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(Na^+-K^+)-ATPase FROM THE FROG BLADDER AND ITS RELATIONSHIP TO SODIUM TRANSPORT

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SUMMARY

1. (Na^+-K^+)-ATPase activity in the bladder and kidney of frog (*Rana catesbiana*) and toad (*Bufo bufo japonicus*) was measured, and the effects of cardiac steroids and temperature on the enzyme activity observed.

2. Active Na^+ transport in the urinary bladder was measured by the short-circuit current (s.c.c.) method in the presence or absence of cardiac steroids and at various temperatures.

3. The systems for measuring the (Na^+-K^+)-ATPase activity from *R. catesbiana* and the s.c.c. shared the following characteristics:

a. Both systems required Na^+ and K^+ together.

b. Both systems were completely inhibited by ouabain and digitoxigenin at a concentration of $1 \cdot 10^{-5}$ M. The s.c.c. was inhibited when these substances were added to the serosal surface of the membrane.

c. Inhibition by digitoxigenin was reversible in both systems.

d. Concentrations for half-maximal inhibition by digitoxigenin in the presence of Na^+ *plus* K^+ were $3 \cdot 10^{-7}$ M for the enzyme and $4 \cdot 10^{-7}$ M for the s.c.c. at 22°.

e. Both systems showed the same characteristic temperature dependency. Q_{10} values were 2.1 (over 13°) and 3.7 (under 13°) for the enzyme, and 2.0 (over 13°) and 3.5 (under 13°) for the s.c.c. respectively.

f. The electrothermodynamic work needed for the s.c.c. did not exceed the amount of free energy released from hydrolysis of ATP by the (Na^+-K^+)-ATPase system.

4. In conclusion, the greater part of the active Na^+ transport through the urinary bladder of *R. catesbiana* was closely related to (Na^+-K^+)-ATPase activity.

INTRODUCTION

The close relationship between active Na^+ transport and (Na^+-K^+)-ATPase has been well established since SKOU[1] discovered the enzyme. On the other hand, many other alternative transport systems have also been proposed even among

Abbreviation: s.s.c., short-circuit current.

mammalian tissues, such as proton excretion in the stomach, cation transport through the mitochondrial membranes, Na^+ reabsorption during urine formation in the urinary tubules, *etc.* Particularly, it has been suggested that a part of sodium reabsorption is condensed through another pathway in urine formation[2,3]. The active Na^+ transport through the urinary bladder of toad, which is thought to be an equivalent tissue to the distal segment of the nephron in the mammalian kidney from the morphological and physiological point of view, was not inhibited completely by cardiac glycosides even at a concentration of $1 \cdot 10^{-4}$ M (ref. 4).

The present study was aimed at investigating the possibility of an alternative active transport system. Some characteristics of (Na^+-K^+)-ATPase obtained from the urinary bladder of toad as well as of *Rana catesbiana*, which is far more susceptible to cardiac glycosides, will be described and compared with the short-circuit current through the urinary bladder membrane.

METHODS

Na^+ transport in urinary bladder

The sodium transport in urinary bladder was measured by a method based on the short-circuit current (s.s.c.) technique of USSING AND ZERAHN[5]. It has been reported that the s.c.c. of toad bladder or frog skin is equivalent to the net sodium flux[5,6]. *Bufo bufo japonicus* (Bufo) and *R. catesbiana* (Rana) were used after they had been maintained in shallow fresh water at room temperature for several days. The bladder from Bufo or Rana was isolated and mounted in a reaction chamber 2 or 3 h after decapitation of the amimal. The area of cross-section of the chamber orifice was 1.2 cm^2. To prevent mechanical distortion of the bladder membrane, both orifices of the chamber were covered with nylon mesh. The composition of Ringer's solution was as follows: 111 mM NaCl, 3.45 mM KCl, 0.45 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.9 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 11 mM glucose; the pH was adjusted to 7.4. This solution in the reaction chamber was continuously adjusted to the desired temperature by a thermister device. Compressed air was used to aerate the solution.

