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p-NITROPHENYL PHOSPHATASE ACTIVITY IN THE MICROSOMAL FRACTION OF TURTLE BLADDER MUCOSAL CELLS

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SUMMARY

The microsomes of turtle bladder epithelium contain a K^+-stimulatable, ouabain- and/or Na^+-inhibitable *p*-nitrophenyl phosphatase with an absolute requirement for Mg^{2+}. The optimal pH range was 7.2–7.3 in the imidazole–histidine buffer system used. Treating the data in terms of Michaelis–Menten kinetics, the apparent K_m's were as follows: for *p*-nitrophenyl phosphate, 0.6 mM; for Mg^{2+}, 0.6 mM; and for K^+, 1.0. Substrate inhibition was observed for *p*-nitrophenyl phosphate, Mg^{2+}, and K^+ at concentrations exceeding 4.0, 4.0 and 10 mM, respectively. 0.1 mM ouabain, or 100 mM Na^+, completely inhibited the *p*-nitrophenyl phosphatase activity, and each inhibitor reacted competitively with K^+ for enzymes sites (K_i for ouabain = 25 nM and K_i for Na^+ = 3.0 mM). The inhibitory action of *N*-ethylmaleimide was dependent upon the time of pre-incubation of the microsomes with *N*-ethylmaleimide. Related to the question of whether *p*-nitrophenyl phosphatase is the same as or distinct from ($Na^+ + K^+$)-ATPase, is the fact that *p*-nitrophenyl phosphatase activity is inhibited competitively in the presence of ATP.

INTRODUCTION

Microsomal preparations from many tissues have a ($Na^+ + K^+$)-dependent ATPase[1–4], which has been related to the transport of Na^+ and K^+ across the cell membrane on the basis of evidence recently reviewed by SKOU[5], HEINZ[6] and ALBERS[7]. Microsomes also contain other enzymatic activities including a K^+-stimulated acetyl phosphatase and a K^+-stimulated *p*-nitrophenyl phosphatase. Acetyl phosphatase has been found in kidney and brain microsomes[8,9], and *p*-nitrophenyl phosphatase has been found in the electric organ of eels[10], red blood cells[11] and white blood cells[12].

In all cases reported[8–12], both acetyl phosphatase and *p*-nitrophenyl phosphatase were inhibited by ouabain and Na^+. Whereas the relationship between either of the two phosphatases and transport is not yet clarified, WOODIN AND WIENEKE[12] has claimed that the K^+-dependent *p*-nitrophenyl phosphatase may be related to K^+ transport in the white blood cells.

The purposes of the present paper were: to investigate the K^+-dependent *p*-nitrophenyl phosphatase in the microsomal fraction isolated from mucosal cells

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of turtle bladder; to evaluate the kinetic parameters such as K_m, K_i and v_{max} of *p*-nitrophenyl phosphatase activity with respect to the concentration of *p*-nitrophenyl phosphate and with respect to the concentrations of Mg^{2+} and K^+; to determine the effect of pH; and to determine the effect of various inhibitors such as Na^+, *N*-ethylmaleimide, ATP, and sodium azide.

METHODS

The procedures for isolating mucosal cells from freshly excised turtle bladders together with those for the ultracentrifugation, fractionation, and isolation of the microsomal pellet have been described in detail previously[1]. Protein concentration of the microsomes, determined by the method of LOWRY *et al.*[13], was used as the normalizing parameter for enzyme activity.

The composition of reaction mixture, expressed in terms of final millimolar concentration was: the disodium salt of *p*-nitrophenyl phosphate, 4.0 (only in case of Fig. 1); or usually the imidazole–histidine salt of *p*-nitrophenyl phosphate, 4.0; $MgCl_2$, 4.0; imidazole, 20; histidine, 20; KCl, 10 mM. The final pH was 7.3, and an enzyme aliquot of 0.5 ml containing 150–300 μg of protein was added to the incubation mixture, the total volume of which was 5 ml. The final concentrations of imidazole and histidine were increased from 20 to 30 mM by the addition of substrate, the imidazole–histidine salt of *p*-nitrophenyl phosphate. The final concentrations of imidazole and histidine were also 30 mM when the disodium salt of *p*-nitrophenyl phosphate was used as substrate.

