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EFFECT ON *P*-NITROPHENYL PHOSPHATE ON THE SHORT-CIRCUITING CURRENT IN THE TURTLE BLADDER

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SUMMARY

1. Addition of *p*-nitrophenyl phosphate (final concentration, 4.0 mM) to a glucose-enriched serosal fluid of the short-circuited bladder of *Pseudemys scripta* is followed by a decrease in the short-circuiting current (I_{sc}) to half the control level after about 45 min; and concomitantly, in the generation of *p*-nitrophenol which reaches concentrations of 0.03–0.06 mM in the serosal fluid after 45–60 min. The pattern of decline of I_{sc} follows that of accumulation of *p*-nitrophenol in the serosal fluid.

2. Identical additions of *p*-nitrophenyl phosphate to glucose-free serosal fluids have no effect on I_{sc} over and above the usual decline occasioned by removal of glucose *per se*.

3. Addition of *p*-nitrophenyl phosphate to the mucosal bathing fluid is not followed by decreases in I_{sc}, and fails to induce the appearance of *p*-nitrophenol.

4. Direct addition of the phenols, *p*-nitrophenol or 2,4-dinitrophenol, to the serosal or to the mucosal bathing solution is also followed by a decrease in I_{sc} to half its control level after 50–60 min; an effect elicited when the final concentration of *p*-nitrophenol is 1.0 mM, but not when the final concentration is 0.1 mM.

5. The mean times required for 50% inhibition of I_{sc} by *p*-nitrophenyl phosphate, *p*-nitrophenol and 2,4-dinitrophenol: 42, 58, and 44 min respectively are not significantly different from one another ($P > 0.1$)

6. It is suggested that serosal *p*-nitrophenyl phosphate is hydrolyzed by a membrane-bound phosphatase to its phenolic product *p*-nitrophenol which interferes with mitochondrial ATP production and consequently with the energy supply for maintenance of Na^+ transport.

INTRODUCTION

The *in vitro* urinary bladder of *Pseudemys scripta* contains active mechanisms for the transport of Na^+ (refs. 1–7), Cl^- (refs. 3, 4, and 8) and HCO_3^- (refs. 9, 10, 6,

Abbreviation: PD, potential difference.

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and 11) from the mucosal to the serosal bathing medium. Ouabain, added to the serosal fluid to a concentration of $1 \cdot 10^{-4}$ M results in a complete and irreversible inhibition of net Na^+ transport (I_{Na^+}), without changing the concomitantly occurring net transport of Cl^- (I_{Cl^-}) or HCO_3^- ($I_{HCO_3^-}$)[7]. Subsequent work showed that acetazolamide (at a final serosal concentration of $1 \cdot 10^{-5}$ M) results in a reversible inhibition of I_{Cl^-}-and $I_{HCO_3^-}$ (refs. 12, 13). Thus ouabain action is specific for the Na^+ transport mechanism, while acetozolamide acts on the anion transport mechanisms in the turtle bladder.

As is the case in many tissues[14], certain properties of the ATPase found in turtle bladder microsomes (*e.g.* Na^+, K^+ stimulatability, ouabain sensitivity, and intra-membrane location)[7,15] can be related to parallel properties of the Na^+ transport process in the intact bladder.

In addition to the ($Na^+ + K^+$)-stimulatable ATPase, a K^+-stimulatable, ouabain-sensitive Mg^{2+}-dependent *p*-nitrophenylphosphatase has been found in the turtle bladder microsomes[16]. In contrast to ATPase, the *p*-nitrophenylphosphatase activity is inhibited rather than stimulated by Na^+ (ref. 16). Whether or not the ($Na^+ + K^+$) stimulatable ATPase and the K^+-stimulatable *p*-nitrophenylphosphatase are parts of the same protein molecule remains to be determined.

