an aldosterone-induced protein) is a direct effect on sodium transport and not secondary to an effect of the hormone on energy production.

glycolytic pathway is also stimulated by the hormone, as reflected by increased glycogenolysis (4) and increased oxidation of 6-3C-glucose (5). This study is concerned with the mechanism of the stimulation of glycolysis by aldosterone.

Experiments are described indicating that the effect of aldosterone on glycolysis appears to be coupled to its effect on sodium transport as previously noted for the effect of the hormone on oxidative metabolism. Measurement of the concentration of glycolytic intermediates in extracts of bladders stimulated by aldosterone reveals evidence of increased activity in situ of the enzymes phosphofructokinase and pyruvate kinase. There was no change in the concentration of ATP in tissue incubated with aldosterone, but there was a significant fall in the concentration of creatine phosphate. The latter is interpreted as evidence against the suggestion (6) that the aldosterone-induced stimulation of sodium transport is primarily the result of stimulation of energy metabolism.

METHODS

*Bufo marinus* originating in the Dominican Republic were purchased from National Reagents, Bridgeport, Connecticut. Toads were kept on Sanicel (Paxton Processing Company, Paxton, Illinois) moistened with tap water until 1 to 3 days before use when they were kept in a shallow bath of 0.6% NaCl to minimize aldosterone secretion. Urinary bladders were dissected from pithed toads and incubated at room temperature in an amphibian Ringer’s solution composed of 90 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1 mM CaCl2, 0.5 mM KH2PO4, 0.5 mM MgSO4, 5 mM glucose, 0.1 mg per ml of streptomycin, and penicillin, and gassed with a mixture of 97% O2-3% CO2 (final pH = 7.0, 220 mosm per kg of H2O).

Experiments were begun in the afternoon and concluded the following morning. After two initial changes of Ringer’s solution during the 1st hour of incubation, the tissue was incubated overnight in abundant (20 mM) glucose, with or without 10-7 M aldosterone. The next morning, the bathing media were replaced twice with fresh Ringer’s solution (5 mM glucose), and the effects of aldosterone were observed.

The rate of sodium transport was estimated by intermittently short circuiting the bladders using the technique of Ussing and Zerahn (7) in chambers that permitted study of two contiguous 3.2-cm2 areas of the same hemibladder. Bladders with initial open circuit potentials of less than 10 mv were discarded. An initial stable short circuit current was recorded for each area of bladder before the addition of aldosterone. The effect of aldo-
sterone was observed the next morning by measuring the short circuit current (at 18 hours) after the two changes of Ringer's solution. Short circuit current was stable for several hours at this time (18 hours after starting the experiment). The effect of inhibitors on these preparations was examined subsequently by measuring the short circuit current intermittently over a 90-min period after the addition of the inhibitor. The short circuit current at 18 hours (SCC_{18 hr}) was related to the initial stable short circuit current (SCC_0) for each area and the ratio

$$\frac{\text{Aldosterone} \text{ SCC}_{18 \text{ hr}}}{\text{Control} \text{ SCC}_0}$$

was used as an estimate of the effect of aldosterone compared with "steroid depletion." The same relationship between the short circuit current after addition of inhibitor to the initial short circuit current was calculated for each preparation.

Glycolytic flux and the concentration of glycolytic intermediates and cofactors were measured at a time corresponding to the 18 hour short circuit current measurement. These incubations were carried out in Erlenmeyer flasks in a metabolic shaker, but otherwise followed the same protocol for composition and changes of the Ringer's solution. The rate of lactate production was estimated by sampling the Ringer's solution at the end of a 60-min period. The rate of glucose oxidation was estimated by adding uniformly labeled 14C-glucose (0.05 PCi per ml) to the Ringer's solution, and collecting all of the 14CO_2 produced over an hour using techniques described previously (8). When glycolytic flux was measured in bladders incubated anaerobically or with ouabain, the tissue was incubated under these conditions for 60 min and then transferred to fresh anaerobic Ringer's solution or Ringer's solution containing ouabain for the 60-min glycolytic flux period.