When the s.c.c. became stable, 0.1 ml of physiological solution containing various inhibitors was added to the serosal side of the membrane. The current was measured every 5 min since a steady current could be obtained for about the next hour. After replacing the serosal and the mucosal solutions three times with fresh solution, it was noted whether or not the s.c.c. returned to its initial value.

ATPase preparation from kidney and bladder

Five bilobed bladders (from Bufo or Rana) and five coupled kidneys (Rana) were removed from the animals in the cold room and rinsed 3 times in distilled water which contained 1 mM Tris–EDTA (pH 7.5). All further procedures were also carried out in the cold room. The bladders were extended on a glass plate and using a glass cover-slip, their mucosal cells scraped off into a Petri dish containing the solution mentioned below. The mucosal tissue of the bladders or the kidneys were then homogenized separately using an ice-cold Teflon homogenizer ten full strokes of the pestle in 10 ml of 0.25 M sucrose containing 1 mM EDTA, and adjusted to pH 7.4 with Tris–HCl.

Preparation of the microsomal fractions from these organs was carried out by a method based mainly on that of HAYS AND BARLAND[7]. In some experiments the microsomal pellet was treated with NaI according to the method of NAKAO *et al.*[8].

Measurement of ATPase activity

The standard assay system for (Na^+-K^+)-ATPase contained 120 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 3 mM Tris–ATP, 50 mM Tris–HCl (pH 7.8) and 10–20 μg of the enzyme protein in a total volume of 1.0 ml. Tris–ATP was prepared with Na^+–ATP by passing a solution of ATP (sodium salt) through a Dowex-50 (H^+) column and subsequently neutralizing with Tris. After incubation at 37° or indicated temperature, the reaction was terminated by the addition of 1.0 ml of 10% trichloroacetic acid. The orthophosphate liberated was determined by a modification of the method of FISKE AND SUBBAROW[9]. The quantity of protein was measured by the method of LOWRY *et al.*[10].

RESULTS

Effect of cardiac steroids on s.c.c. of bladder (Bufo and Rana)

The typical effect of ouabain on the s.c.c. of the bladder of Bufo is shown in Fig. 1. Ouabain at a concentration of $1 \cdot 10^{-4}$ M depressed the s.c.c. to about 30–40% of the initial value. Only at a very high concentration of ouabain, $2 \cdot 10^{-3}$ M, was the s.c.c. inhibited completely. On changing Ringer's solution in both chambers to a fresh one the s.c.c. returned gradually to its initial level.

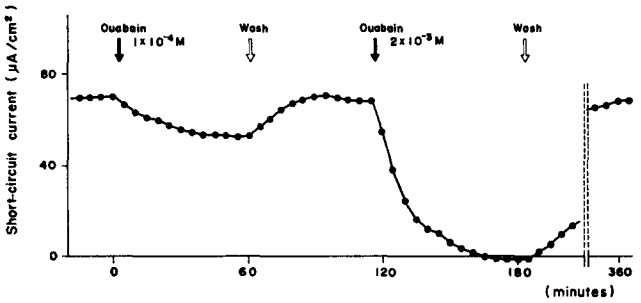

Fig. 1. Demonstration of effect of ouabain on s.c.c. (*B. bufo japonicus*). Black arrows indicate the addition of ouabain to the serosal bathing solution and white arrows indicate the removal of ouabain by washing three times with fresh Ringer's solution. The composition of Ringer's solution is described in METHODS. Upon changing to a fresh solution, the s.c.c. returned to its initial value. Temperature was kept at 21 ± 1°.

In the case of Rana, ouabain and digitoxin at the low concentration of $1 \cdot 10^{-7}$ M were effective in reducing the s.c.c., but the recovery after washing was not observed at any concentration in the range $1 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ M (Figs. 2 and 3). On the other hand, digitoxigenin reduced the s.c.c. completely at a concentration of $1 \cdot 10^{-5}$ M at 22°, and the initial value was restored after changing the solution (Fig. 4).