Similarities between ATPase and *p*-nitrophenylphosphatase are also reflected in the data on Na^+ transport across erythrocyte ghosts. Thus, HOFFMAN[17] showed that ATP on the inner surface is capable of supporting active Na^+ transport across the membrane of erythrocyte ghosts; and ASKARI AND RAO[18] showed that *p*-nitrophenyl phosphate on the outer surface stimulates Na^+ efflux across the membrane of erythrocyte ghosts[18]. In both cases, the Na^+ transport was maintained and stimulated by ATP or *p*-nitrophenyl phosphate in the absence of any other substrate and was inhibited by ouabain[18]. From these findings, one may infer that the interaction of either *p*-nitrophenyl phosphate or ATP and a spatially oriented membrane-bound phosphatase with the resultant hydrolysis provides the free energy for the translocation of Na^+ against its transmembrane gradient of electrochemical potential. However, the exact nature of the translocator mechanism remains obscure.

If the Na^+ pump in the turtle bladder possessed properties like those assigned to the Na^+ pump in erythrocytes, then *p*-nitrophenyl phosphate added to the fluid bathing serosal surface of the turtle bladder would react with the "membrane"-bound (microsomal) K^+ phosphatase, break down hydrolytically, and release free energy which under certain conditions would be utilized to maintain Na^+ transport. A similar addition of ATP, in our hands, failed to evoke an increase in I_{sc} or Na^+ transport and resulted in an inhibition of the transport rate of Na^+ (*i.e.* of I_{sc}). This does not necessarily exclude the possibility that the free energy of its hydrolysis at a membrane site is the immediate source of ion transport energy, especially in view of the spatial orientation of enzymes in the plasma membranes.

Accordingly, experiments on exogenously added *p*-nitrophenyl phosphate were undertaken with the expectation that the active site of intra-membrane-*p*-nitrophenylphosphatase was oriented toward the outer or serosal interface of the membrane. What follows is a presentation of evidence that exogenously added *p*-nitrophenyl phosphate is indeed hydrolyzed to *p*-nitrophenol when added to the serosal fluid (but not to the mucosal fluid), and that this *in vitro* hydrolysis is associated with an unexpected decrease in I_{sc} even in the presence of exogenous glucose.

METHODS

Tissue preparation

Turtles (*P. scripta*), weighing 0.75–1.5 kg and possessing a carapace 15–20-cm wide, were obtained from the Lemberger Co. (Oshkosh, Wisc.). Turtles were sacrificed by decapitation, and bladders were excised and mounted in a double-barreled chamber (modified after USSING AND ZERAHN[19]) by a technique described previously[8].

The double-barreled chamber permitted two hemibladders to be used simultaneously as experimental and control halves which were chosen at random. Each side of each hemibladder was bathed by 12 ml of Na^+-Ringer solution. All bathing media were continuously mixed and circulated past the bladder. Each hemibladder had a surface area of 1.5 cm^2 exposed to the bathing solution, a dry weight of 17.3 $\pm$ 1.6 (S.D.) mg and a tissue-water content of 133 $\pm$ 18 (S.D.) μl. All experiments were performed at 24–26°.

Short-circuiting technique

Isolated bladders were maintained in the short-circuited state using the technique of USSING AND ZERAHN[19]. The short-circuiting current (I_{sc}), transbladder potential difference (PD) and membrane resistance (R) were measured by techniques described previously[8]. The following sign reference was adopted: cations (*viz.*, Na^+) moving in the forward (M $\rightarrow$ S) direction contributed a positive moiety of I_{sc}; anions (*viz.*, Cl^- and HCO_3^-), a negative moiety. In the isolated turtle bladder I_{sc} is positive when the bladder is bathed on both sides by Na^+-Ringer solution, for Na^+ transport is normally greater than anion transport in the short-circuited state.

All values of I_{sc} are given per hemibladder (*i.e.* μA/1.5 cm^2), and values of R are also on the basis of a hemibladder (*i.e.* kΩ–1.5 cm^2). Open circuited potential difference (PD), an intensive parameter, is reported in units of mV.

