Blockage of Gallbladder Tight Junction Cation-Selective Channels by 2,4,6-Triaminopyrimidinium (TAP)

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ABSTRACT The organic cation 2,4,6-triaminopyrimidinium (TAP) specifically inhibits Na⁺ passive permeation (P Na⁺) across gallbladder, small intestine, and choroid plexus without detectable effect on the Cl⁻ permeability, indicating that Na⁺ and Cl⁻ follow different permeation pathways. In bullfrog gallbladder, where it was examined in greater detail, the effect of TAP was shown to be: (a) completely reversible, (b) due only to the protonated form of 2,4,6-triaminopyrimidinium, (c) effective when added to either one or both sides of the membrane (the rate limiting for the delay in the response being the diffusion through the unstirred layers), and (d) exhibiting a typical saturation kinetics, best fitted with the parameters “Km” = 2.6 mM and maximal effect = 100% inhibition. These data, along with the fact that the P Na⁺ blocking action of chemical analogs of TAP increases with their ability to donate protons to form hydrogen bonds, suggest that TAP blocks the cation permeation of the channels by strongly associating, via hydrogen bonds, with the anionic ligands within the channel.

INTRODUCTION

Recently it was shown that the main transepithelial ionic conductance route in Necturus gallbladder is between epithelial cells (through the tight junctions), and not through the cells (Frömter and Diamond, 1972; Frömter, 1972). A physical model of this pathway for cations, devised from studies on the passive ion permeation and selectivity in frog and rabbit gallbladder describes a system of large (about 10- to 20-Å diameter) hydrated “channels,” whose ionized acidic groups render them cation selective. The ligands within the channel (probably the same acidic groups) behave as remarkably strong H-bond proton acceptors (Moreno and Diamond, 1974 a, 1974 b, 1975 a, 1975 b). If this model is correct it might be possible to specifically block cation permeation by introducing a compound which would associate strongly with the anionic ligands within the channel, possibly through strong H-bond...
interaction. With such considerations in mind the organic cation 2,4,6-triaminopyrimidinium (TAP) was selected as a good candidate for the role since it has a strong H-bond proton donor ability and its molecular dimensions would allow it to fit within the channels.

This paper shows that TAP specifically inhibits cation permeability across the tight junctions in gallbladder. The specificity of this action suggests the use of TAP first as a tool for studying the characteristics and pathways of the passive ionic permeation in gallbladder and other leaky epithelia, and second, along with some of its chemical derivatives, as a "probe" for the channel structure. A note including some of these results has been published (Moreno, 1974).

METHODS

Experimental Techniques

Experimental material consisted of gallbladders, small intestines, and choroid plexuses from bullfrog (Rana catesbeiana) weighing ca. 350 g, gallbladders and small intestine (terminal ileum) from white New Zealand rabbits, weighing 2.5-3 kg, and gallbladders from Necturus maculosus, weighing ca. 250 g. Techniques and methods for obtaining an in vitro preparation of gallbladder have been described previously (Moreno and Diamond, 1974 b, 1975 b). Briefly, the gallbladder was removed from the animal, carefully rinsed free of bile, cut open into two halves, and each half mounted as a flat sheet between two Lucite chambers with a window area of 10 or 20 mm². Each chamber contained 2 ml of solution that was vigorously stirred by magnetic stirring bars. Transepithelial potential differences (PD) and conductances (G) were measured as described previously (Moreno and Diamond, 1974 b, 1975 b). The equipment (chambers, stirring and recording devices) was duplicated, so that the two halves of the same gallbladder could be studied simultaneously, one serving as a control.

The standard bathing solution has the following composition (in mM): 150 NaCl, 0.25 CaCl₂, 0.34 K₂HPO₄, 3.42 KH₂PO₄, pH 6.1. Solutions were adjusted to other pH by adjusting the K₂HPO₄/KH₂PO₄ ratio maintaining the K⁺ concentration constant at 4 mM. The solutions containing 2,4,6-triaminopyrimidine or its derivatives were titrated to the appropriate pH with HCl. NaCl dilution potentials were measured by replacing half the NaCl in the mucosal solution isoosmotically with mannitol; biionic potentials were measured by completely replacing NaCl with the Cl⁻ salt of another cation, all of the other components of the Ringer solution remaining constant.

Thirty minutes to 1 h after the tissues were mounted in the chambers, G was measured every 10 min or less. Two NaCl dilution potentials bracketing a K⁺:Na⁺ biionic potential were taken periodically by changing the mucosal solutions. Experiments were discontinued unless the initial NaCl dilution potentials were greater than 9 mV and remained constant; and the initial spontaneous PD between identical solutions in rabbit and frog gallbladders was < 2 mV.

As discussed previously (Moreno and Diamond, 1974 b), the use of the experimental conditions summarized above led to preparation with passive properties (ionic con-
ductances and permeability ratios) which remain constant for many hours, in contrast with the fast-deteriorating gallbladder preparations reported in earlier experiments (e.g. Wright et al., 1971; Barry et al., 1971). At pH 6.1, the passive electrical properties of frog and rabbit gallbladders do not differ significantly from those at pH 7.4 (Moreno and Diamond, 1974 b).

Exceptions to the above experimental techniques were as follows: Intestines were mounted across 1-cm² chamber windows once their muscular layer was removed; frog choroid plexuses were mounted across 7-mm² chamber windows (as described by Wright, 1972); Ringer's solutions containing 100 mM NaCl, instead of 150 mM NaCl were used with choroid plexus and Necturus gallbladder preparations.

