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Chloride and Potassium Movements from Frog's Sartorius Muscle in the Presence of Aromatic Anions

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Summary. The efflux of ^{36}Cl and ^{42}K from frog's sartorius muscles equilibrated in Ringer's fluid with added KCl were measured in the absence and presence of salicylate, benzoate, and acetylsalicylate. The transmembrane potential and resistance were also measured in sartorii under similar conditions. Although the rate coefficient for loss of ^{42}K remained reasonably constant over extended experimental periods for untreated muscles, the rate coefficient for loss of ^{36}Cl fluctuated in many muscles giving rise to minima and maxima. The aromatic anions mentioned increased the efflux of chloride while having no detectable effect on the potassium efflux. The aromatic anion-stimulated chloride efflux was insensitive to alterations of external pH and was markedly reduced when nitrate replaced external chloride. No detectable changes in transmembrane potential or resistance were produced by salicylate, the most extensively studied aromatic anion. The results suggest that salicylate and the other aromatic anions stimulate an exchange diffusion mechanism for chloride.

Starting with Boyle and Conway (1941), the chloride permeation system in frog twitch muscle fibers has been extensively analyzed. From these studies two interesting properties of this system have emerged. First, the presence of any one of the foreign anions in the lyotropic series reduces the fluxes of chloride (Harris, 1958; Adrian, 1961; Hutter & Warner, 1967*c*; Moore, 1969). Second, the chloride permeability is markedly affected by variations in the external pH: in acid fluids the chloride permeability is low, in alkaline fluids it is high (Hutter & Warner, 1967*a*, *b*; Moore, 1969). These two properties provide useful diagnostic measures for studying the chloride permeation system whenever it is altered by one means or another.

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In the experiments reported here, the purpose was to perform studies on chloride and potassium movements in frog muscle using salicylate and other anions of aromatic acids. These experiments were prompted by the reports that in erythrocytes aromatic anions markedly reduce anion permeability (Parpart, 1940; Passow, 1969; Passow & Schnell, 1969). Many of these anions are also known to increase the cation permeability of red cells (Passow & Schnell, 1969; Wieth, 1970*a*; Gunn & Tosteson, 1971). Unexpectedly, for frog muscle, salicylate, benzoate, and acetylsalicylate induced an increase in chloride efflux rather than a decrease. Furthermore, these anions had no detectable effect on potassium efflux. An additional unanticipated finding was that the chloride efflux from some muscles equilibrated in solutions containing high potassium chloride concentrations showed fluctuations in time. It is with these findings that this paper is concerned. A preliminary, brief report has appeared elsewhere (Venosa, Ruarte & Horowicz, 1970).

Materials and Methods

Sartorius muscles from American leopard frogs (*Rana pipiens*) were used in all experiments. During the course of the experiments, frogs were obtained from several dealers located in different regions of the country. Generally, both the left and right sartorius were dissected from each frog. Such a pair was used only if each muscle was without visible damage and free from both parasites and hemorrhages. The muscles were carefully cleaned of fibers from other muscles. At the end of each experiment, the muscles were examined again. Results were only used when the muscles were free of any signs of damage or contractures.

The weights and lengths for a number of muscles were measured and the averages and standard error for these parameters were 93 ± 5 mg and 38 ± 1 mm, respectively. In a few instances the thickness was estimated from the weight and the area of the broad lateral surfaces of the muscles. The average thickness so estimated was 0.9 mm.

The standard equilibrating and bathing solution used in all experiments was one in which KCl was added to Ringer's fluid. The solution adopted had the following composition: (in mmoles/liter) NaCl 115; KCl 100; $CaCl_2$ 1.8; Na_2HPO_4 2.15; and NaH_2PO_4 0.85. The pH was checked frequently and normally had a value in the range of 7.1 to 7.2. In some experiments a fraction or all of the chloride present in the standard solution was replaced by an equivalent amount of nitrate. Solutions containing added salicylate, benzoate, or acetylsalicylate were freshly prepared for each experiment. In some experiments the pH of the standard solution was reduced to 5.2 by replacing the phosphate with 2 mM Tris-maleate buffer. The radioactive loading solutions were made by using either $Na^{36}Cl$ (Amersham/Searle Corp.) or ^{42}KCl (International Chemical and Nuclear Corp.), instead of NaCl or KCl. The specific activities of the loading solutions were about 12 μC/ml for ^{36}Cl and 1.7 mC/ml for ^{42}K.