All three cardiac steroids, however, had no effect on the s.c.c. when they were added to the mucosal side of the membrane in either species. The s.c.c. required

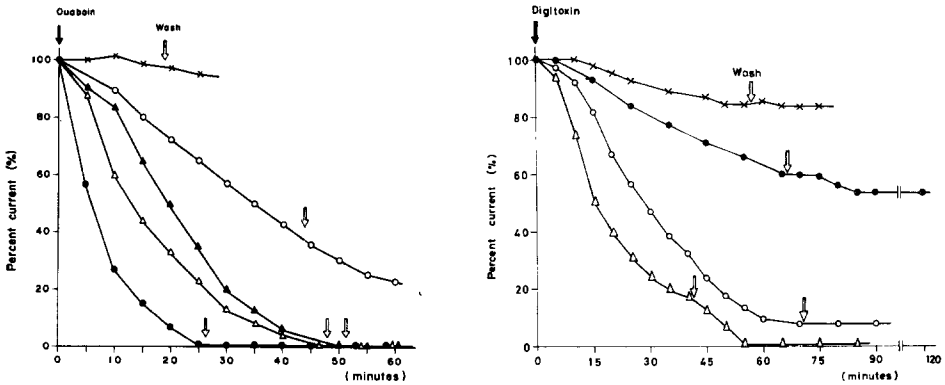

Fig. 2. Effect of ouabain on s.c.c. (*R. catesbiana*). The results of five experiments were plotted. After the s.c.c. became stable, various concentrations of ouabain were added to the serosal bathing solution at 0 time. White arrows indicate the removing of ouabain by washing. The experiments were performed at 21 ± 1°. The concentrations (M) of ouabain were: ×—×, $1 \cdot 10^{-7}$; ○—○, $4 \cdot 10^{-6}$; ▲—▲, $5 \cdot 10^{-6}$; △—△, $1 \cdot 10^{-5}$; ●—●, $1 \cdot 10^{-4}$.

Fig. 3. Effect of digitoxin on s. c. c. (*R. catesbiana*). The results of four experiments were plotted. The procedure is the same as that described in Fig. 2. The concentrations (M) of digitoxin were: ×—×, $1 \cdot 10^{-7}$; ⊙—⊙, $5 \cdot 10^{-7}$; ○—○, $1 \cdot 10^{-6}$; △—△ $1 \cdot 10^{-5}$.

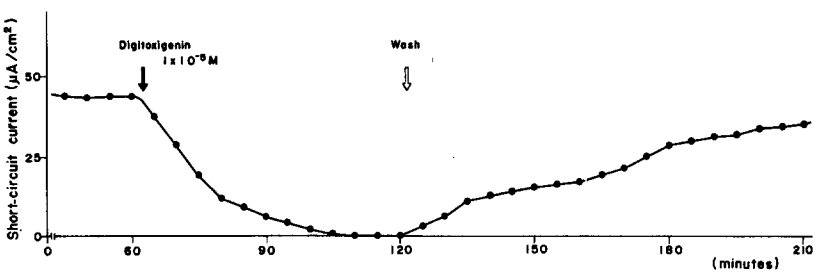

Fig. 4. Demonstration of effect of digitoxigenin on s.c.c. (*R. catesbiana*). Digitoxigenin at a concentration of $1 \cdot 10^{-5}$ M was added to the serosal bathing solution after the s.c.c. became stable. White arrow indicates the removal of digitoxigenin by washing. The procedure is the same as that described in Fig. 1.

sodium and potassium together for its full activation as reported by HAYS AND LEAF[11].

(Na^+-K^+)-ATPase activity of bladder and kidney (Bufo and Rana)

The (Na^+-K^+)-ATPase activity of the bladder of Bufo was measured (Table I) and the effect of ouabain on this ATPase examined. A high concentration of ouabain was needed to inhibit the (Na^+-K^+)-ATPase even at 37° as was also the case with the s.c.c. (Fig. 5). The concentration for half-maximal inhibition was $1 \cdot 10^{-5}$ M.