2,4,6-triaminopyrimidine was obtained from Eastman Organic Chemicals, Rochester, N. Y.; 2,4-diaminopyrimidine, 4,6-diaminopyrimidine, 4,5,6-triaminopyrimidine, and 2,4,6-triamino-1,3,5-triazine (Melamine) from Aldrich Chemical Company, San Leandro, Calif.; and 2-amino-4-dimethylaminopyrimidine, and 4-amino-2-dimethylaminopyrimidine were gifts of Dr. B. Roth from Wellcome Research Laboratories, Research Triangle Park, N.C. Unless it is specifically mentioned, these drugs were added to both sides of the preparations, and the concentration of their monoprotonated form is reported (see pp. 104–106). The experiments were performed at 23°C.

Analysis of the Data

The calculation of the ionic permeabilities (P_i) and conductances (G_i) have been extensively described and discussed elsewhere (Moreno and Diamond, 1974 b, 1975 b). Briefly, permeability ratios were extracted from measured dilution and bionic potentials by means of the Goldman-Hodgkin-Katz equation. In gallbladder, for the common monovalent inorganic chloride salt solutions the ratio of cation to anion permeabilities is equal to its ratio of ionic conductances (Barry et al., 1971; Wright et al., 1971), for a NaCl salt solution:

\[ \frac{P_{Na}}{P_{Cl}} = \frac{G_{Na}(total)}{G_{Cl}}, \quad (1) \]

and the total transepithelial conductance, G:

\[ G = G_{Na}(total) + G_{Cl}. \quad (2) \]

Therefore,

\[ G_{Cl} = G/1 + (P_{Na}/P_{Cl}). \quad (3) \]

The low Cl conductance of frog and rabbit gallbladder may be assumed to be entirely in a shunt in which the ratio of Na⁺ and Cl⁻ transport numbers equals the free solution mobility ratio \( u_{Na}/u_{Cl} \), i.e.:

\[ G_{leakage} = G_{Cl}(1 + u_{Na}/u_{Cl}). \quad (4) \]

Evidence for this interpretation presented elsewhere (Barry et al., 1971; Moreno and Diamond, 1974 b, 1975 a, 1975 b) may be summarized as follows, and further evidence
will be given on p. 110: (a) the chloride conductance of a freshly dissected rabbit gallbladder is negligible; (b) when after dissection, $G$ increases and $P_{Na}/P_{Cl}$ decreases, the increase in $G_{Cl}$ and $G_{Na}$ are in the free solution mobility ratio: $G_{Na}/G_{Cl} = u_{Na}/u_{Cl}$; (c) most of the apparent variation in cation-to-cation permeability ratios in different preparations, or at different times in the same preparation disappear if the ratios are corrected for a free solution shunt whose conductance is estimated from the measured $G_{Cl}$ (Eq. 4); (d) organic cations whose molecular structure indicates that they should be impermeant in the native membrane, show a $P_{X}/P_{Cl} \approx u_{X}/u_{Cl}$ independently of the actual value of $G_{Cl}$; (e) the $Q_{in}$ of $G_{Cl}$ is not significantly different from the one of $u_{Cl}$ while the $Q_{in}$ of $G_{Na}$ is considerably higher; and (f) pH variations from 9 to 3 can modify $G_{Na}$ by 80% without affecting $G_{Cl}$. Therefore, the Na$^+$ membrane conductance corrected by the Cl shunt or leakage current,$^1$ $G_{Na}$:

$$G_{Na} = G - G_{Cl}(1 + u_{Na}/u_{Cl}). \quad (5)$$

The ratio of cation permeabilities was also corrected for the Cl shunt, for two cations $X$ and $Y$:

$$\frac{P_{X}}{P_{Y}} = \frac{P_{X}/P_{Cl} - u_{X}/u_{Cl}}{P_{Y}/P_{Cl} - u_{Y}/u_{Cl}}, \quad (6)$$

where $(P_{X}/P_{Y})$ is the cation permeability ratio corrected for the leakage pathway, $P_{X}/P_{Cl}$ and $P_{Y}/P_{Cl}$ were obtained from biionic and dilution potentials using the Goldman-Hodgkin-Katz equation, and the ratios of free solution mobilities $u_{X}/u_{Cl}$ and $u_{Y}/u_{Cl}$ were obtained from Robinson and Stokes (1970). The derivation and justification for the use of Eq. 6 has been published elsewhere (Moreno and Diamond, 1974 b, 1975 b). Calculations were carried out on an IBM/360/91 computer (IBM Corp., Armonk, N. Y.) using APL language. Unless explicitly mentioned errors are expressed as standard errors of the mean.

$^1$ More generally, for two monovalent inorganic ions $X$ and $Y$, $P_{X}/P_{Y} = G_{X}/G_{Y}$ (Barry et al., 1971), and the partial ionic conductance of the ion $i$, $G_{i} = G \times [P_{i}/P_{X}] \times [G_{i}/G_{X}]$, where $i$ represents any permeable ion and $G_{X}$ its concentration. Eqs. 1–5 are, however, valid approximations for the conditions used here, since K$^+$, phosphate, and Ca$^{++}$ have much lower concentrations and/or permeabilities than Na$^+$ and Cl$^-$. The error introduced by using Eqs. 3 and 5, i.e. omitting K$^+$, vs. including K$^+$ (the most permeable and concentrated of those ions) in the calculation of $G_{Na}$ and $G_{Cl}$ for frog gallbladder is $<3\%$. When ratios of two partial ionic conductances are taken, this error introduced by the omission of K$^+$ becomes $<1\%$.

