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ABILITY OF DIPHOSPHONATES TO BIND CALCIUM AND THEIR EFFECT ON OSMOTIC PERMEABILITY OF THE FROG BLADDER WALL

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Calcium plays an exceptionally important role in the regulation of various physiological processes, stabilization of membranes, and phenomena of cell adhesion [13]. Diphosphonates have begun to be used in recent years in the treatment of disturbances of calcium metabolism [1, 9, 11]. Together with other effects, the possibility of binding of calcium with these substances in biological systems must probably be taken into account in the mechanism of their action. To study the degree of extraction of calcium from biological structures by diphosphonates, changes in permeability of the wall of the isolated frog urinary bladder for water were determined. This object has been widely used in recent years in experimental physiology in order to study membrane transport [6].

The object of the present investigation was to compare the effect of diphosphonates on the osmotic permeability of the frog's urinary bladder, depending on their ability to bind calcium ions.

EXPERIMENTAL METHOD

The action of a series of organophosphorus complexones with different stability constants with calcium ions and being analogs of methylenediphosphonic acid (Table 1, compounds Nos. 2-6), and also of compound No. 7, similar in its structure to ethylenediaminetetraacetic acid (compound No. 1) was studied. The experiments were conducted as follows. The isolated urinary bladders of frogs (*Rana temporaria*) were filled with Ringer's solution diluted with water (1:10) and were placed in aerated Ringer's solution. After various time intervals the bladders with their contents were weighed, and the decrease in weight was used to calculate the volume of water which had passed through the bladder wall along the osmotic gradient [7]. Complexones were added to the Ringer's solution on the side of the serous membrane.

EXPERIMENTAL RESULTS

Under ordinary conditions the bladder wall possesses very low osmotic permeability and water transport along the osmotic gradient amounts to 0.03-0.05 $\mu l/cm^2 \cdot min$. At the beginning of the experiment inside the bladder there was 700-800 µl of water and the loss of water dur-

Laboratory of Evolution of the Kidney and Water and Salt Metabolism, I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry, Academy of Medical Sciences of the USSR, Leningrad. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 93, No. 6, pp. 71-74, June, 1982. Original article submitted November 4, 1981.

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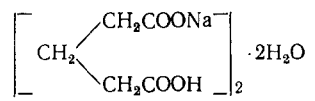
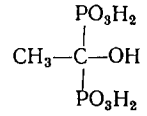
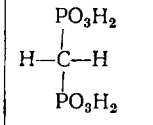
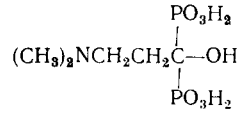
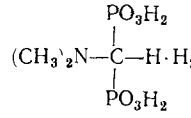
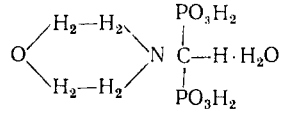
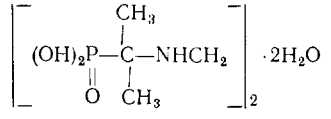
TABLE 1. Logarithms of Stability Constants of Complexes with Calcium Ions

Compound No.	Formula	Empirical formula	Ca^{2+}		Literature source
			MHL	ML	
1		$C_{10}H_{18}O_{10}N_2Na_2$	3,55	10,61	[12]
2		$C_2H_8O_7P_2$	3,58	6,04	[4]
3		$CH_6O_6P_2$	3,88	6,03	[4]
4		$C_5H_{15}NO_7P_2$	5,57	5,71	[5]
5		$C_3H_{13}O_7NP_2$	4,56	4,78	[2]
6		$C_5H_{13}O_7NP_2$	4,05	4,18	[2]
7		$C_8H_{26}O_8N_2P_2$	—	2	[3]

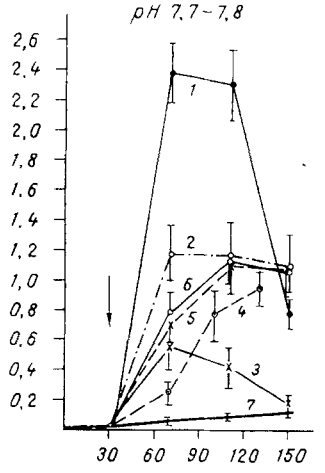


Fig. 1

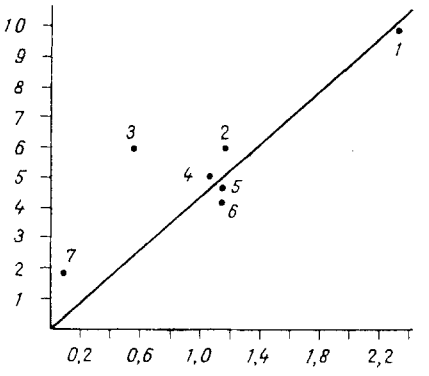


Fig. 2

Fig. 1. Time course of permeability of frog urinary bladder wall for water after addition of EDTA or diphosphonates to calcium-free solution on side of serous membrane in dose of 1 mM. 1-7) No. of compound. Abscissa, time of experiment (in min); ordinate, flow of water along osmotic gradient (in $\mu l/cm^2$). Arrow indicates time of addition of compound.