Na^+-Ringer solution

Both surfaces of each hemibladder were bathed at all times by a buffer system which had the following composition (in mM): Na^+, 101; K^+, 4.8; Ca^{2+}, 2.0; Mg^{2+}, 0.8; Cl^-, 92; HCO_3^-, 17; $H_2PO_4^-$, 0.07; HPO_4^{2-}, 0.73; SO_4^{2-}, 0.80; CO_2, 0.33. D-Glucose, when present, was 11 mM. Osmolality was 221 mosmoles/kg and ionic strength was 0.116. During the experiment, the isolated preparation was continuously replenished with O_2 by bubbling both serosal and mucosal media with a mixture of O_2–CO_2 (99:1, by vol.) pre-saturated with water vapor. The pH varied between 7.4 and 7.5.

Reagents

p-Nitrophenyl phosphate (disodium salt), *p*-nitrophenol and 2,4-dinitrophenol (Grade II) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Stock solutions of *p*-nitrophenyl phosphate, *p*-nitrophenol and 2,4-dinitrophenol were made in Na^+-Ringer solution; final concentrations were 40, 20 and 20 mM, respectively. The addition of 1.3, 0.65 or 0.65 ml of *p*-nitrophenol phosphate, *p*-nitrophenol or 2,4-dinitrophenol respectively to either the mucosal or serosal bathing solutions (volume, 12 ml) of the experimental hemibladder resulted in final concentrations of

4.1 or 1 mM, respectively. The other hemibladder of the same tissue served as a time control.

Washing of *p*-nitrophenyl phosphate, *p*-nitrophenol or 2,4-dinitrophenol from the chamber was accomplished by complete removal of bathing fluids from both the mucosal and serosal sides, by rinsing both twice with 12-ml aliquots of fresh Na^+-Ringer solution, and by refilling both sides with 12 ml of fresh solution. This maneuver of washing was performed on the control hemibladder as well as on the experimental hemibladder.

Determination of p-nitrophenyl phosphate hydrolysis

To determine the concentration of *p*-nitrophenol in the bathing media, 0.5-ml aliquots were removed from the mucosal and serosal baths at 15-min intervals and added to 2 ml of 100 mM Tris buffer (pH 9.2). The absorbance was measured at 400 mμ in a Zeiss Model PMQ II spectrophotometer and the extinction coefficient (ε) for *p*-nitrophenol was taken as 18000 $M^{-1} \cdot cm^{-1}$ at 400 mμ and pH 9.2 (ref. 20).

RESULTS

General comments

Paired halves of a given turtle bladder, mounted simultaneously in a double chamber, were interposed between identical Na^+-Ringer solutions. The use of paired hemibladders permitted simultaneous measurements of I_{sc}, PD and R in both the experimental and control halves.

After mounting in the double-barreled chamber and application of the externally delivered short-circuiting current to a mated pair of hemibladders, the levels of I_{sc}, PD, and R varied for about 1–1.5 h, and then reached steady levels during which period the various agents were added to the experimental but not to the paired control hemibladder.

After addition of *p*-nitrophenyl phosphate, *p*-nitrophenol or 2,4-dinitrophenol, there occurred a decrease in PD and an increase in R concomitantly with the inhibition of I_{sc} in all experiments reported herein. These electrophysiological changes were reversible, insofar as the parameters returned toward the control levels after removal of the inhibitor. In what follows, the only electrophysiological effects shown will be those on I_{sc}.