As will be shown in the Results section, TAP inhibits $G_{Na}$, and therefore the assumption that the cellular pathway of ion permeation is negligible as compared with the extracellular pathway when a considerable fraction of $G_{Na}$ has been blocked by TAP, should be taken critically. The relative importance of the extracellular vs. the cellular pathway of ion permeation has only been assessed quantitatively in gallbladders from Necturus where it was shown that nearly 95% of the current follows the extracellular route (Frömter, 1972). This value could be tentatively used for frog gallbladder (see also p. 110). TAP 10 mM inhibits $80\%$ of $G_{Na}$ in frog gallbladder, and since in the untreated gallbladder $G_{leakage} \approx 25\%$ of $G$, after 10 mM TAP, $G$ is reduced to ca. 40% of its original value. Still in these circumstances $\approx 80\%$ of the current would follow the extracellular route. (see also Moreno, 1975, pp. 123–24). A definite, quantitative picture in this respect will probably be given by microelectrode analysis.
RESULTS

Action of TAP on Na+ and Cl Permeabilities

Fig. 1 illustrates an experiment in which the action of 10 mM TAP was tested on the electrical parameters of a frog gallbladder. The three main features of TAP action shown in the figure are: (a) TAP decreases the total transepithelial conductance (G); (b) it does not affect the Cl- partial ionic conductance (Gcl); and (c) the effect is completely reversible.

In 15 frog gallbladder experiments such as the one illustrated in Fig. 1 10 mM TAP reduced G from 9.1 ± 0.8 mmho cm-² to 3.8 ± 0.5 mmho cm-², the NaCl dilution potential from 10.1 ± 0.8 to 2.7 ± 0.4 mV, and the $P_{Na}/P_{Cl}$ ratio from 5.0 to 1.5. These effects of TAP were reversible in the 10 experiments in which it was checked. After calculating the partial ionic conductances it can be seen that the effects are due to a specific inhibition of $G_{Na}$: in the 15 experiments mentioned, 10 mM TAP reduced $G_{Na}$ from 6.5 to 1.3 mmho cm-², i.e. by 80%, while $G_{Cl}$ remained constant ($G_{Cl} = 1.5$ mmho cm-² in the control and during TAP exposure).

Let us define the fractional inhibition ($\alpha$) of the partial conductance of an ion $X$ ($\alpha_X$) as

$$\alpha_X = 1 - \frac{G_X(\text{TAP})}{G_X(\text{control})},$$

where $G_X(\text{control})$ and $G_X(\text{TAP})$ are the conductances of the ionic species $X$ in the control period and in the presence of TAP, respectively. Fig. 2 (below) shows the experimental values of $\alpha_{Na}$ as a function of TAP concen-
Effect of TAP on $G_{Na}$ and $G_{Cl}$ in frog gallbladder. The fractional inhibition of $G_{Na}$, $\alpha_{Na}$ (O, below) and $G_{Cl}$, $\alpha_{Cl}$ (C, above) are plotted against TAP concentration. Each point corresponds to one experimental value. Notice that $G_{Na}$ is strongly inhibited ($\alpha_{Na}$ increases) as TAP concentration increases, whereas $G_{Cl}$ is unaffected. The line drawn through the $\alpha_{Na}$ values is the least-mean-squares fit to Eq. 9 yielding $K_m = 2.6$ mM. The line through the $G_{Cl}$ values is the horizontal line $\alpha_{Cl} = 0$.

Each point represents one experimental value of $\alpha_{Na}$ obtained in a single gallbladder. As TAP concentration increases, $\alpha_{Na}$ increases ($G_{Na}$ decreases). At 19 mM TAP $\alpha_{Na} = 0.92$, i.e., 92% of $G_{Na}$ is inhibited. The form of the dependence of $\alpha_{Na}$ on TAP concentration suggest a saturation kinetics. Therefore the experimental values were fitted by the equation:

$$\alpha_{Na} = \frac{C[\alpha_{Na \text{ (max)}}]}{K_m + C},$$

where $C$ represents TAP concentration and $K_m$ and $\alpha_{Na \text{ (max)}}$ are two constant parameters. The curve drawn through the $\alpha_{Na}$ points of Fig. 2 is based on the least-mean-squares fit of the experimental values to Eq. 8. The fitted parameters and their standard deviations are $K_m = 2.56 \pm 0.09$ mM, and $\alpha_{Na \text{ (max)}} = 1.02 \pm 0.02$. This indicates that 100% of $G_{Na}$ can be inhibited by TAP at sufficiently high concentrations, and that the inhibitory process can be described by a single constant:

$$\alpha_{Na} = \frac{C}{K_m + C}.$$

The situation for $G_{Cl}$ illustrated in the upper part of Fig. 2 is very differ-
ent: at all TAP concentrations tested $\alpha_{c1} \approx 0$, the average value of $\alpha_{c1}$ for the 32 experiments illustrated in the figure is $0.01 \pm 0.05$, and the regression line which defines the relation between $\alpha_{c1}$ and TAP concentration does not differ significantly from the horizontal line $= \alpha_{c1} 0$.

**Time-Course of the Effect of TAP**

Fig. 3 shows the time-course of $G_{Na}$ after addition of 7 mM TAP in a frog gallbladder. After TAP is added to both sides there is a rapid (i.e. completed in less than 10 s) decrease in $G_{Na}$, followed by a slower inhibition which takes about 10 min to reach a steady-state level. When TAP is added only to the mucosal solution, only the fast response occurs, and when it is added subsequently also to the serosal side, an additional, but slower re-

![Figure 3. Effect of TAP on $G_{Na}$ in frog gallbladder. Between the arrows 7 mM TAP was added at the times and to the sides indicated. Notice that the response to the addition of TAP at both sides is the sum of a fast response (associated with addition to the mucosal side) and a slower one (associated with addition of TAP to the serosal side).](image)

response appears. The same observations hold for the time-course of the recovery of $G_{Na}$, when TAP is withdrawn from the solutions.