The ATPase activities of the bladder and kidney of Rana were also measured (Table II). The (Na^+-K^+)-independent ATPase activity of the bladder was abundant in comparison to the (Na^+-K^+)-dependent ATPase activity and was not eliminated easily by treatment with NaI. On the other hand, most of the (Na^+-K^+)-independent ATPase activity of the kidney was removed by NaI treatment (Table III). However, both the (Na^+-K^+)-dependent ATPase of the bladder and kidney showed the same

TABLE I

ATPASE ACTIVITIES OF BLADDER MICROSOMES FROM *B. bufo japonicus*

Total ATPase activity was assayed at the indicated temperature under the standard conditions as described in METHODS. (Na^+-K^+)-independent ATPase activity was determined under the same conditions, but in the absence of $Na^+ + K^+$. (Na^+-K^+)-dependent ATPase activity was obtained from the difference between the total and (Na^+-K^+)-independent ATPase determinations.

Tissue	*Temp.*	*Specific activity (μmoles P_i/mg protein per h)*	
		(Na^+-K^+)-independent ATPase	*(Na^+-K^+)-dependent ATPase*
Bladder	37°	20.1	6.3
	20°	12.4	1.6

TABLE II

ATPASE ACTIVITIES OF BLADDER AND KIDNEY MICROSOMES FROM *R. catesbiana*

Each activity was assayed at the indicated temperature under the standard conditions. (Na^+-K^+)-independent ATPase activity was determined by the addition of 0.1 mM ouabain, and (Na^+-K^+)-dependent ATPase activity was obtained from the difference between the total and (Na^+-K^+)-independent ATPase determinations.

Tissue	*Temp.*	*Specific activity (μmoles P_i/mg protein per h)*	
		(Na^+-K^+)-independent ATPase	*(Na^+-K^+)-dependent ATPase*
Bladder	37°	22.6	6.4
	22°	16.5	1.7
Kidney	37°	10.9	43.3
	22°	6.6	10.2

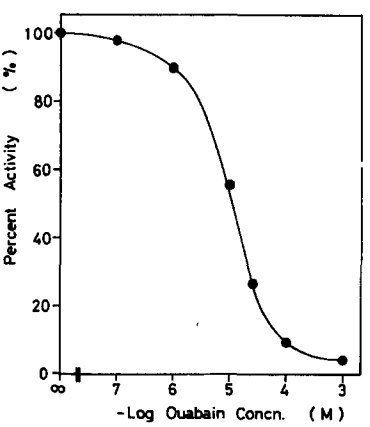

Fig. 5. Effect of ouabain on bladder (Na^+-K^+)-ATPase activity (*B. bufo japonicus*). (Na^+-K^+)-ATPase activity was obtained from the difference between the total and (Na^+-K^+)-independent ATPase determinations under standard conditions except for the addition of various concentrations of ouabain. Incubation was carried out at 37°.

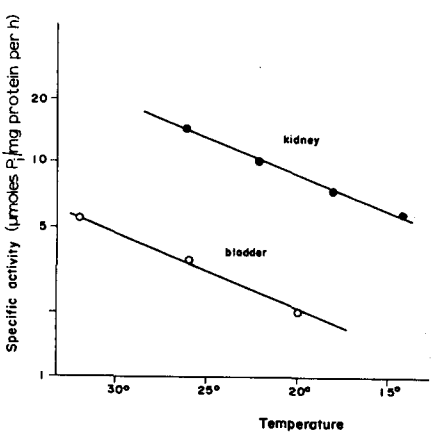

Fig. 6. Effect of temperature on (Na^+-K^+)-ATPase (*R. catesbiana*). Both (Na^+-K^+)-ATPase activities of the bladder and kidney were assayed under the standard conditions except for changing of incubation temperature. The same thermodependency was observed between bladder and kidney enzymes.

TABLE III

PURIFICATION OF THE ATPASE FROM *R. catesbiana* BY NaI TREATMENT

Microsomes obtained from the kidneys and bladders were treated with NaI (see ref. 8). Activities of the treated enzymes were determined under the standard conditions. Recoveries of protein and (Na^+-K^+)-dependent ATPase activities were calculated from the values before NaI treatment as 100% (see Table II).