After 5 min of pre-incubation, the reaction was started by addition of *p*-nitrophenyl phosphate, and incubated at 38° for 20–30 min. The reaction was stopped by addition of 5 ml of cold $HClO_4$ 6 %. Control tubes, carried through all incubations, were of two types: those without *p*-nitrophenyl phosphate and those without the enzyme. The non-catalytic rate of hydrolysis of *p*-nitrophenyl phosphate was zero, but the lot of *p*-nitrophenyl phosphate used contained P_i amounting 2–3 % of the concentration of *p*-nitrophenyl phosphate. Aliquots of the final mixture were analyzed routinely for P_i by the method of BERENBLUM AND CHAIN[14].

The initial reaction velocity was determined by measuring the increment of P_i released into the incubation flask after 30 min of incubation of the appropriate amount of microsomes with *p*-nitrophenyl phosphate. For an initial concentration of *p*-nitrophenyl phosphate of 4 mM, no more than 15 % of total *p*-nitrophenyl phosphate was hydrolyzed during the first 30 min of incubation. The amount of P_i released was proportional to the time of incubation for as long as 40 min of incubation at 38°. For a given batch of microsomes at a given time, the rate of P_i release was directly proportional to the amount of protein (150–300 μg) in each incubation flask. After storage of the microsomes at −20°, the *p*-nitrophenyl phosphatase activity decreased with time. The half-life of *p*-nitrophenyl phosphatase, estimated from the time-dependent logarithmic decrease of the activity, was 18–19 days.

During the experiments on the ATP-induced inhibition of *p*-nitrophenyl phosphatase, the *p*-nitrophenol released (instead of the P_i) was measured spectrophotometrically in order to insure that the hydrolytic product originated solely from *p*-nitrophenyl phosphate, and not from the added ATP. The spectrophotometric measurements were made at 25° in a Beckman DU monochromator fitted with a

Gilford Model 220 absorbance indicator and a Varian Model G-14 strip chart recorder. The concentrations of the reactants were the same as in the phosphate experiments. The substrate was added, mixed, and the change in absorbance measured at 348 mμ (ref. 15), the isosbestic point for *p*-nitrophenol and *p*-nitrophenolate ion, where $\varepsilon = 5.4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for both species. The reaction was linear for 1–10 min, and the initial slope was taken for the calculation of the enzymatic rate.

Preparation of the imidazole–histidine salt of p-nitrophenyl phosphate

1 g of the disodium salt of *p*-nitrophenyl phosphate (Sigma) was dissolved in 20–30 ml of water and added gradually to a 10-cm column of Dowex 50 X8 (H^+ form). Then the column was washed 3 times with 10-ml aliquots of water. Flame photometric measurements indicated that passage through the column replaced more than 99 % of the Na^+ with H^+. The entire effluent (pH 2.0) was collected, and its pH adjusted to 7.3 by the addition of a mixture containing equimolar amounts of imidazole and histidine hydroxide.

RESULTS

Table I presents values on mean rates of hydrolysis of *p*-nitrophenyl phosphate in four experiments on microsomes under the conditions denoted in the first column.

Under all conditions except Condition d, small increments of P_i (over and above the non-enzymatic blank) were released into the incubation fluid. Apart from the significance of the small, but detectable releases of P_i, the largest of the small increments (Condition e) was but 3 % of the amount of P_i released, 12.2 μmoles/mg per h, in the presence of $Mg^{2+} + K^+$ (Condition d).