In three frog gallbladders TAP (7 mM) added only to the mucosal side produced inhibition of $G_{Na}$ with a half time $< 10$ s reaching a steady-state level at $\alpha_{Na} = 0.37 \pm 0.04$ (3). In another three experiments (in the other halves of these gallbladders) 7 mM TAP was added only to the serosal side, in these cases half of the maximal action was obtained at $130 \pm 9$ s; the effect was nearly an exponential function of time and $\alpha_{Na}$ reached the steady-state level at $\alpha_{Na} = 0.32 \pm 0.04$. In the same three gallbladders, the unstirred layer thickness, calculated from the half time of sucrose streaming potentials (as in Diamond, 1966) was $110 \pm 9 \mu m$ (3) at the mucosal side and $480 \pm 67 \mu m$ (3) at the serosal side. With these values and the value of the free solution diffusion coefficient of TAP ($6.25 \times 10^{-4} \text{cm}^2 \text{s}^{-1}$, estimated from equivalent conductance measurements at $25^\circ \text{C}$ in a 50 mM
TAP·Cl solution, as in Moreno and Diamond, 1975 b, Appendix), one can predict a half time of 7.4 ± 0.6 and 140 ± 20 s for the buildup of TAP concentration after its addition to the mucosal and serosal sides, respectively, in agreement with the actual half times observed for the action of TAP (< 10 and 130 s). This indicates that in frog gallbladder the unstirred layers can explain completely the time-courses of the action of TAP. These results also indicate that TAP has effects from both mucosal and serosal sides, and that when TAP is added to one side its effect at t = ∞ does not depend on which side it is added. One would expect this symmetry for properties depending on the tight junctions, which contact both serosal and mucosal solutions.

**Active Form of 2, 4, 6-Triaminopyrimidine**

In the preceding pages, we have assumed that the monocationic form of 2,4,6-triaminopyrimidine (TAP) is the active form, and consequently we have been reporting the concentration of this form rather than those of the total added base. The validity of this assumption will be now examined in detail.

2,4,6-triaminopyrimidine is a base that exists in solution mainly as a neutral species (B) at high pH, as a monocation (BH+) in the moderately acid region, and as a dication (BH2+) in the strong acid region:

\[
\text{H}_2\text{N} \quad \overset{+\text{H}^+}{\longrightarrow} \quad \text{H}_3\text{N} - \text{NH} \quad \overset{+\text{H}^+}{\longrightarrow} \quad \text{H}_4\text{N}^- \quad \text{NH}^+ + \text{NH}_2^+ \quad \text{NH}_2^+ \quad \text{NH}_2^+.
\]

The acid dissociation constants being \(pK_1 = 6.74\) and \(pK_2 = 1.31\) (Roth and Strelitz, 1969, 1970). To know which one of the three forms (B, BH+, or BH2+) is responsible for the action on \(G_{Na}\) in frog gallbladder, the effect of 10 mM 2,4,6-triaminopyrimidine (total added base concentration, \(Bt = B + BH^+ + BH_2^{2+}\)) added to both sides of frog gallbladder preparations was studied at pH's ranging from 5.5 to 8.5. The result of the experiment is showed in Fig. 4. Each point represents a value of \(\alpha_{Na}\) obtained in one frog gallbladder at a particular pH (indicated on the abscissa). As the pH increases, the value of \(\alpha_{Na}\) decreases. Since \(G_{Na}\) does not vary significantly

\[^2\] The maximal action of 7 mM TAP added to only one side (\(\alpha_{Na} \approx 0.35\)) is, however, significantly smaller than the action of the same amount added to both sides (\(\alpha_{Na} \approx 0.73\)), or to the action expected for half that concentration added to both sides (\(\alpha_{Na} \text{ predicted for 3.5 mM TAP on both sides} = 0.57\)). The reason for that is not clear, and might be due to a dissipative diffusion of TAP at the membrane, which reduces its concentration at the active sites. That could be the situation if TAP barriers to diffusion at each border of the active sites are affected by TAP itself so that the barrier in contact with the higher TAP concentration becomes relatively less permeant to TAP than the other barrier in contact with the lower concentration, and therefore at the active sites between the barriers, TAP concentration is effectively lower than half the concentration at the side at which TAP is added.
Figure 4. \( \alpha_{Na} \) produced by 10 mM 2,4,6-triaminopyrimidine (total base added concentration) in frog gallbladder as a function of the pH of the solutions. Each point corresponds to one experimental value. The variation in \( \alpha_{Na} \) as a function of pH is due to variations in the concentrations of the \( B, BH^+, \) or \( BH_2^{++} \) forms of the organic base. The lines represent the theoretical lines assuming \( B \) (---), \( BH^+ \) (----), or \( BH_2^{++} \) (■■■) as the only active forms from Eqs. 13, 14, and 15, respectively. The experimental points only show agreement with the second possibility.

Within the range of pH studied here (Moreno and Diamond, 1974 b, Fig. 5), and furthermore, in each experiment the control, TAP action, and recovery periods were performed at the same pH, the variations of \( \alpha_{Na} \) as a function of pH are correlated with the variations in the relative concentrations of the three forms of 2,4,6-triaminopyrimidine with pH, indicating that they are differently active.

At any given pH the concentration (represented by brackets) of the \( B, BH^+, \) and \( BH_2^{++} \) forms of 2,4,6-triaminopyrimidine will be given by the following equations:

\[
[B] = \frac{[B_t]}{1 + 10^{-a}(1 + 10^b)}, \tag{10}
\]

\[
[BH^+] = \frac{[B_t]}{1 + 10^a + 10^{-b}}, \tag{11}
\]

\[
[BH_2^{++}] = \frac{[B_t]}{1 + 10^b(1 + 10^a)}, \tag{12}
\]

where, \( a = pH - pK_a, b = pH - pK_b, \) and \([B_t] = [B] + [BH^+] + [BH_2^{++}]\).