Once muscles were ready for use, they were exposed for 10 min to the standard inactive high KCl solution described above and were then soaked for an additional 2 to 3 hr in similar solutions containing either $^{36}Cl^-$ or $^{42}K^+$. After this loading period, the muscles were removed without either blotting or draining from the active solutions and fastened to glass frames which had not been exposed to the active solutions. The frames were constructed to allow exposure of the muscles to the solutions on both sides. Once

fastened to their frames, the muscles were bathed in a series of tubes each containing 4 ml of inactive solution. The tubes were rotated by a motor to insure adequate stirring. As a rule, the collection periods were of 5-min duration. In some of the longer experiments, when the total activity in the muscles was at a low value, the collection period was increased to 10 min.

At the end of the ^{36}Cl efflux experiments, each muscle was transferred to a tube containing 4 ml of distilled water for 1 hr. It was then left overnight in a second tube containing 4 ml of distilled water. In general, most of the radioactivity remaining in the muscle at the end of the experiment appeared in the first tube. At the end of the ^{42}K efflux experiments the muscles were removed from the glass frames and placed with 4 ml of distilled water in tubes similar to those used for counting the efflux of potassium. After the muscles had been in the distilled water for 1 to 2 hr, the tubes containing them were counted.

The 4-ml collection samples of $^{36}Cl^-$ were counted in a liquid scintillation spectrometer by adding the entire aqueous sample to 16 ml of scintillator solution. The scintillator solution contained 250 mg of 1,4-*bis*-2-(4-methyl-5-phenyl-oxazolyl)-benzene; 5 g of 2.5-diphenyl-oxazole; 500 ml of ethanol; 2,000 ml of toluene; and 1,250 ml of Triton X-100. The aqueous samples of ^{42}K were counted directly in a crystal well gamma spectrometer.

The efflux is expressed as the average fraction of the total muscle ^{36}Cl lost per minute during each collection period. The total amount of ^{36}Cl in the muscle during any collection interval was estimated by the sum of the activity found in all the subsequent collection samples, the activity of the distilled water samples, and one-half of the activity leaving the muscle during the given collection period. A similar procedure was followed for the ^{42}K experiments after correction for decay.

The membrane conductance measurements were made by determining the space constant and 'input resistance' on surface fibers. Two microelectrodes filled with 3 M KCl were inserted in a given fiber. One of them was used for passing rectangular constant current pulses and the other for recording the changes in membrane potential at different distances from the current electrode. Both the current pulse and the membrane potential change were displayed on a Tektronix 502 Dual Beam Oscilloscope and photographed. A standard cable analysis using the equations given by Fatt and Katz (1951) was performed on the measurements obtained.

Hyperpolarizing pulses of 100- to 200-msec duration were used. The change of membrane potential at zero separation between current-passing and voltage-recording electrodes was generally about 15 mV. The current electrode was displaced from the fixed voltage-recording electrode. Five separations in steps of about 200 μ, were used in each determination of the space constant. The separations were measured by a calibrated eyepiece micrometer using a Wild dissecting microscope. A complete sequence of measurements took about 6 min.

All experiments were carried out at room temperature (20 to 24 °C).

Results

Resting Chloride Efflux from Muscles Equilibrated in Solutions with High KCl Concentrations

To measure chloride efflux over extended periods of time, muscles were equilibrated in solutions made by adding 97.5 mM KCl to the conventional Ringer's fluid. In these solutions the fibers are depolarized, the internal

potassium and chloride concentrations are increased while the fiber volume remains constant (Boyle & Conway, 1941). Hence, for any given specific activity of chloride, more radioactivity can be incorporated into the muscles. In addition, the time taken for a given fraction of the internal ^{36}Cl to exchange is lengthened. This has the favorable effect of allowing a more distinct separation of the extracellular loss from the intracellular loss of ^{36}Cl.