TABLE I

EFFECT OF CATIONS AND OUABAIN ON *p*-NITROPHENYL PHOSPHATASE ACTIVITY OF MICROSOMES

Each value represents the mean ± S.E. in four experiments. Final concentrations of additions in incubation mixtures were: imidazole, 20 mM; histidine, 20 mM; Mg^{2+}, 4.0 mM; K^+, 10 mM; Na^+, 100 mM; and ouabain, 0.1 mM. In all cases, final concentration of the imidazole–histidine salt of *p*-nitrophenylphosphate was 4.0 mM and the final pH was 7.3 (see METHODS).

Additions	*p-Nitrophenyl phosphatase activity* (*μmoles P_i/mg protein per h*)
(a) Buffer alone	0.15 ± 0.08
(b) Buffer + Mg^{2+}	0.37 ± 0.20
(c) Buffer + K^+	0.20 ± 0.10
(d) Buffer + Mg^{2+} + K^+	12.2 ± 0.84
(e) Buffer + Mg^{2+} + K^+ + Na^+	0.40 ± 0.25
(f) Buffer + Mg^{2+} + K^+ + ouabain	0.45 ± 0.15

The activity of the $(Mg^{2+} + K^+)^-$ dependent, ouabain-sensitive *p*-nitrophenyl phosphatase of the turtle bladder microsomes, was considerably greater than that found in the microsomes of other tissues[10–12]. Not shown in Table I are similar results from sixteen other experiments on freshly prepared bladder microsomes where

the mean value ± S.E. for K^+-dependent *p*-nitrophenylphosphatase was 10.1 ± 0.7 μmoles/mg protein per h. The mean values for the Mg^{2+} and (Na^+ + K^+)-dependent ATPase, concomitantly determined in two of the four experiments of Table I, were 26.9 and 40.8 μmoles/mg per h, respectively. The next set of experiments was designed to determine the kinetic parameters of the K^+-dependent, ouabain-sensitive microsomal *p*-nitrophenyl phosphatase.

Kinetic parameters

The microsomal *p*-nitrophenyl phosphatase activity is a polycomponent reaction, the velocity of which depends upon the concentration of co-factors such as Mg^{2+}, and K^+ as well as upon that of the substrate, *p*-nitrophenyl phosphate. Therefore, the measured or apparent values of kinetic parameters such as K_m and v_{max} are functions of both co-factor and substrate concentrations. In what follows (Figs. 1–3), the apparent values for K_m and v_{max} are those set by the experimental conditions defined for each case.

Fig. 1, a Lineweaver–Burk plot of values of reciprocal activity in arbitrary units *versus* reciprocal concentration of *p*-nitrophenyl phosphate, was obtained from a set of incubation mixtures in which the concentration of microsomes, Mg^{2+} and K^+, were fixed while those of *p*-nitrophenyl phosphate varied from 0.4 to 15 mM. Treating the data as if they followed simple Michaelis–Menten kinetics, the apparent K_m (K_s) (between the limits of concentration of 0 and 4 mM *p*-nitrophenyl phosphate) was 0.6 mM; and the v_{max} between the same concentration limits was 8.3 μmoles/mg per h. The plot also indicates a pronounced substrate inhibition for concentrations of *p*-nitrophenyl phosphate in excess of 4 mM. Not shown is a plot for the same data in the form of $1/v$ *versus* substrate concentration from which the apparent K_i (or K_{ss}) for *p*-nitrophenyl phosphate was estimated to be 16 mM.

Fig. 2, a plot of values of reciprocal activity in arbitrary units *versus* reciprocal Mg^{2+} concentration, was obtained from a set of incubation mixtures in which the concentrations of microsomes, K^+ and *p*-nitrophenyl phosphate were fixed, while