From Eqs. 9, 10, 11, and 12, for the hypothetical cases in which \( B, BH^+, \) or \( BH_2^{++} \) are, respectively, the only active forms, \( \alpha_{Na} \) will depend on pH following the equations

\[
\alpha_{Na} = \frac{1}{1} + K_m(1 + 10^{-a}[1 + 10^b]) \div [B_t], \tag{13}
\]

\[
\alpha_{Na} = \frac{1}{1} + K_m(1 + 10^a + 10^{-b}) \div [B_t], \tag{14}
\]

\[
\alpha_{Na} = \frac{1}{1} + K_m(1 + 10^b[1 + 10^a]) \div [B_t]. \tag{15}
\]
The parameters $K_n$ of Eqs. 13-15 are given by the concentration of each form at pH $\approx 7.15$, since at this pH (see Fig. 4) $\alpha_{Na} = 0.5$ ($[B] = 7.3 \times 10^{-3}$ M; $[BH^+] = 2.6 \times 10^{-3}$ M; and $[BH^{2+}] = 3.3 \times 10^{-4}$ M).

The lines traced in Fig. 4 represent the predicted $\alpha_{Na}$ vs. pH correlations calculated from Eqs. 13-15, i.e. assuming $B$ (broken line), $BH^+$ (full line), or $BH^{2+}$ (dotted line) are the only active forms. The experimental data strongly suggest that only the monoprotonated form is responsible for the effect. This conclusion does not depend on the particular pH's or $[Bt]$ used: at pH 5.5 and $[Bt] = 1$ mM, Eqs. 13-15 predict $\alpha_{Na} = 0.95, 0.27,$ or $0.00$ depending on whether $BH^{2+}, BH^+$, or $B$ is, respectively, the only active form. The result of three experiments in frog gallbladder under such conditions is $\alpha_{Na} = 0.26 \pm 0.05 (3)$, i.e. as predicted if only $BH^+$ is the active form.

These results indicate that the monoprotonated form of 2,4,6-triaminopyrimidine is more active than the neutral form, and that from pH 5.5 to 8.5 the monoprotonated form is fully responsible for its action on $G_{Na}$. These results do not necessarily imply, however, that the $B$ or $BH^{2+}$ forms are completely inactive, nor that at equal concentrations, the $BH^+$ form would be more active than the $BH^{2+}$ form, since at the pH's used here $[BH^+] /[BH^{2+}] > 10^4$.

Effect of TAP Analogs

Table I lists analogs or derivatives of TAP which also inhibit $Na^+$ permeability in frog gallbladder. Experimental $\alpha_{Na}$ values were obtained at various concentrations of these compounds in a similar manner as for TAP in frog gallbladder (pp. 101-102). These values were fitted into Eq. 9 by the least-mean-squares method, and the parameter $K_n$ (the extrapolated concentration at which $\alpha_{Na} = 0.5$), estimated in this way is shown in the last column of Table I. The most effective compound is TAP, and the sequence of effectiveness correlates with the number of nonblocked $-NH_2$ radicals of the molecule, suggesting that these radicals play an important role in the interaction of TAP with the channel sites (see pp. 111-112). Other cations tested but which did not produce detectable inhibition of $G_{Na}$, were (name of the compound followed by the maximum concentration tested): ammonium 10 mM, potassium 10 mM, guanidinium 8 mM, methylammonium 10 mM, ethylammonium 10 mM, trimethylguanidinium 10 mM, biguanidinium 10 mM, tetramethylammonium 10 mM, and tetraethylammonium 5 mM.

Effect of TAP on the Permeability of Different Cations

To determine whether TAP affects the permeability of cations other than $Na^+$ the effect of 6.4 mM TAP on the $X$:Na biionic potential ($X$ being $Li^+$,
TABLE I
EFFECT OF TAP DERIVATIVES OR ANALOGS ON $G_{Na}$ IN FROG GALLBLADDER

| Compound                              | $\text{No. of } -\text{NH}_2$ | $\sigma_H$ | $K_m$  |
|---------------------------------------|-------------------------------|----------|--------|
| (1) 2,4,6-triaminopyrimidinium (TAP)   | 3                             | 7        | 2.6±0.1 (48) |
| (2) 2,4,6-triamino-1,3,5-triazinium    | 3                             | 7        | 3.4±0.1 (3)  |
| (3) 4,5,6-triaminopyrimidinium         | 3                             | 7        | 5.5±1.4 (3)  |
| (4) 2,6-diaminopyrimidinium            | 2                             | 5        | 7.7±0.6 (6)  |
| (5) 4,6-diaminopyrimidinium            | 2                             | 5        | 8.2±1.1 (6)  |
| (6) 2-amino-4-dimethylaminopyrimidinium| 1                             | 3        | 11.2±1.8 (5) |
| (7) 4-amino-2-dimethylaminopyrimidinium| 1                             | 3        | 11.2±1.7 (5) |

First column: names of the compounds; second column: number of nonblocked $-\text{NH}_2$ radicals; third column: number of donor protons for H-bond ($\sigma_H$). The experimental values of $\alpha_{Na}$ obtained at different concentrations of the compounds were fitted into Eq. 9 by the method of least-mean-squares, and the value ±SD (number of observations) of the fitted parameter $K_m$ is presented in the fourth column. Only the concentration of the monoprotonated form of the compounds was considered in the calculations. The chemical formulae of the compounds (neutral form) are:

![Chemical structures](image)

$\text{Cs}^+$, $\text{H}_2\text{C}N\text{H}_4^+$, or $(\text{H}_2\text{N})_2\text{C} = \text{NH}_2^+$ was studied in frog gallbladder. Table II shows the ratios $P_x/P_{Na}$ obtained with and without 6.4 mM TAP. The control values are similar to those obtained previously (Moreno and Diamond 1974 b, 1975 b), and none was significantly affected by TAP. In these gallbladders $G_{Na} = 7.7±1.4$ mmho cm$^{-2}$ was reduced by TAP to $2.5±0.7$ mmho cm$^{-2}$, i.e., by 68%, this indicates that $G_x$ was also reduced by nearly 68%.