Effect of p-nitrophenyl phosphate

In glucose-rich bathing fluids. Fig. 1, a double-panelled plot of values of short-circuiting current (I_{sc}) and concentration of *p*-nitrophenol *versus* time, depicts data from one out of six similarly designed experiments. The upper portion of the figure shows the effect on the I_{sc} of addition of *p*-nitrophenyl phosphate (final concentration, 4 mM) to the glucose-containing serosal bathing fluid of one hemibladder, but not to that of the other which was used as a time control. Addition of *p*-nitrophenyl phosphate was followed by a decrease in I_{sc} from 295 to 110 μA over the next 50 min after which the *p*-nitrophenyl phosphate-induced inhibition was terminated by substitution of fresh Na^+-Ringer solutions for the *p*-nitrophenyl phosphate-containing serosal and mucosal fluids. The time required for 50% inhibition of I_{sc} (from 295 to 148 μA) was 40 min.

The lower portion of Fig. 1 shows the time-course for the accumulation of *p*-nitrophenol (the hydrolytic product of *p*-nitrophenyl phosphate) in the serosal medium. The constant rate of increase in the concentration of serosal *p*-nitrophenol was 35 μM/h which corresponds to a rate of hydrolysis of (0.035 mM/h)·(12 ml) = 0.42 μmole/h for the hemibladder.

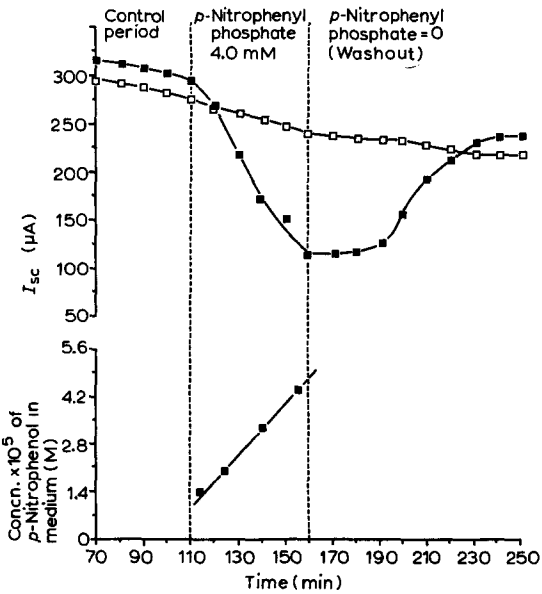

Fig. 1. Effect of addition and washout of *p*-nitrophenyl phosphate on the pattern of I_{sc} *versus* time and on the concentration of *p*-nitrophenol in the serosal medium *versus* time. ■, data from the experimental hemibladder; □, concomitant data from the mated control hemibladder.

In three other experiments, the rate of increase in the concentraton of *p*-nitrophenol in the serosal fluid following the serosal addition of *p*-nitrophenyl phosphate varied from 30 to 60 μM/h.

The phenolic product, appearing first in the serosal fluid, later began to appear in the mucosal fluid, a phenomenon detected by the direct observation of the yellow color in all experiments and by chemical determination of the phenol in three of the six experiments.. The amount of *p*-nitrophenol accumulating in the mucosal fluid 60 min after the addition of *p*-nitrophenyl phosphate was 5–15% of the amount which had accumulated concomitantly in the serosal fluid.

Fig. 1 also shows that inhibition of I_{sc} by *p*-nitrophenyl phosphate was terminated by removal of *p*-nitrophenyl phosphate and its product of hydrolysis from the bathing media; and that subsequent to the removal, there was complete recovery of I_{sc} in the experimental hemibladder relative to its control hemibladder.

Table I presents mean values with statistical parameters from six experiments wherein complete recovery of I_{sc} followed the removal of the *p*-nitrophenyl phosphate and *p*-nitrophenol from and the replacement of both bathing fluids with fresh Ringer solutions. In all six experiments, the inhibition was terminated when I_{sc} had decreased to 50–70% of its value at the time of addition of *p*-nitrophenyl phosphate.

The time required for 50% inhibition of I_{sc} ranged between 30 and 80 min with a mean value of 42 ± 8 (S.E.) min.