The method of preparing extracts of tissue for estimation of the concentration of metabolites was designed to minimize changes during the extraction procedure, and has been described previously, as have the analytic techniques (9). Except for the colorimetric measurement of creatine, these are enzymatic assays coupled to the oxidation or reduction of pyridine nucleotides which were estimated spectrophotometrically or fluorometrically. Inorganic phosphate was measured enzymatically (10) in this study and yielded values about 30% lower than those estimated colorimetrically. Recovery of P_i added to extracts was close to 100% with each method. Presumably acidification during the colorimetric assay resulted in hydrolysis of some organic phosphates.

Aldosterone and ouabain were purchased from Calbiochem, antimyein A from Sigma.

RESULTS

The effect of 18 hours of incubation with and without aldosterone on short circuit current is indicated in Figs. 1 and 2. Compared to base-line (initial short circuit current), there was a slight fall in short circuit current in the control preparation and a 2- to 4-fold rise in the preparation incubated with the hormone. Thus, aldosterone caused a 4- to 6-fold stimulation of short circuit current compared with paired control ("steroid depleted") tissue. At the same time, the glycolytic flux was approximately doubled in the aldosterone-treated tissue, as estimated by the rate of lactate production and glucose oxidation (Table I, Experiment A).

The relationship between the effects of aldosterone on glycolysis and on sodium transport was examined when the effects of the hormone were well established by adding ouabain, an inhibitor of sodium transport. Following the addition of ouabain at 18 hours, short circuit current fell to about the same level in both preparations (Fig. 1), as did the rate of glycolysis (Table I, Experiment B). A similar relationship between the effect of aldosterone on sodium transport and on the oxidation of a number of tricarboxylic acid cycle substrates has been demonstrated previously (11). Indeed, it has been suggested that aldosterone acts by stimulating the synthesis of an enzyme involved in oxidative metabolism with the resulting enhancement of energy production directly causing the aldosterone enhancement of sodium transport (6). The validity of this assumption was tested by making the bladders anaerobic after the 18-hour aldosterone effect had occurred. When the gas
TABLE I

Effect of aldosterone on glycolysis

The values for aldosterone are the mean differences ± standard error from paired control tissue.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lactate production</th>
<th>Uniformly labeled 14C-glucose → 14CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of experiments</td>
<td>Control</td>
</tr>
<tr>
<td>A*</td>
<td>16</td>
<td>8.96</td>
</tr>
<tr>
<td>B* (10^-4 M ouabain)</td>
<td>8</td>
<td>4.28</td>
</tr>
<tr>
<td>C* (N2 + 0.5 mM CN-)</td>
<td>14</td>
<td>62.8</td>
</tr>
</tbody>
</table>

* Ringer's solution equilibrated with 97% O2-3% CO2.

* Ringer's solution equilibrated with 97% N2-3% CO2.