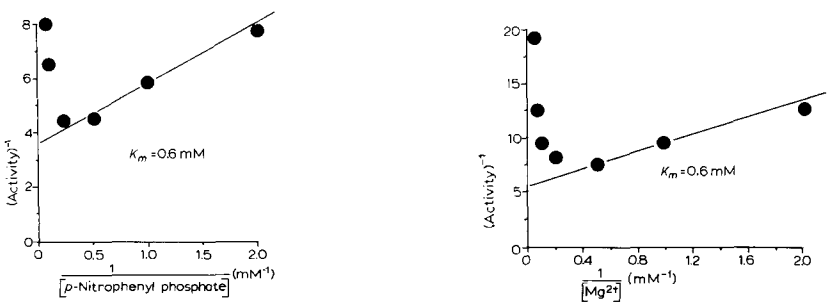


Fig. 1. Lineweaver–Burk plot of reciprocal *p*-nitrophenyl phosphatase activity in arbitrary units *versus* reciprocal millimolar concentration of *p*-nitrophenyl phosphate. The concentrations of buffer, Mg^{2+}, K^+ and the pH were the same as those described in Table I and in METHODS. $(Activity)^{-1}$ is in arbitrary units, taken from raw absorbances prior to correcting for dilution, aliquot, time of incubation and protein concentration, all of which were constant in any single set of experiments.

Fig. 2. Lineweaver–Burk plot of reciprocal *p*-nitrophenyl phosphatase activity in arbitrary units *versus* reciprocal millimolar concentration of Mg^{2+}. Other than the Mg^{2+}, the concentrations of the constituents were the same as those described in Table I and in METHODS.

those of Mg^{2+} varied from 0.4 to 25 mM. Between the limits of Mg^{2+} concentration of 0.0 to 4 mM, the apparent K_m was 0.6 mM and the v_{max} was 7.2 μmoles/mg per h. Pronounced substrate (Mg^{2+}) inhibition was observed for Mg^{2+} concentrations in excess of 4 mM and the apparent K_i for Mg^{2+} was 16 mM.

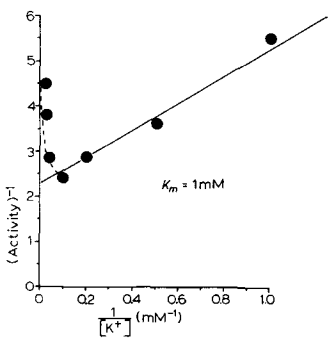
On the basis of data in Figs. 1 and 2, the optimal activity in 10 mM K^+ occurs when the concentration ratio, $[Mg^{2+}]/[p\text{-nitrophenyl phosphate}] = 1.0$. This is similar to the $[Mg^{2+}]/[ATP]$ ratio required for optimal activity of the Mg^{2+} and $(Na^+ + K^+)$-dependent ATPase activity in several tissues[4,5,7].

Fig. 3, a plot of values of reciprocal activity in arbitrary units *versus* reciprocal concentration of K^+, was obtained from a set of incubation mixtures in which the concentrations of microsomes, Mg^{2+}, and *p*-nitrophenyl phosphate were kept constant while those of K^+ varied from 0.5 to 100 mM.

Between the limits of K^+ concentration of 0.5–10 mM, the apparent K_m was 1 mM and the v_{max} was 14.1 μmoles/mg per h. Substrate (K^+) inhibition was observed for K^+ concentrations in excess of 10 mM, and the apparent K_i for K^+ was 100 mM.

Since the addition of 100 mM Na^+ resulted in nearly complete inhibition of the K^+-dependent *p*-nitrophenyl phosphatase (see Table I), we decided to examine the effect of lower concentrations of Na^+ upon the kinetic pattern of the K^+-dependent *p*-nitrophenyl phosphatase illustrated in Fig. 3. Therefore, the *p*-nitrophenyl phosphatase activity *versus* K^+ concentration in the presence of a fixed concentration of Na^+ was determined for a family of such Na^+ concentrations, *e. g.* in the presence of 0, 5, 10, 20 and 50 mM. The K^+ concentration was kept below 10 mM to avoid the inhibitory effect of K^+ itself.