Effects of TAP on Other Epithelia

Besides frog gallbladder, TAP was tested in the following “leaky” (Frömter and Diamond, 1972) epithelia: rabbit and *Necturus* gallbladder, rabbit and
TABLE II
EFFECT OF TAP (6.4 mM) ON THE CATION PERMEABILITY RATIOS IN FROG GALLBLADDER

| Cation       | Control   | TAP       |
|--------------|-----------|-----------|
| Li⁺         | 0.40±0.02 | 0.36±0.03 |
| Cs⁺         | 1.08±0.05 | 0.91±0.08 |
| H₂CNH⁺      | 0.65±0.04 | 0.56±0.05 |
| (H₂N)₂C=NH⁺ | 0.81±0.07 | 0.78±0.07 |

The values are averages (±SE) based on the measurements of bidirectional potential in four frog gallbladders. None of the values in the control period is significantly different from those in the TAP period.

bullfrog small intestines (terminal region), and bullfrog choroid plexus. The results are expressed in Table III. As in frog gallbladder, in all the cases 10 mM TAP decreased \( G \) and \( P_{Na}/P_{Cl} \) and the effect could be completely attributed to an inhibition of \( G_{Na} \) without affecting \( G_{Cl} \) (when calculated through Eqs. 3 and 5). The data indicate that in the epithelia listed, as in frog gallbladder, TAP specifically inhibits \( G_{Na} \).

Effects of TAP on Rabbit Gallbladder

Of the epithelia listed in Table III the TAP effect was studied in greater detail in the rabbit gallbladder. Fig. 5 shows the relation between \( \alpha_{Na} \) and TAP concentration in rabbit gallbladder. As in frog gallbladder the experimental \( \alpha_{Na} \) values were fitted by the method of the least-mean-squares to Eq. 8. The fitted parameters and their standard deviations are \( K_m = 3.6 \pm 0.3 \) mM, and \( \alpha_{Na}(\text{max}) = 1.04 \pm 0.02 \). This indicates that at sufficiently high TAP concentrations nearly 100% of \( G_{Na} \) can be inhibited by TAP, and that the process can be described by a single constant (Eq. 9), as in frog gallbladder. These similarities between the responsiveness of frog and rabbit gallbladders to TAP correlate well with studies indicating similar routes, mechanisms of ion permeation, selectivities, and chemical specificity of the sites in the cation channels of both species (Moreno and Diamond, 1974 b, 1975 a, 1975 b).

The time-course of \( G_{Na} \) inhibition by TAP is, however, different in both species: when 10-18 mM TAP is added simultaneously to the serosal and mucosal sides of rabbit gallbladders, half of the full \( G_{Na} \) inhibition is achieved in \( 17.3 \pm 3.5 \) min (n = 10). In similar experimental conditions in frog gallbladders, the half time is less than 10 s (Fig. 3). Since the unstirred layer thickness of rabbit and frog gallbladder preparations under the present experimental conditions are almost identical (Moreno, 1975), in rabbit gallbladder the rate-limiting factor in the time response is not given, as it is in...
This table shows the effect of 10 mM TAP on the membrane conductance ($G$), $G_{Na}$, $G_{Cl}$ (expressed in millimho square centimeters) and $P_{Na}/P_{Cl}$. The values of $G_{Na}$ and $G_{Cl}$ are calculated from Eqs. 5 and 3, i.e. assuming the existence of a leakage pathway with the characteristics described by Eq. 4. This assumption has only been proved valid for rabbit and frog gallbladder (pp. 99-100). Notice that TAP reduces $G$, $P_{Na}/P_{Cl}$, and $G_{Na}$ without apparently affecting $G_{Cl}$, as in the case of frog gallbladder.

![Graph](image)

**Figure 5.** Effect of TAP on $G_{Na}$ in rabbit gallbladder. The fractional inhibition of $G_{Na}$, $\alpha_{Na}$ is plotted against TAP concentration. Each point corresponds to one experimental value. The line drawn through the $\alpha_{Na}$ values is the least-mean-squares fit to Eq. 9 yielding $K_m = 3.6$ mM.

frog gallbladder, by the unstirred layers, but by some other barrier, present in rabbit but not in frog gallbladder.²

² It is noticeable that the van der Waals radius of the larger cross section of TAP is 4 Å, a value close to the cross-sectional equivalent pore cylindrical radius attributed to rabbit cation channels (4.4 Å), and considerably smaller than the equivalent radius (8.1 Å) attributed to frog channels (Moreno and...
**DISCUSSION**

The discussion will be limited to two general points: the routes of ion permeation in gallbladder in relation with the action of TAP, and the possible mechanism through which TAP produces this action.

**Routes of Ion Permeation in Gallbladder**

In *Necturus* gallbladder 95% of the passive ion permeation bypasses the epithelial cells through the tight junctions (Frömtter, 1972; Frömtter and Diamond, 1972). Many indirect evidences indicate that in frog and rabbit gallbladder (whose transepithelial electrical resistance is 3–10 times smaller than in *Necturus*) the main route is also through the tight junctions (Barry and Diamond, 1970; Barry et al., 1971; Wright et al., 1971; Machen et al., 1972; Moreno and Diamond, 1975 a). The fact that TAP has qualitatively the same action on gallbladders from the three species is in accordance with this idea of similar ion permeability routes in them.