In five experiments on paired hemibladders, short circuited as above, *p*-nitrophenyl phosphate was added to a final concentration of 4 mM to the mucosal fluid rather than to the serosal fluid of one hemibladder, while the paired hemibladder served as a time control. In no case was any significant inhibition of I_{sc} (other than that ascribable to time alone) found. Moreover, there was no apparent appearance of *p*-nitrophenol over and above the small amount initially present in the solution of *p*-nitrophenyl phosphate added to the mucosal fluid, and there was no apparent appearance of the phenol in the serosal fluid.

In glucose-free bathing fluids. Six experiments were performed on paired hemibladders under short-circuiting conditions identical to those described above except that glucose was not included in the bathing fluids. In three experiments *p*-nitrophenyl phosphate was added to the serosal fluid (final concentration, 4 mM); and in three experiments *p*-nitrophenyl phosphate at the same concentration was added to the mucosal fluid. The level of I_{sc} in both experimental and control hemibladders declined progressively toward zero with time. The addition of *p*-nitrophenyl phosphate (either to the serosal or to the mucosal fluid) had no significant effect on the rate of decline of I_{sc}. In other words, the plot of values of I_{sc} *versus* time (not shown) in the *p*-nitrophenyl phosphate-treated group was indistinguishable from that of the paired control group. Therefore, under the present conditions, exogenously added *p*-nitrophenyl phosphate is unable to serve as a substrate to stop or to reverse the decline in I_{sc}. On the other hand, the declining level of I_{sc} was reversed toward control levels and recovery ensued after removal of the *p*-nitrophenyl phosphate-containing fluid and replacement by glucose-enriched Ringer solution. A similar glucose-induced

TABLE I

RECOVERY OF I_{sc} AFTER REMOVAL OF *p*-NITROPHENYL PHOSPHATE

Mean values ± standard error of I_{sc} immediately before inhibition by *p*-nitrophenyl phosphate (see "prelim.") and the maximal values achieved after removal of *p*-nitrophenyl phosphate (see "after washout") are shown for six pairs of experimental and mated control hemibladders. Also shown are relative values of I_{sc} ("washout" relative to "prelim.") and percent maximal recovery ("experimental" relative to "control"). The probability values, *P*, shown were derived from experimental and control values which were compared by means of the unpaired *t*-test; and the percent maximal recovery was compared with 100% by means of the unpaired *t*-test.

Experimental condition	*Before p-nitrophenyl phosphate*	*After washout of p-nitrophenyl phosphate*	$\frac{I_{sc}(washout)}{I_{sc}(prelim.)}$ (%)	$\frac{I_{sc}(experimental)}{I_{sc}(control)}$ (%)
	(μA)			
Experimental ($n = 6$)	222 ± 22	182 ± 21	82 ± 6	103 ± 3
Paired control ($n = 6$)	232 ± 24	185 ± 21	80 ± 5	
Probability values for completeness of recovery of I_{sc} after washout				
P(experimental = control)	> 0.7	> 0.9	> 0.8	—
P(experimental/control = 100)	—	—	—	> 0.9

recovery has been reported previously in bladders which had been bathed in glucose-free media, but which had not been treated with *p*-nitrophenyl phosphate[8].

In sodium-free, glucose-rich serosal fluid. Three experiments were performed on paired hemibladders under short-circuiting conditions identical to those described above except that the serosal fluid contained choline instead of Na^+ while the mucosal fluid was the usual Na^+-rich Ringer solution. This format was prompted by the previously reported data showing that 100 mM Na^+, inhibited completely the K^+-stimulated *p*-nitrophenylphosphatase activity in turtle bladder microsomes[16]. Since such phosphatase activity was detected by observing the appearance of *p*-nitrophenol in the serosal fluid of the intact bladder bathed by Na^+-rich Ringer (see lower portion of Fig. 1), we anticipated an increase in the rate of phenol appearance after removal of Na^+ from the serosal fluid.