Fig. 4 shows five Lineweaver–Burk plots of values of reciprocal activity in arbitrary units *versus* reciprocal K^+ concentration in the presence of no Na^+ (the lowermost line which is in the same activity range as that of Fig. 3), and in the presence of 5,



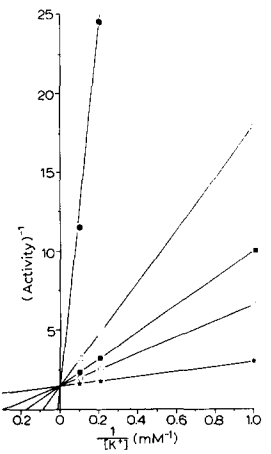


Fig. 3. Lineweaver–Burk plot of reciprocal *p*-nitrophenyl phosphatase activity in arbitrary units *versus* reciprocal millimolar concentration of K^+. Other than that of K^+, the concentrations of the constituents were the same as those described in Table I and in METHODS.

Fig. 4. Lineweaver–Burk plot of reciprocal *p*-nitrophenyl phosphatase activity *versus* reciprocal millimolar concentration of K^+ for a family of Na^+ concentrations. ✱—✱, $[Na^+] = 0$; ○—○, $[Na^+] = 5$ mM; ■—■, $[Na^+] = 10$ mM; □—□, $[Na^+] = 20$ mM; ●—●, $[Na^+] = 50$ mM.

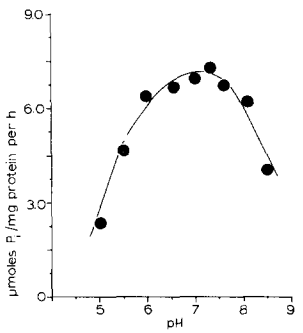
10, 20, and 50 mM Na^+. Data shown were taken from two consecutive experiments, one covering Na^+ concentrations of 0, 5 and 10 mM; and the other covering Na^+ concentrations of 0, 20, and 50 mM. The line for 100 mM Na^+, not shown, is co-linear with the ordinate.

The five plots shown in the figure indicate that the higher the Na^+ concentration, the lower the *p*-nitrophenyl phosphatase activity over the range of K^+ concentrations used (1–10 mM); and that the v_{max} for all five concentrations of Na^+ was 10.4 μmoles/mg per h, which suggests that Na^+ competes with K^+ for the K^+-activation sites on the microsomal protein. The K_i for Na^+, 3 mM, was estimated graphically from the extrapolation of the plot of apparent K_m for K^+ *versus* Na^+ concentration. This suggests that the affinity of the *p*-nitrophenyl phosphate-related protein for Na^+ was approx. one-third of that for K^+ (K_m for K^+ = 1.0 mM; see Fig. 3).

Effect of pH

The effect of pH (over the range 5–8.5) on *p*-nitrophenyl phosphatase activity was determined in the presence of 8 mM Na^+ and 20 mM K^+. The concentration of K^+ used, 20 mM, was twice that of the other experiments on (Mg^{2+} + K^+)-dependent *p*-nitrophenyl phosphatase. This K^+ concentration was found sufficient to eliminate the inhibitory effect of the 8 mM Na^+ contained in the disodium salt of *p*-nitrophenyl phosphate, and provided a convenient means of varying pH (by substitution of equimolar amounts of KOH for KCl) without changing the final concentrations of K^+, imidazole, histidine, Mg^{2+}, Na^+ or *p*-nitrophenyl phosphate.

Fig. 5 is a plot of values of *p*-nitrophenyl phosphatase activity *versus* final pH of the incubation mixture in one of three parallel experiments on microsomes. It can be seen that a pH of 7.2–7.4 was required for optimal activity under the conditions used. At pH 5, the activity was 38 % of that at 7.3; and at pH 8.5 the activity was 52 % of that at 7.3. Data from the two other experiments, not shown in the figure, fell about a closely similar pattern.



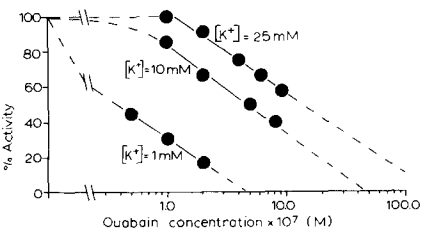


Fig. 5. *p*-Nitrophenyl phosphatase activity *versus* pH of the incubation mixture. The millimolar concentrations of cations were: Mg^{2+}, 4; K^+, 20; Na^+, 8; and *p*-nitrophenyl phosphate, 4 .