Is the extracellular pathway unique? Do anions and cations cross through the same or different routes? The fact that TAP can block Na⁺ permeability without affecting Cl⁻ permeability demonstrates that Na⁺ and Cl⁻ pathways can be separated pharmacologically. This strongly suggests that Cl⁻ and Na⁺ are crossing through different channels or pathways, since it is difficult to imagine that in a single channel permeable to both Na⁺ and Cl⁻, TAP would only affect the Na⁺ permeability. The extrapolation of the data of Fig. 2 at infinite TAP concentration leads to $\alpha_{Na} = \alpha_{Na}(\text{max}) = 1.0$, and $\alpha_{Cl} = 0$, i.e., under these hypothetical circumstances (closely matched in actual experiments at 19 mM TAP, in which $\alpha_{Na} = 0.92$ and $\alpha_{Cl} = 0.01$) $G_{Cl}$ is unaffected and $G_{Na}$ is completely blocked, and therefore, from Eqs. 1–4, $G = G_{\text{leakage}}$, and $(P_{Na}/P_{Cl}) = (u_{Na}/u_{Cl})$. That is, at sufficiently high TAP concentrations where all the specific channels for Na⁺ would be blocked, what still will remain is a pathway responsible for 100% of $G_{Cl}$ with the characteristics of a free solution shunt. As mentioned on pp. 99–100 the existence of separate pathways for Na⁺ the Cl⁻, the Cl⁻ pathway having the characteristics of a free solution shunt, has been indicated previously in the light of several experimental findings (summarized on pp. 99–100).

A similar picture (Na⁺ permeating through cation-selective channels at the tight junctions, Cl⁻ through a parallel leakage pathway) might in principle be valid also for small intestine and choroid plexus; they behave as

Diamond, 1975 b). Perhaps the differences in time-courses of TAP action are somehow connected to the difference in channel-equivalent radius: the sites in rabbit might be less accessible to TAP than those in frog channels. Alternatively, the first molecules of TAP acting in the rabbit channels might retard the process of diffusion of the forthcoming molecules to sites deeper in the channel, before they block $G_{Na}$. 
leaky epithelia (Frizzel and Schultz, 1972; Frömter and Diamond, 1972; Wright, 1972) and, as in gallbladder, TAP inhibits their $G_{Na}$ without apparently affecting $G_{Cl}$ (Table III). However, these epithelia have been studied only at one TAP concentration, and their passive $Cl^-$ and $Na^+$ permeabilities have not been studied in the same detail as for gallbladder.

**Mechanism of Action of TAP**

The blockage of the gallbladder cation permeability by TAP could, in principle, be due to conformational changes induced by TAP which lead to a new state of the channels impermeable to cations or, alternatively, to a direct block of the cation channels and/or sites by TAP. Although no conclusive proof has been presented in this paper there is evidence to suggest that the action of TAP is mediated directly by its binding to sites inside the tight junction cation channels, and that the sites of binding are possibly those which control the channel's cation permeation and selectivity.

The cation permeability and selectivity in frog and rabbit gallbladders is thought to be mediated by hydrated and relatively large channels (the estimated equivalent cylindrical diameters are 9 Å in rabbit and 16 Å in frog gallbladder). Their cation selectivity depends, in both species, on the presence of ionized acidic groups whose apparent $pK_a$ is 4.4. The channel ligands (probably the same acidic groups) behave as strong H-bond proton acceptor sites that distinguish very sharply (probably more than water) between nitrogenous cations on the basis of their number of protons available for H-bond formation: cations with greater H-bond donor ability are more permeable, presumably because they have more affinity to the sites (Moreno and Diamond, 1974a, 1974b, 1975a, 1975b).

TAP is a highly water-soluble organic cation of mol wt = 126.14 and van der Waals dimensions of $8 \times 8 \times 4$ Å. 2,4,6-triaminopyrimidine protonates at one of its symmetrical ring nitrogens, but the net charge is delocalized by resonance and shared with the two-, four-, and six-amino groups (Roth and Strelitz, 1969, 1970) the resonant structures being:

![Resonant structures of TAP](image)

Therefore, in its protonated form, 2,4,6-triaminopyrimidine can donate a large number of partially ionized protons to form H-bonds with suitable acceptors.

The fact that the sites in the cation tight junction channels behave as
strong proton acceptors, and that TAP is a strong proton donor suggests that TAP interacts with those sites within the channel and that this association is via H-bonds. This idea is further supported by the following facts: (a) Only the cationic form is active (p. 106). As was mentioned above the net charge of 2,4,6-triaminopyrimidine will increase its proton donor ability and its energy of interaction with the negatively charged proton acceptor sites at the channels. (b) There is a rather specific ion-pair formation between protonated 2,4-diaminopyrimidine (an analog of TAP also effective in gallbladder) and phosphate anions (acidic groups like the sites in the channel) in solutions at relatively low concentration (Roth and Strelitz, 1969). (c) All the TAP derivatives found effective in blocking the channels (Table I) have proton donor ability, and the blocking effectiveness sequence coincides with the sequence of the maximum number of protons that each molecule can donate to form H-bonds with proton acceptors (Table I). The reversibility of the action of TAP would also be in accord with a H-bond or ionic interaction of TAP and the sites (instead of a covalent bond). Several other facts are also in agreement with the idea that the action of TAP takes place directly inside the cation channels; from its molecular dimensions TAP would fit into the equivalent cylindrical radius attributed to the cation channels; TAP is another hydrophilic organic cation in many respects comparable to those studied previously (Moreno and Diamond, 1975 b) whose permeability and selectivity characteristics indicate migration through the tight junction cation channels; and TAP acts, and is equally active, from both serosal and mucosal sides of the gallbladder.