However, after addition of *p*-nitrophenyl phosphate to the Na^+-free serosal fluid (4 mM), both the inhibition of I_{sc} and the rate of appearance of *p*-nitrophenol were essentially the same as those found after addition of *p*-nitrophenyl phosphate to a Na^+-rich serosal fluid (see Fig. 1).

The effect of *p*-nitrophenyl phosphate on the intact bladder in Na^+-free serosal fluid, together with its effect in Na^+-rich serosal fluid and together with the inhibitory effect of Na^+ on microsomal *p*-nitrophenylphosphatase[16] suggests the following: (a) that the active site of *p*-nitrophenylphosphatase in the intact bladder is not accessible to *p*-nitrophenyl phosphate at the serosal fluid interface of the serosal membrane; and (b) that *p*-nitrophenyl phosphate in the serosal fluid must enter the intact cell prior to its hydrolysis and prior to the onset of decrease of I_{sc}; or that (c) the enzyme in an intact cell, unlike the isolated enzyme, is not sensitive to Na^+.

Effect of phenols

p-Nitrophenol. Fig. 2 shows that addition of *p*-nitrophenol when $t = 120$ min to a final concentration of 0.1 mM to the serosal bathing fluid (glucose-enriched) of the experimental hemibladder did not result in any significant inhibition of I_{sc}. However, subsequent addition of *p*-nitrophenol when $t = 180$ min to a final concentration of 1 mM resulted in a decrease in I_{sc} from 210 to 85 μA over the ensuing 70

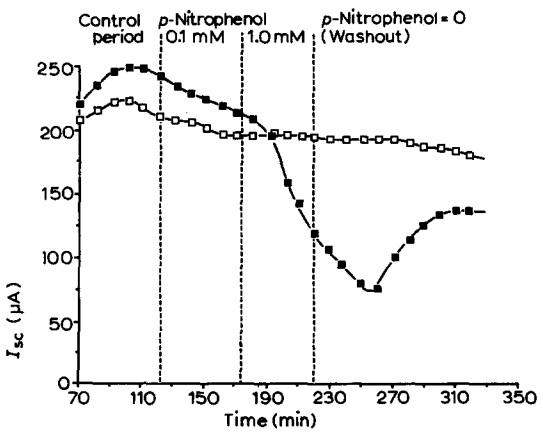

Fig. 2. Effect of addition and washout of *p*-nitrophenol on the pattern of I_{sc} *versus* time. ■ and □ correspond to data from the experimental and mated control hemi-bladder, respectively.

min. The time required for 50% inhibition of I_{sc} (from 210 to 105 μA) was 50 min; and during this time, the phenol migrated from the serosal to the mucosal fluid as checked by chemical measurement and the naked eye.

After removal of *p*-nitrophenol and subsequent replacement of both bathing media when $t = 220$ min, I_{sc} continued to decrease for an additional 30 min before the process of recovery started. I_{sc} of the experimental hemibladder recovered to 70% of the control (□).

In two additional experiments, addition of *p*-nitrophenol to a final serosal concentration of 0.1 mM resulted in no significant inhibition of I_{sc}. However, in six other experiments, addition of *p*-nitrophenol to a final concentration of 1 mM resulted in a significant inhibition of I_{sc}. Hence, the effective inhibitory concentration of *p*-nitrophenol, with whatever cell site it reacts, lies between 0.1 and 1.0 mM. The time required for 50% inhibition of I_{sc} in each of the six experiments ranged between 20 and 90 min with a mean value of 58 ± 6 (S.E.) min. There was no significant inhibition of I_{sc} when the concentration of serosal *p*-nitrophenol was 0.1 mM.