A, an inhibitor of electron flow in the cytochrome system has also been reported to eliminate the aldosterone effect on short circuit current (3). Therefore, in another group of experiments, 2 × 10^-6 M antimycin A was added at the same time that the gas phase was changed to 97% N2-3% CO2. The short circuit current ratio before addition of inhibitors was 5.43; 60 min later it was 3.78 (N = 4). The previously reported anaerobic experiments were performed in a low (2.4 mM) bicarbonate Ringer's solution gassed with air or 100% N2, final pH 8.1. Therefore, the protocol of Fig. 2 was repeated using a 2.4 mM HCO3- medium (12). Under these conditions, the results were similar to those in the literature in that the open circuit potential and short circuit current were lower than those observed when the 25 mM HCO3- Ringer's solution was used, particularly during anaerobic incubation. Mean values in the two groups of experiments are summarized in Table II. Detailed comparison between the two sets of experiments are not valid since the two groups of data were not obtained from paired tissue. Other differences in experimental protocol between these and experiments reported by others include the use of abundant glucose throughout the experiments (rather than none until 30 min after anaerobiosis was started (1)) and the imposition of anaerobiosis after prolonged exposure to aldosterone (rather than after 3 hours of incubation with the hormone (6)). Finally, although the gas used in the anaerobic experiments of Fig. 2 was certified by the supplier (Air Products and Chemicals, Inc.) to contain less than 0.07% oxygen, it is conceivable that complete anaerobiosis was not achieved. However, since 0.5 mM cyanide or 2 × 10^-4 M antimycin A was added to the vigorously gassed Ringer's solution, it seems reasonable to assume that oxidative metabolism was completely eliminated. The effect of cyanide and anaerobiosis was completely reversible (Fig. 2). Under the anaerobic conditions of Fig. 2, the rate of lactate production was elevated in both preparations as compared to the rate under aerobic conditions (Table I, Experiment A), and there was still a greater rate of lactate production in the tissue incubated with aldosterone (Table I, Experiment C). Thus, under the conditions of these experiments, stimulation of short circuit current by aldosterone was still apparent in the absence of oxidative metabolism, and was associated with enhanced glycolysis. In these experiments, whenever aldosterone stimulated sodium transport, there was also stimulation of glycolysis.

The control of glycolysis during stimulation by aldosterone under aerobic conditions was examined by measuring the concentration of glycolytic intermediates and cofactors in the tissue at a time corresponding to 18 hours in Figs. 1 and 2. The
TABLE II

Comparison of results during anaerobiosis with Ringer's solution containing 25 mM HCO₃⁻ and 2.4 mM HCO₃⁻

Mean values for open circuit potential and short circuit current, initially (0 hours), after 18 hours of incubation with (+) and without (−) aldosterone, and after 60-min anaerobic incubation with 0.5 mM cyanide or 2 × 10⁻⁴ M antimycin A.

<table>
<thead>
<tr>
<th></th>
<th>0 hours</th>
<th>18 hours</th>
<th>Anaerobiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Open circuit potential</td>
<td>Short circuit current</td>
<td>Open circuit potential</td>
</tr>
<tr>
<td></td>
<td>μV</td>
<td>μA</td>
<td>μV</td>
</tr>
<tr>
<td>Ringer's solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mM HCO₃⁻</td>
<td>41</td>
<td>26</td>
<td>101</td>
</tr>
<tr>
<td>2.4 mM HCO₃⁻</td>
<td>35</td>
<td>31</td>
<td>53</td>
</tr>
</tbody>
</table>

* N = 11.

** N = 7.

Fig. 3. The effect of aldosterone on the concentration of glycolytic intermediates. The ordinate is the concentration of each intermediate in tissue incubated for 18 hours with aldosterone divided by the concentration in paired control tissue, expressed as a percentage. Vertical lines, mean ± 1 S.E. G6P, glucose-6-P; F6P, fructose-6-P; FDP, fructose-1,6-di-P; 3PG, 3-P-glycerate; PEP, P-enolpyruvate; PYR, pyruvate; LACT, lactate.

concentration of glycolytic intermediates in tissue incubated with aldosterone compared with the paired control tissue is illustrated in Fig. 3. In aldosterone-treated tissue there was a fall in the concentration of glucose-6-P and fructose-6-P and a rise in the concentration of fructose-1,6-di-P and triose-P (dihydroxyacetone-P + glyceraldehyde-3-P) indicating activation of the enzyme phosphofructokinase. Similarly, the fall in the concentration of P-enolpyruvate and rise in the concentration of pyruvate indicate increased activity of pyruvate kinase in tissue incubated with aldosterone. These have been shown previously to be glycolytic control points in the toad bladder (9). The concentrations of glycogenic intermediates in bladders examined after prolonged incubation with glucose were higher in this study than in the previous study in which bladders were incubated for a shorter period in the absence of added substrate. The difference in metabolite levels is primarily the result of incubation with glucose. The addition of glucose to Ringer's solution causes an elevation in the concentration of metabolites in tissue examined after just a few hours of incubation in vitro to concentrations similar to those in Fig. 3.*

* J. S. Handler, A. S. Preston, and J. Orloff, unpublished observations.