Fig. 2. Relationship between logarithm of stability constants of complex with Ca^{++} ion and change in osmotic permeability of bladder wall. Abscissa, flow of water (in $\mu l/cm^2 \cdot min$) 60 min after addition of compound (No., see Table 1); ordinate, log $K_{Ca^{++}}$ (Table 1). Remainder of legend as to Fig. 1.

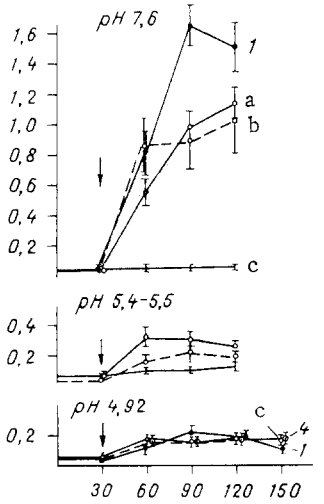


Fig. 3. Effect of EDTA and diphosphonates on osmotic permeability of bladder wall in physiological saline with different pH values of medium. Abscissa, time of experiment (in min); ordinate, flow of water along osmotic gradient (in $\mu l/cm^2 \cdot min$). a) Ethylenediaminetetramethylphosphonic acid ($C_6H_{20}N_2O_8P_4$); b) nitrylotrimethylphosphonic acid ($C_2H_{12}N_1O_9P_3$); c) control in Ringer's solution and corresponding pH value. Remainder of legend as to Fig. 1.

ing 15 min was under 1% of its total volume in the bladder. Addition of antidiuretic hormone to the Ringer's solution on the side of the serous membrane of the bladder or removal of calcium from this solution by means of EDTA sharply increased permeability for water [8]. After addition of EDTA to calcium-free Ringer's solution the loss of fluid gradually increased during the first 30 min and reached a maximum in the period from 30 to 60 min of the experiment: permeability increased 50-fold or more (Fig. 1).

Experiments were carried out by a similar scheme with diphosphonates. In calcium-free Ringer's solution, pH 7.7-7.8, complexone No. 4 (Table 1) gave an effect very similar in its kinetics to that of EDTA, but weaker (Fig. 1). Permeability for water was increased 90 min after addition of $C_5H_{15}NO_7P_2$ (1 mM) and its flow along the osmotic gradient increased from 0.041 ± 0.006 to 1.09 ± 0.17 $\mu l/cm^2 \cdot min$ ($n = 8$). An increase in the concentration of compound No. 4 to 2 and 4 mM did not potentiate the effect: 0.99 ± 0.13 $\mu l/cm^2 \cdot min$ ($n = 7$) and 1.1 ± 0.18 $\mu l/cm^2 \cdot min$ ($n = 6$), respectively. Consequently, the degree and velocity of extraction of calcium from the tissue reached a maximum after addition of only 1 mM of the compound, and the time course of the reaction (the same with all concentrations) was determined by penetration of diphosphonate into the tissue, absorption of calcium from the structures, and, finally, changes in tissue structures leading to increased permeability. A study of the effect of different diphosphonates on permeability for water when the pH of the medium was 7.7-7.8 showed good correlation between the calcium binding constant and the degree of increase of permeability of the frog bladder wall for water (Fig. 2).

A stable level of H^+ concentration within limits of 7.36-7.40 is maintained very accurately in blood and extracellular fluid. At the same time, in certain organs such as the stomach and in the terminal portions of the renal tubules, acidification of the fluid may take place with a shift of pH much below 5.0. Because of the possibility of finding diphosphonates with organ-specific action, it was decided to analyze the character of calcium binding with EDTA and diphosphonates in media with different pH values. For this purpose Ringer's solution was prepared without sodium bicarbonate and 5 ml buffer (0.1 M Na_2HPO_4 + citric acid; the pH of the solution was 6.0-6.3 and 5.4-5.5) or acetate buffer, pH 4.92, was added to 95 ml of this solution. The results showed that the effect of all compounds on permeability for water became much weaker when the pH shifted to the acid side (Fig. 3). EDTA had actually a slightly stronger action than diphosphonates in solution with pH 6.0-6.3, but later, on acidification, the action of all compounds was reduced even more sharply and they changed permeability practically equally weakly.

Analysis of the physiological action of diphosphonates must therefore take into consideration the possibility of their effect on cellular and membrane processes as a result of calcium binding from biological structures, which depends on the pH of the biological fluid. This may evidently account for the ability of diphosphonates to prevent calcification of soft tissues, to modify the activity of certain enzymes, and to cause other physiological effects.

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