To determine whether the early rapid efflux of ^{36}Cl for the conditions chosen could be ascribed to the efflux from the extracellular space, the total ^{36}Cl content in the muscles was examined during the first half-hour of efflux. From the results of Hodgkin and Horowicz (1959), Adrian (1960; 1961), Harris (1963; 1965), Hutter and Warner (1967*b*) and Moore (1969), it is reasonable to assume that both the intracellular and extracellular compartments have the same specific activity of ^{36}Cl after equilibrating in active solutions for the 2- to 3-hr periods routinely employed in this study. When equilibration with active solutions was ended, muscles were transferred unblotted and undrained into the first efflux collection tube. From inulin determinations made on eight muscles the extracellular space for this kind of transfer is $43.6 \pm 2.2\%$ (mean $\pm$ SEM). This is consistent with the value of $29.1 \pm 0.78\%$ found by Adrian (1960) for unblotted but drained muscles. Using Adrian's dry-to-wet-weight ratios one can estimate that the intracellular water was 43.3% of the total wet weight. Thus, the intracellular and extracellular water were equal for the type of transfer used in this study. With an internal potential of about -17 to -18 mV, the internal chloride concentration is about half of its external concentration. Therefore, about one-third of the total ^{36}Cl in an equilibrated muscle should be associated with the intracellular water and two-thirds with extracellular water. Fig. 1 shows how total ^{36}Cl content averaged from 23 muscles, declined with time during the first 30 min of efflux into inactive solutions. After a rapid phase, the ^{36}Cl content declined as an exponential function of time. Extrapolating this component to zero time gives a value of 0.46. As Huxley (1960) has pointed out, this sort of extrapolation leads to an over-estimate of the initial presumptive intracellular ^{36}Cl, because this procedure does not reckon with diffusion in the extracellular space. Using Huxley's model of a single intracellular compartment in series with a single extracellular compartment the extrapolations of the slow phase over-estimates the intracellular radioactivity by about 12%. Allowing for this extrapolation error, one obtains an estimate of about 0.41 for the fraction of total ^{36}Cl initially present in the presumptive intracellular compartment. This is reasonably close to the expected value. Thus, the early, fast component of the chloride efflux can

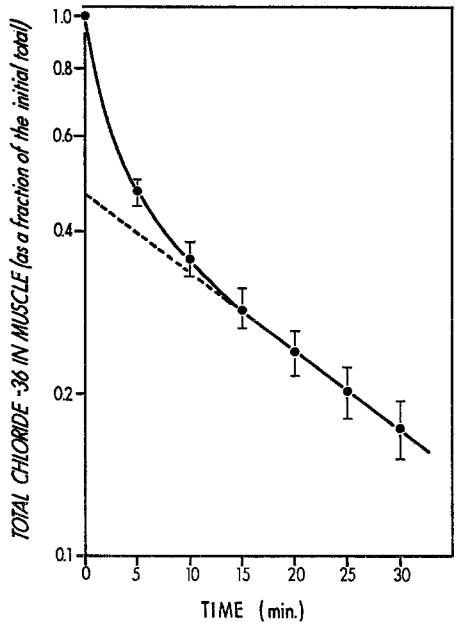

Fig. 1. $^{36}Cl^-$ in muscle as a function of time for the first 30 min of the washout. Mean $\pm$ SEM from 23 controls

be ascribed to the extracellular space. The slower component of chloride efflux with a rate coefficient in the range of 0.03 to 0.04 min^{-1} represents the kinetic behavior of the intracellular chloride pool.

If the efflux of chloride is constant and the internal chloride is confined to a single intracellular compartment, then the fractional loss per unit time of ^{36}Cl should remain constant for extended periods. In a number of muscles this behavior was observed. Part *A* of Fig. 2 illustrates one such experiment. After the initial rapid loss of ^{36}Cl, the fractional loss of chloride per unit of time remained constant for the remainder of the experiment. In this experiment the loss of ^{36}Cl was exponential for a period of about four time constants.