TABLE III

Effect of aldosterone on concentration of some cofactors and metabolites

The mean concentration for each cofactor in tissue incubated without aldosterone (control) is tabulated, as well as the mean ratio of the concentration in tissue incubated with aldosterone to the concentration in paired control tissue ± the standard error; N = 7.

<table>
<thead>
<tr>
<th>Cofactors</th>
<th>Mean concentration in control (μmoles/g dry wt %)</th>
<th>Aldosterone:control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>8.44</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>ADP</td>
<td>1.93</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>0.177</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>P_i,</td>
<td>9.77</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>Creatine-P</td>
<td>11.70</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>Creatine</td>
<td>6.04</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>2,3-Di-P-glycerate</td>
<td>0.663</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>0.436</td>
<td>235 ± 16</td>
</tr>
</tbody>
</table>

The effect of aldosterone on the concentration of some cofactors and other metabolites is summarized in Table III. Aldosterone had no significant effect on the concentration of ATP, ADP, 5'-AMP, or inorganic phosphate. Changes in the concentration of these cofactors are known to affect the activity of phosphofructokinase (13) and pyruvate kinase (14), and appear to be responsible for their activation in the toad bladder when glycogenolysis is stimulated by anaerobiosis (9). Aldosterone did cause a fall in the concentration of creatine-P and a rise in the concentration of creatine, changes that appear to reflect changes in the rate of sodium transport (see "Discussion"). Sharp et al. (15) found that aldosterone had no effect on the concentration of creatine-P, ATP, or ADP in the toad bladder. They reported concentrations about 80% lower than those in Table II. Their lower values and their failure to detect a change in creatine-P are presumably the results of loss during extraction. Aldosterone also caused a fall in the concentration of 2,3-di-P-glycerate and a rise in the concentration of α-glycerophosphate. The change in α-glycerophosphate probably reflects a shift in the concentration of cytoplasmic DPN-DPNH as do the changes in lactate relative to pyruvate (Fig. 3).
DISCUSSION

The stimulation by aldosterone of sodium transport and glucose oxidation in these experiments is generally similar to that reported by other investigators (1, 5, 12). The effect of aldosterone on lactate production has not been examined previously. Since ouabain, an inhibitor of sodium transport, eliminated the effect of aldosterone on glycolysis (Fig. 1 and Table I, Experiment B), it would appear that the effect of the hormone on glycolysis is coupled to its effect on sodium transport. In view of the fall in short circuit current during anaerobiosis there can be no doubt that oxidative metabolism is necessary for high rates of sodium transport by the toad bladder and for full expression of the effect of aldosterone. Assuming that oxidative metabolism was completely blocked, the data in Fig. 1 indicate that whatever the effect or effects of aldosterone or the aldosterone-induced protein, it does not depend entirely upon oxidative metabolism.

The data in Fig. 3 indicate that the stimulation of glycolytic flux by aldosterone is a result of activation of the enzymes, phosphofructokinase and pyruvate kinase. The same enzymes were shown to be rate-controlling in the toad bladder when sodium transport and glycogenolysis were stimulated by arginine vasotocin under aerobic or anaerobic conditions, and when sodium transport and glycogenolysis were inhibited by ouabain or by the substitution of Tris+ for all sodium in the Ringer's solution. Kirsten et al. (16) have assayed the activity of aldolase, pyruvate kinase, and lactate dehydrogenase in homogenates of epithelial cells of bladders exposed to aldosterone. They found no change in the activity of these enzymes at a time when they could detect changes in the activity of some mitochondrial enzymes. The discrepancy between their observations and the activation of pyruvate kinase shown in the present study probably results from their estimation of enzyme activity in vitro under optimal conditions, whereas measurement of the concentration of intermediates as employed in Fig. 3 reflects the activity of enzymes in situ.