Unlike *p*-nitrophenyl phosphate, the addition of *p*-nitrophenol to a final concentration of 1 mM in the mucosal bathing fluid resulted in a significant inhibition of I_{sc}. The characteristics of this inhibition were similar to those observed when *p*-nitrophenol was added to the serosal fluid. In three experiments, the time required for 50% inhibition ranged between 20 and 50 min; and during this time the phenol migrated from the mucosal to the serosal fluid as determined chemically and visually.

2,4-Nitrophenol. Fig. 3 shows the effect on I_{sc} of addition of 2,4 dinitrophenol to a final concentration of 1 mM in the serosal bathing fluid (glucose-enriched) of one hemibladder, while the other hemibladder served as a time control in one out of eight similar experiments on paired hemibladders. Addition of 2,4-dinitrophenol at $t =$ 120 min resulted in a decrease in I_{sc} from 305 to 45 μA over the ensuing 110 min. The time required for 50% inhibition of I_{sc} (from 305 to 152 μA) was 25 min; and during

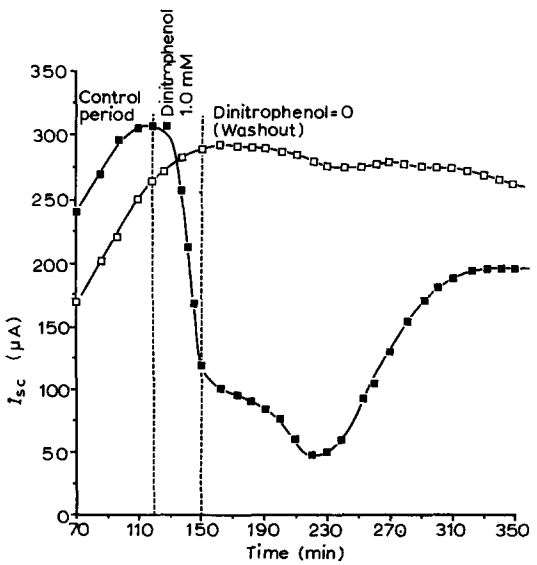

Fig. 3. Effect of addition and washout of 2,4 dinitrophenol on the pattern of I_{sc} *versus* time in experimental (■) and in mated control hemibladder (□).

this time the phenol migrated from the serosal to the mucosal fluid as seen by the naked eye. Even after removal of the 2,4-dinitrophenol and subsequent replacement of both bathing media with 2,4-dinitrophenol-free Ringer solution when $t = 150$ min, I_{sc} continued to decrease for an additional 45 min before the process of recovery started. During the recovery period, the level of I_{sc} of the experimental hemibladder increased to 74% of the concomitant level of the control hemibladder.

In the eight experiments, addition of 2,4-dinitrophenol to a final concentration of 1 mM (four additions to the serosal and four to the mucosal bathing fluid) resulted in a 50% inhibition of I_{sc} within 20–60 min. The mean value of the time required for 50% inhibition was 44 ± 6 (S.E.) min. Lower concentrations of 2,4-dinitrophenol were without effect upon the I_{sc}.

When added to the mucosal fluid, 2,4-dinitrophenol migrated across the bladder to the serosal fluid as seen by the naked eye.

Apparently, both phenols, (*p*-nitrophenol or 2,4-dinitrophenol), penetrated the bladder wall equally well in either direction (M to S or S to M) and both phenols exerted quantitatively similar inhibitory effects whether added to the serosal or to the mucosal fluid.

DISCUSSION

A K^+-dependent *p*-nitrophenyl phosphatase has been demonstrated in the "microsomal" membranes of a number of tissues wherein the active transport of Na^+ occurs[14]. This activity is found in the same microsomal fraction as that containing the ($Na^+ + K^+$)-dependent moiety of ATPase activity[21,22] and attempts to separate microsomal K^+-dependent phosphatase and ($Na^+ + K^+$)-ATPase have not been successful. Both activities, ATPase[7,15,23] and *p*-nitrophenylphosphatase[16] have been found in the microsomal fraction of isolated mucosal cells from urinary bladders of the fresh-water *Ps. scripta*.