Fig. 6. Semi-log plot of percentage activity of *p*-nitrophenyl phosphatase *versus* concentration of ouabain for three levels of K^+ concentration: 1, 10, and 25 mM. Other than ouabain and K^+, the concentrations of the constituents in the incubation mixtures were the same as those described in Table I and in METHODS.

Effect of ouabain

The next set of experiments was designed to examine the dependence of ouabain inhibition upon the concentration of K^+ in the incubation mixture.

The microsomal pellet in the presence of Mg^{2+}, K^+, appropriate buffer, and ouabain was pre-incubated at 38° for 5 min before starting the reaction by addition of *p*-nitrophenyl phosphate. Increasing the time of the aforementioned pre-incubation from 5 to 30 min did not significantly change the degree of ouabain-induced inhibition.

Fig. 6 presents a plot of normalized values of *p*-nitrophenyl phosphatase activity *versus* the logarithm of ouabain concentration for a family of three different K^+ concentrations. Data presented were taken from one of two identically designed experimental sets. In each set, the K^+ concentrations of the incubation mixtures were fixed at the three levels (1, 10 and 25 mM) denoted in the figure.

For each of the three concentrations of K^+ used, the plots of percent activity *versus* log ouabain concentration were linear over the range of ouabain concentrations denoted along the abscissa. The linear nature of the three logarithmic functions indicates that the greater the K^+ concentration (from 1 to 25 mM) the greater the concentration of ouabain required for any degree of inhibition between the limits of 10–90 % of the control level. This pattern resembled that expected of a competitive inhibition between K^+ and ouabain. As anticipated, the plot of 1/activity *versus* $1/K^+$ concentration (for each of the ouabain concentrations used) generated a set of straight lines intersecting at a common value on the 1/activity ordinate, where v_{max} for K^+ was approximately the same as that in Figs. 2 and 3; and where the graphic extrapolation of the plot of K_m for K^+ *versus* ouabain concentration indicated that the K_i for ouabain was 25 nM. Nearly complete inhibition of K^+-dependent *p*-nitrophenyl phosphatase was achieved in the presence of 0.1 mM ouabain, at all concentrations of K^+ used in this study.

Effect of N-ethylmaleimide

The addition of *N*-ethylmaleimide is known to inhibit $(Na^+ + K^+)$-ATPase, K^+-stimulated acetyl phosphatase, and K^+-stimulated *p*-nitrophenyl phosphatase from various tissue sources[2,7,8]. Moreover, the degree of inhibition was partly dependent upon the time of pre-incubation of microsomes with the inhibitor.

Current experiments on *N*-ethylmaleimide in the turtle bladder microsomes were first focused on the relation between the time of pre-incubation of the microsomes with *N*-ethylmaleimide and the subsequent degree of inhibition of *p*-nitrophenyl phosphatase activity. Two sets of experiments, each involving a control and four concentrations of *N*-ethylmaleimide, were performed. For each level of *N*-ethylmaleimide, the time of exposure of enzyme to *N*-ethylmaleimide, Mg^{2+}, K^+ and buffer (before starting the reaction with addition of *p*-nitrophenyl phosphate) was 5, 20 and 30 min. In each case, the same times of pre-incubation and incubation were applied to the three control mixtures which contained enzyme and all of the aforementioned constituents except *N*-ethylmaleimide.

Fig. 7 presents a plot of values of percent activity *versus* concentration of *N*-ethylmaleimide for a family of three pre-incubation periods (5, 20 and 30 min) in one of the two sets of aforementioned experiments.