Although changes in the concentration of ATP, ADP, 5'-AMP, and inorganic phosphate may contribute to the activation of phosphofructokinase and pyruvate kinase in the toad bladder during anaerobiosis (9), the concentration of these cofactors was unchanged when these enzymes were activated during incubation with aldosterone (Fig. 3 and Table III). There was also no change in the concentration of these cofactors in other experiments in which changes in the rate of sodium transport were associated with parallel changes in the activity of phosphofructokinase and pyruvate kinase which were reflected in changes in the rate of glycogenolysis (9). The experiments with aldosterone thus provide further evidence of a link between the rate of active sodium transport by the toad bladder and the activity of phosphofructokinase and pyruvate kinase. Since the techniques used in these studies measure the concentration of metabolites in the entire tissue rather than in the microenvironment of an enzyme, it is possible that physiologically significant changes in the concentration of these cofactors are associated with changes in the rate of sodium transport and were not detected. Other metabolites such as citrate have been shown to exert regulatory effects upon phosphofructokinase activity (17–19), and may be responsible for the change in enzyme activity in these experiments.

The shift in the concentration of creatine-P and creatine in tissue incubated with aldosterone (Table III) is similar to that seen in other experiments in which sodium transport by the toad bladder was stimulated by arginine vasotocin under aerobic and anaerobic conditions, and is in the opposite direction to that observed when sodium transport was inhibited by ouabain (9). These changes are summarized in Table IV. When sodium transport was stimulated (arginine vasotocin-02, arginine vasotocin-04, and aldosterone), the concentration of creatine-P fell, and when sodium transport was inhibited by ouabain, the concentration of creatine-P rose with reciprocal changes in the concentration of creatine. These changes in the concentration of creatine-P and creatine are those which are to be expected if energy expenditure by sodium transport precedes the regeneration of high energy phosphate by metabolism. Thus, the fall in the concentration of creatine-P is tissue incubated with aldosterone is not compatible with the suggestion (6) that the rate of sodium transport is energy limited in steroid-depleted tissue and that aldosterone stimulates sodium transport primarily by stimulating the production of energy. It seems more likely that aldosterone (or an aldosterone-induced protein) directly affects sodium transport in addition to whatever effects it may have on metabolic processes in the toad bladder.

### Table IV

**Correlation between changes in rate of sodium transport and changes in concentration of creatine phosphate and creatine**

The data, in part from Reference 9, are the concentration in experimental tissue divided by the concentration in paired control tissue, ± the standard error, expressed as a percentage. The arrows represent the direction of change of short circuit current in the experimental tissue.

<table>
<thead>
<tr>
<th></th>
<th>AVT+Ou</th>
<th>AVT-Na</th>
<th>Ouabain</th>
<th>Aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short circuit current</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Creatine-P</td>
<td>91 ± 2</td>
<td>90 ± 6</td>
<td>110 ± 3</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>Creatine</td>
<td>111 ± 3</td>
<td>102 ± 1</td>
<td>86 ± 3</td>
<td>119 ± 4</td>
</tr>
<tr>
<td>No. of experiments</td>
<td>12</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

*Arginine vasotocin.
*Arginine vasotocin during anaerobic incubation.

There were no changes in the concentration of creatine-P and creatine in experiments in which sodium transport was inhibited by substituting Tris+ for sodium in Ringer's solution (9). These results are not included in the tabulation. As discussed previously (9), it is likely that in tissue incubated with Tris+, metabolism is affected by a number of factors in addition to the diminished rate of sodium transport.

### References

The Effect of Aldosterone on Glycolysis in the Urinary Bladder of the Toad
Joseph S. Handler, Agnes S. Preston and Jack Orloff


Access the most updated version of this article at [http://www.jbc.org/content/244/12/3194](http://www.jbc.org/content/244/12/3194)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/244/12/3194.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/244/12/3194.full.html#ref-list-1](http://www.jbc.org/content/244/12/3194.full.html#ref-list-1)