The binding of TAP to the sites in the channel could inhibit its Na⁺ permeability because the association of Na⁺ to the TAP sites constitutes a requirement to Na⁺ permeability, or simply because the binding of TAP to the sites builds an impassable energy barrier to Na⁺ ions. If one assumes that the affinity of the channel ligands for TAP is much larger than for Na⁺ ($K_{Na} \gg K_{TAP}$), then the inhibition of $G_{Na}$ as a function of TAP concentration will depend only on $K_{TAP}$, and in the simplest case the dependence will follow a Michaelis Menten kinetics, i.e. Eq. 9. The value of $K_{m} = 2.56$ mM from Fig. 2 would therefore represent the affinity constant of TAP for the sites, or $K_{TAP}$.

The membrane conductance-NaCl concentration relation is essentially linear up to at least 400 mM in rabbit gallbladder (Wright et al., 1971) and in frog gallbladder (Bindslev et al., 1975) indicating that up to this concentration Na⁺ ions are not competing significantly with each other for the sites in the channel (they do not saturate the sites). This implies that if we were able to define the affinity constant of Na⁺ for the sites in the channel, $K_{Na}$, it would probably be > 400 mM. Then, considering the value of the parameter $K_{m} = 2.56$ in frog gallbladder as $K_{TAP}$, $K_{Na}/K_{TAP} > 160$, i.e. TAP affinity for the sites would be more than 160 times larger than Na⁺ affinity.
In four frog gallbladders I measured the TAP:Na bionic potentials (75 mM TAP·Cl in the mucosal side, vs. 75 mM NaCl in the serosal side, with the other components of the Ringer as it was stated in the Methods section). The calculated (Eq. 6) \( P_{\text{TAP}}/P_{\text{Na}} \) ratio was 0.22 ± 0.03 (4). In general, these \( P \) ratios obtained from bionic potentials can be expressed (in systems where permeation is limited by diffusion within the membrane interior rather than by interfacial processes) by:

\[
\frac{P_{\text{TAP}}}{P_{\text{Na}}} = \frac{K_{\text{Na}}}{K_{\text{TAP}}} \times \frac{u_{\text{TAP}}^*}{u_{\text{Na}}^*},
\]

where \( u_{\text{Na}}^* \) and \( u_{\text{TAP}}^* \) are the mobilities of Na⁺ and TAP inside the channel. (Karreman and Eisenman, 1962; Eisenman, 1962, 1968; Barry and Diamond, 1971), using this equation and from the estimations for \( P_{\text{TAP}}/P_{\text{Na}} = 0.22 \) and \( K_{\text{Na}}/K_{\text{TAP}} > 160 \), the mobility ratio \( u_{\text{Na}}^*/u_{\text{TAP}}^* > 730 \). Then the sequence and \( K_{\text{Na}}/K_{\text{TAP}} > 160 \), the mobility ratio \( u_{\text{Na}}^*/u_{\text{TAP}}^* > 730 \). Then the sequence \( P_{\text{Na}} > P_{\text{TAP}} \) extracted from the bionic potential is determined by the mobility (and not the affinity) sequence: the ion with higher affinity is the one with less permeability. This is in contrast with what apparently happens in this system with other ions (Barry et al., 1971; Moreno and Diamond, 1974 b) and in other systems with other ions (Eisenman, 1962, 1968; Diamond and Wright, 1969; see however Hille, 1975). This peculiarity of the behavior of TAP, evidenced by its high affinity and low mobility is probably the basis of its unique blocking properties.

Possibly TAP could associate with the acidic sites in the channel simply by a double H-bond between the two partially negatively charged oxygens of the group as proton acceptors, and the protonate ring nitrogen and a neighbor amino radical of the aminopyrimidine as donors:

\[
\begin{align*}
\text{H} & \quad \text{O}\quad -\quad \text{H}\quad -\quad \text{N}\quad -\quad \text{NH}_2 \\
\text{H} & \quad \text{O}\quad -\quad \text{H}\quad -\quad \text{N}\quad -\quad \text{NH}_2
\end{align*}
\]

or

\[
\begin{align*}
\text{H} & \quad \text{O}\quad -\quad \text{H}\quad -\quad \text{N}\quad -\quad \text{NH}_2 \\
\text{H} & \quad \text{O}\quad -\quad \text{P}\quad -\quad \text{O}\quad -\quad \text{H}\quad -\quad \text{N}\quad -\quad \text{NH}_2
\end{align*}
\]

This double H-bond structure has been proposed as the one responsible for the strong ion pair formation seen in solutions of 2,4-diaminopyrimidinium phosphate at relatively low concentrations (Roth and Strelitz, 1969); and could explain qualitatively the sequence of effectiveness of TAP analogs (Table I), since this sequence is similar to the sequence of statistical possibilities that the analogs have to form such double H-bond arrangement. A carboxilic or phosphate (mono- or dianionic) group could in principle form this interaction with TAP, either one of these groups can match the two H-bonds with almost ideal angles between the internuclear lines.

This scheme has the attractive simplicity of only assuming an ionized group.
to explain the specific binding and action of TAP. However it does not allow one to understand why other organic cations which could donate more protons for H-bond, and would allow double H-bond formations (e.g. biguanidinium, triaminoguanidinium) do not have any detectable action on $G_{Na}$. Possibly, the double H-bond illustrated represents an essential part of the interactions, but other ligands in the channel also play important roles.

TAP is expected to have chemical specificity for similar structures and/or conformations in other systems rather than for a particular anatomical entity like the tight junctions.

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