It can be seen that the degree of inhibition of the K^+-dependent *p*-nitrophenyl phosphatase activity increased with increasing time of pre-incubation of the enzyme

with the inhibitor over the entire range of concentrations (0.01–5 mM) of *N*-ethylmaleimide used. For a concentration of inhibitor of 1 M, the inhibition was 25 % after 5 min, 52 % after 20 min, and 73 % after 30 min of pre-incubation. The concentrations of *N*-ethylmaleimide required to induce 50 % inhibition of activity were: >5 mM for 5 min of pre-incubation; 1.25 mM for 20 min; and 0.38 mM for 30 min of pre-incubation.

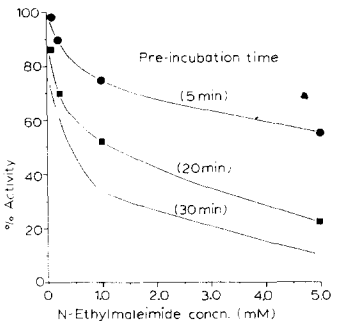


Fig. 7. Percentage activity of *p*-nitrophenyl phosphatase *versus* concentration of *N*-ethylmaleimide. Pre-incubation times are shown within the parentheses over each curve.

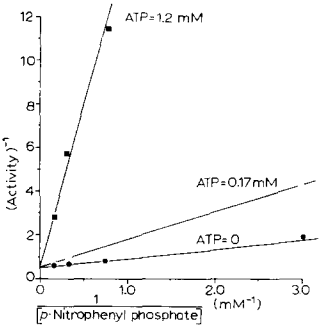


Fig. 8. Lineweaver–Burk plot of reciprocal *p*-nitrophenyl phosphatase activity in arbitrary units *versus* reciprocal millimolar concentration of *p*-nitrophenyl phosphate in the absence and in the presence of ATP, the concentration of which is indicated next to each line.

Of interest are previously reported data[1] showing that 45 % inhibition of the $(Na^+ + K^+)$-ATPase was obtained with 0.1 mM *N*-ethylmaleimide after 30 min of pre-incubation of enzyme with inhibitor, a result quite similar to that shown (40 %) for inhibition of the K^+-dependent *p*-nitrophenyl phosphatase with the same amount of inhibitor and the same time of pre-incubation (see the lower of Fig. 7).

Of further interest is the fact, reported previously from this laboratory[1], that *N*-ethylmaleimide (final concentration, 0.1 mM) reduced the short-circuiting current of the turtle bladder to zero 30 min after its addition to the bathing media.

Effect of ATP

Because of certain similarities between the reactions of the inhibitors, ouabain and *N*-ethylmaleimide, on K^+-dependent *p*-nitrophenyl phosphatase and $(Na^+ + K^+)$-ATPase it was decided to measure the activity of the former (*p*-nitrophenyl phosphatase) in the presence of ATP. In each of two experimental sets, *p*-nitrophenyl phosphatase activity was measured in the absence and in the presence of two concentrations of ATP. The *p*-nitrophenyl phosphatase activity in the presence of ATP was assayed by measuring the *p*-nitrophenol released as described in METHODS.

Fig. 8, a plot of values of reciprocal activity on arbitrary units *versus* reciprocal *p*-nitrophenyl phosphate concentration, was obtained from a set of incubation mixtures in a Na^+-free medium in which the concentrations of microsomes, K^+, and Mg^{2+} were fixed for two levels of ATP (0.17 and 1.2 mM) while the concentration of *p*-nitrophenyl phosphate varied from 0.33 to 6 mM. The three lines generated between these limits of substrate concentration intersected at the same point on

the ordinate ($1/v_{max}$), suggesting that ATP competes with *p*-nitrophenyl phosphate for occupation sites on the enzyme. The value of v_{max} shown here was obtained at 25°, and consequently was smaller than that estimated in other experiments at 38° (see Fig. 1).

Effect of sodium azide

Addition of sodium azide to a final concentration range of 0.01–1.0 mM had no detectable effect on the K^+-dependent *p*-nitrophenyl phosphatase, even after 30 min of pre-incubation.

DISCUSSION

Some of the properties of K^+-dependent *p*-nitrophenyl phosphatase reported in this study are similar to certain properties of $(Na^+ + K^+)$-ATPase that have been reported by us[1] and others[2,5].