Tissue	*Temp.*	*Specific activity (μmoles) P_i/mg protein per h*			
		(Na^+-K^+)-independent ATPase	*(Na^+-K^+)-dependent ATPase*	*Recovery of protein (%)*	*Recovery of (Na^+-K^+)-ATPase (%)*
Bladder	37°	8.7	6.9	33	38
Kidney	37°	4.6	90.3	42	87
	22°	3.8	21.1	42	87

susceptibility to cardiac steroids and thermodependency as shown in Fig. 6. Thus the properties of the (Na^+-K^+)-dependent ATPase from the two sources were essentially the same, and more accurate determination being possible in the case of the kidney enzyme. For this reason, the enzyme after treatment with NaI was mainly used in the further experiments.

Effect of digitoxigenin on s.c.c. of bladder (Rana)

The effect of digitoxigenin at various concentrations from $1 \cdot 10^{-9}$ to $1 \cdot 10^{-4}$ M was tested on the s.c.c. at 22° and at 14°. There was a definite difference between

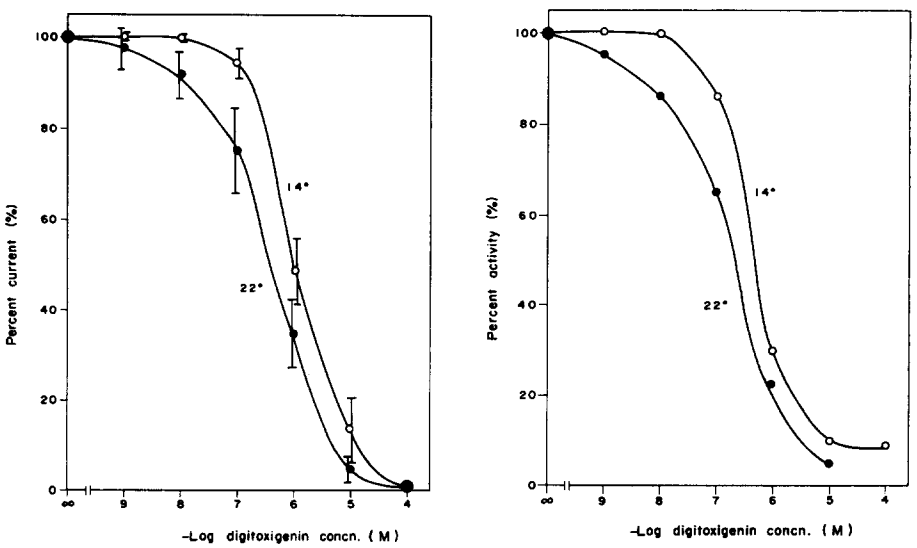

Fig. 7. Effect of digitoxigenin on s.c.c. (*R. catesbiana*). The procedure is described in METHODS. Various concentrations of digitoxigenin were added to the standard reaction chamber. ○—○, at 14°; ●—●, at 22°. The vertical bars represent ± 1 S. E.

Fig. 8. Effect of digitoxigenin on (Na^+-K^+)-ATPase activity (*R. catesbiana*). The standard assay conditions were employed except for varied concentrations of digitoxigenin. Incubation temperatures were ○—○, at 14°; ●—●, at 22°.

the concentration of digitoxigenin that produced half-maximal inhibition at 22° and that at 14° (Fig. 7). The concentration for half-maximal inhibition at 22° was $4 \cdot 10^{-7}$ M, while the concentration for half-maximal inhibition at 14° was $8 \cdot 10^{-7}$ M.

At concentrations over $1 \cdot 10^{-5}$ M, inhibition of the s.c.c. was almost complete. On changing the solution, however, the s.c.c. recovered its initial value in all cases as described before (Fig. 4).