In searching for a role of the K^+-dependent *p*-nitrophenylphosphatase in Na^+ transport, ASKARI[18] found that *p*-nitrophenyl phosphate, on the outer surface of the erythrocyte ghost supported the Na^+ efflux in the absence of any other substrate. However, data shown herein indicate that exogenously added *p*-nitrophenyl phosphate inhibits the I_{sc} (and presumably the Na^+ transport) across the turtle bladder in the presence of serosal glucose, and fails to affect the progressive decline in I_{sc} in the absence of ambient glucose. The *p*-nitrophenyl phosphate-induced inhibition of I_{sc}, not expected on the basis of ASKARI's results[18], could have been ascribed to the high concentration of serosal Na^+ which is known to inhibit K^+-dependent *p*-nitrophenylphosphatase in the isolated microsomes of the bladder epithelium[16]. However, present data demonstrate that *p*-nitrophenyl phosphate in Na^+-free serosal fluid has the same inhibitory effect on I_{sc} as does *p*-nitrophenyl phosphate in Na^+-rich serosal fluid.

When *p*-nitrophenyl phosphate is added to the serosal surface of the turtle bladder, the rate of hydrolysis of *p*-nitrophenyl phosphate is sufficient to raise the serosal concentration of *p*-nitrophenol to 0.05 mM in 45 min, during which time there occurs a 58% inhibition of the I_{sc}. In order to produce a similar degree of inhibition of I_{sc} by the direct addition of *p*-nitrophenol to the serosal bathing fluid, it is necessary to raise the concentration of the phenol to 1.0 mM, or to a level greater than that accu-

mulating after endogenous hydrolysis of *p*-nitrophenyl phosphate added to the serosal fluid. This could be explained by the following assumptions: (a) that *p*-nitrophenol is the inhibitor of a reaction associated directly or indirectly with Na^+ transport; and (b) that its concentration at the effective site (wherever that may be in the cell, or cell membrane, or mitochondria or other sites) determines the degree of inhibition of transport. Thus, the concentration of *p*-nitrophenol accumulating at the effective site from the hydrolysis of *p*-nitrophenyl phosphate is greater than that measured in the serosal fluid (*e.g.* $5 \cdot 10^{-5}$ M in the present report) into which the hydrolytically produced phenol diffuses. Similarly, the serosal concentration of exogenously added phenol (*e.g.* $1.0 \cdot 10^{-3}$ M in the present report) is greater than that at the effective cellular site toward which the phenol diffuses until it reaches whatever concentration is required for any specified degree of inhibition. In short, the phenolic concentration at the effective cell site (not that in the serosal fluid), determined the degree of inhibition of Na^+ transport. Neither the exact concentration of phenol at the site nor the exact cellular location of the site are known. Nevertheless, the data in this report suggest that it must be less than $1 \cdot 10^{-3}$ M and greater than $5 \cdot 10^{-5}$ M.

Whether or not the phenolic product acts directly on the phosphatase of the intact system is not apparent from these data. In isolated microsomes, 0.1 mM 2,4-dinitrophenol has no effect upon the $(Na^+ + K^+)$-ATPase, and 0.1 mM *p*-nitrophenol has no effect upon the *p*-nitrophenylphosphatase suggesting that the inhibition of I_{sc} by 2,4-dinitrophenol and *p*-nitrophenol in the intact system may be due to an effect on an energy-yielding reaction other than the microsomal phosphatase reaction. Since phenols are known uncouplers of oxidative phosphorylation their inhibitory effects on I_{sc} may be mediated by way of their interaction with the appropriate mitochondrial sites. In this connection, data shown here demonstrate that 2,4-dinitrophenol or *p*-nitrophenol penetrate the bladder wall readily (from M to S or from S to M), thereby making possible the interaction between such phenols and any cytoplasmic organelle including the mitchondrion.

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