In the case of the turtle bladder microsomes, the pH, the Mg^{2+}/substrate ratio, and the Mg^{2+} and K^+ concentrations required for optimal activity of either *p*-nitrophenyl phosphatase or ATPase were about the same[1]. At the optimal K^+ concentration, the concentration of ouabain needed for 50 % inhibition of either activity, K^+-*p*-nitrophenyl phosphatase or $(Na^+ + K^+)$-ATPase, was 0.3–0.5 μM, a sensitivity to ouabain of 10–100-fold greater than that reported for 50 % inhibition of either microsomal activity in other tissues[8,16,17]. The ouabain sensitivity of acetyl phosphatase was only 20 % less than that of $(Na^+ + K^+)$-ATPase in guinea pig kidney and brain[9], but was approx. 10-fold greater than that of $(Na^+ + K^+)$-ATPase in beef brain[8].

Other similarities between the two activities can be made with respect to the effects of *N*-ethylmaleimide and the competition between ATP and *p*-nitrophenyl phosphate. Thus, pre-incubation of microsomes with 0.1 mM *N*-ethylmaleimide for 30 min resulted in a 40–45 % inhibition of either $(Na^+ + K^+)$-ATPase or K^+-*p*-nitrophenyl phosphatase; and ATP appeared to compete with *p*-nitrophenyl phosphate for occupation sites on the protein (see Fig. 8).

The main difference in the cationic effect on the two enzyme activities was that of Na^+ which stimulated the activity of the $(Na^+ + K^+)$ATPase, but which inhibited the activity of K^+-*p*-nitrophenyl phosphatase. This qualitative difference suggests that the hydrolysis of ATP and *p*-nitrophenyl phosphate is catalyzed *via* two mechanisms despite all of the aforementioned similarities. However, data reported here and elsewhere are not sufficient to determine whether each substrate, ATP or *p*-nitrophenyl phosphate, binds with a common site on the enzyme protein or even whether such binding entails two sites on the same protein, two sub-unit sites on a polymeric protein or two independent protein units. All possibilities can be made consistent with any of the available data. The solution of the problem will probably require the isolation, solubilization, and characterization of one or more pure enzymatically active proteins.

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REFERENCES

1 Y. E. SHAMOO AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 111.
2 J. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
3 S. L. BONTING AND L. L. CARAVAGGIO, *Biochim. Biophys. Acta*, 101 (1963) 37.
4 A. K. SEN AND K. L. POST, *J. Biol. Chem.*, 339 (1964) 345.
5 J. C. SKOU, *Ann. Rev. Physiol.*, 45 (1965) 596.
6 E. HEINZ, *Ann. Rev. Physiol.*, 29 (1967) 21.
7 R. W. ALBERS, *Am. Rev. Biochem.*, 36 (1967) 727.
8 Y. ISRAEL AND E. TITUS, *Biochim. Biophys. Acta*, 139 (1967) 450.
9 H. BADER AND A. K. SEN, *Biochim. Biophys. Acta*, 118 (1966) 116.
10 R. W. ALBERS AND G. J. KOVAL, *J. Biol. Chem.*, 241 (1966) 1896.
11 A. ASKARI AND D. KOYAL, *Biochem. Biophys. Res. Commun.*, 32 (1968) 227.
12 A. M. WOODIN AND A. A. WIENEKE, *Biochem. Biophys. Res. Commun.*, 33 (1968) 558.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND J. R. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, (1938) 295.
15 J. MCD. ARMSTRONG, D. V. MYERS, J. A. VERPOORTE AND J. T. EDSALL, *J. Biol. Chem.*, 241 (1966) 5137.
16 R. W. ALBERS AND G. J. KOVAL, *J. Biol. Chem.*, 241 (1966) 1896.
17 G. G. BERG AND J. SZEKERCZES, *J. Cellular Comp. Physiol.*, 67 (1966) 487.