Effect of digitoxigenin on (Na^+-K^+)-ATPase activity (Rana)

A highly specific (Na^+-K^+)-ATPase obtained from the kidney by NaI treatment was inhibited by digitoxigenin; the inhibition curve is shown in Fig. 8. Similarly, in the case of the s.c.c., there was a definite difference between the inhibitory concentration of digitoxigenin at 22° and that at 14°. The concentrations for half-maximal inhibition were $3 \cdot 10^{-7}$ and $5 \cdot 10^{-7}$ M, respectively. Complete inhibition was observed at concentrations of $1 \cdot 10^{-5}$ M and over.

Effect of temperature on s.c.c. (Rana)

After the s.c.c. had reached an almost steady value, the temperature of both parts of the bathing solution was changed from high to low or from low to high. In all cases the s.c.c. was observed to be thermoreversible.

A plot of the logarithm of s.c.c. ($\mu A \cdot cm^{-2}$) against the reciprocal of the absolute temperature ($1/T$) over a range of 4–24° is shown in Fig. 9. It is seen that all the points fall with satisfactory approximation on a biphasic Arrhenius curve, the breaking point being around 13°. The Q_{10} was 2.0 in the high temperature region and 3.5 in the low temperature region.

Effect of temperature on (Na^+-K^+)-ATPase activity (Rana)

The standard assay system was used to observe the effect of temperature on the (Na^+-K^+)-ATPase activity. The concentration of enzyme protein was the same at various temperatures, but the incubation time varied from 40 to 80 min.

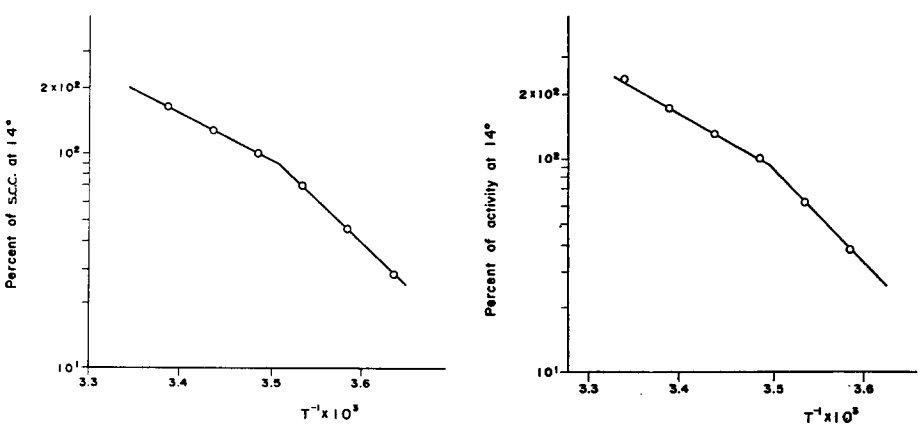

Fig. 9. Arrhenius plot for s.c.c. (*R. catesbiana*). The reciprocal of the absolute temperature is plotted as the abscissa, the logarithm of the s.c.c. at the corresponding temperature expressed as per cent of the s.c.c. at 14° as the ordinate. The standard Ringer's solution was employed. The temperature was changed from high to low and low to high.

Fig. 10. Arrhenius plot for (Na^+-K^+)-ATPase activity (*R. catesbiana*). The standard assay conditions were employed. Incubation temperature and time were varied.

The Arrhenius plot for the (Na^+-K^+-)ATPase is shown in Fig. 10. The reciprocal of the absolute temperature was plotted as the abscissa, and the logarithm of the specific activity at the corresponding temperature, expressed as per cent of the specific activity at 14°, as the ordinate. This curve showed the same change in slope as that of the s.c.c. at about 13°. The Q_{10} was 2.1 in the high temperature region and 3.7 in the low temperature region.

Energetics for s.c.c. and hydrolysis of ATP

The minimum energy output of the bladder of Rana for the Na^+ transport can be calculated according to LINDERHOLM[12]. The mean electrical current of 13 bladders was 70 $\mu A \cdot cm^{-2}$ at 22°, and the electrical potential between the two surfaces of the membrane produced by Na^+ was 18 mV. Other ions are passive in the short-circuited bladders. Thus, the electrical energy released was 0.3 $\mu cal \cdot cm^{-2} \cdot sec^{-1}$.

Standard free energy of the hydrolysis of ATP to ADP and phosphate depends on pH and concentration of magnesium salts. The value is accepted to be near -7000 $cal \cdot mole^{-1}$ (ref. 13) under physiological conditions. If the free energy of the hydrolysis of ATP in the bladder of Rana is assumed to be -7000 $cal \cdot mole^{-1}$ in the given concentrations of ATP, ADP and P_i at physiological pH, the following calculation may be possible. From Table II, it may be noted that the (Na^+-K^+)-ATPase activity of the bladder was 1.7 μmoles $P_i \cdot (mg\ protein)^{-1} \cdot h^{-1}$ at 22°. An average value of the protein obtained was 125 $\mu g/cm^2$ of the bladder (5 bladders). Therefore, the free energy yielding of bladder with ATP hydrolysis was 0.41 $\mu cal \cdot cm^{-2} \cdot sec^{-1}$.

DISCUSSION

A strict parallel was observed between the characteristics of (Na^+-K^+)-ATPase and the s.c.c. through the urinary bladder membrane as follows: (1) Both required Na^+ and K^+ simultaneously. (2) Both were completely inhibited by ouabain and digitoxigenin and half-maximal inhibition concentrations were well corrected in both cases of Rana and Bufo, although the absolute values of half-maximal inhibition concentration were 100 times greater in the case of Bufo than those of Rana. (3) The temperature coefficients of both phenomena coincided at the high temperature region as well as at the low temperature region. The inflection points on Arrhenius plot were quite similar. (4) Although digitoxigenin inhibited the active Na^+ transport completely, the active Na^+ transport was restored to almost normal level after washing the membrane. A similar recovery was also observed in the experiment of digitoxigenin binding to NaI-treated enzyme preparation[18]. (5) Electrothermodynamical work needed for the s.c.c. did not exceed the amount of free energy yielding by hydrolysis of ATP with the (Na^+-K^+)-ATPase system.

These considerations may lead us to the conclusion that the greater part of the active Na^+ transport through the urinary bladder of Rana was closely related to the (Na^+-K^+)-ATPase. No alternative pathway could be observed in the present experiments. However, s.c.c. was also decreased by tetrodotoxine[14], hydrochlorothiazide, pteridine derivatives[15], amiloride–HCl[16], sodium azide, monoiodoacetic acid[17], 2,4-dinitrophenol[6], *etc.* Some of these are thought to be effective on the Na^+ transport by changing the membrane permeability and some by inhibiting the

ATP synthesis. These findings suggest that additional factors may exist in the active Na^+ transport system besides (Na^+-K^+)-ATPase, and the relation between the two may be not parallel, but serial. Future experimentation is necessary in this regard.

The species variation in the susceptibility of ATPase to cardiac glycosides is not clear at the present time. The difference, however, might be due to the topographical variation at the outer surface of cell membrane. Though the species difference for cardiac glycoside inhibition was rather pronounced in the present experiments, the reversibility of aglycon inhibition and the irreversibility of glycoside inhibition were observed in the s.c.c. of bladder of Rana. The findings were quite consistent with the experiment of binding aglycon and glycoside to NaI-treated enzyme preparation[18].

Sodium transport from the mucosal to the serosal surface of the urinary bladder was inhibited by a cardiac steroid when it was added to the serosal side of the membrane. This is consistent with the finding in red blood cells[19] in respect to the direction of sodium transport and the effects of cardiac steroids. This may be of interest when considering the topographical aspects of the membrane.

The minimum energy output of the urinary bladder of Rana for the sodium transport was calculated. The calculation can, as LINDERHOLM[12] mentioned, only be a rough approximation of energy used for active transport of ions, owing to the incomplete knowledge of the essential electrical characteristics of the tissues. However, the free energy of the hydrolysis of ATP to ADP and phosphate was thought to be more than enough to explain the work needed for the Na^+ transport calculated by this method.

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