On the other hand, with heterogeneous populations where the rate constant varies from fiber to fiber, the fractional loss per unit time for the entire population might be expected to be high during the early stages of an experiment with a gradual decline occurring as time passes. Part *B* of Fig. 2 illustrates an experiment in which the efflux behaved in this fashion. In this muscle, despite the constancy of the external conditions, there was a smooth, monotonic decline in the rate coefficient.

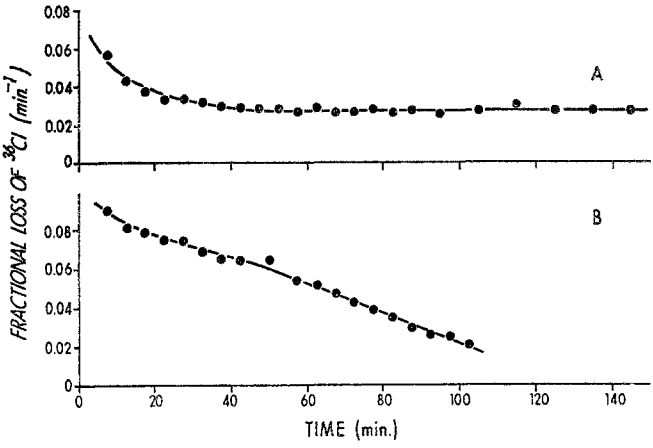

Fig. 2. Two patterns of $^{36}Cl^-$ efflux from frog sartorius muscle. (*A*) Almost constant rate coefficient (Exps. 11, 3, 70; Muscle no. 2). (*B*) Monotonic decline of the rate coefficient lasting 105 min (Exps. 10, 2, 69; Muscle no. 2)

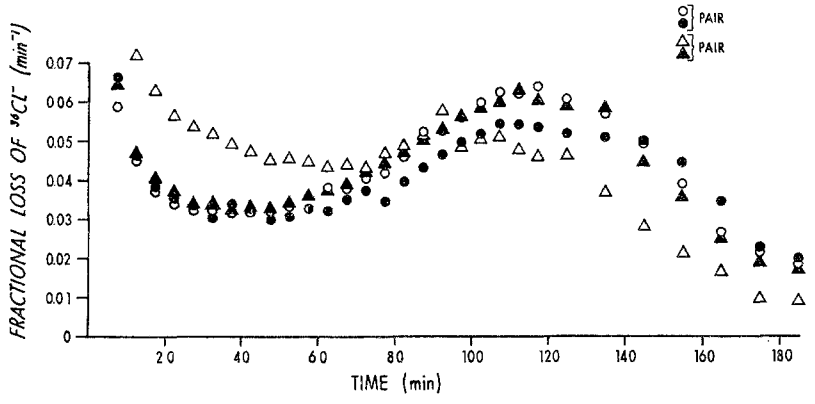

Fig. 3. Fluctuating pattern of $^{36}Cl^-$ efflux rate coefficient from two pairs of sartorius muscles. *See* text (Exps. 3, 25, 69; Muscle nos. 1–2–3–4)

Unexpectedly, a third type of efflux pattern was exhibited by some of the muscles studied. This pattern is shown in Fig. 3. In the experiment illustrated, the efflux of ^{36}Cl was measured from two pairs of sartorius muscles taken from two frogs. It is apparent that in these muscles there was what might be termed an 'oscillatory' pattern in the rate coefficient for ^{36}Cl efflux. After the initial extracellular washout, there was a minimum in the rate coefficient at a level of 3 ot 4% per min. This was followed by an increase to a maximum value of 5 to 6% per min with a final gradual decline during the last hour of the 3-hr experimental period. The transition from the minimum to the maximum was smooth and gradual. While the cause

of these oscillations remains unknown, they did not have any apparent effect on the action of the aromatic anions described below. In fifty untreated muscles, 16 showed clear signs of 'oscillatory' behavior; 8 had very shallow, broad minima; 16 had constant rate coefficients; and 10 had monotonically declining rate coefficients. In those muscles exhibiting clear oscillations, the maximum rate coefficient was on the average 1.47 times greater than the first minimum.

Chloride Efflux in the Presence of Salicylate, Acetylsalicylate, and Benzoate

An experiment in which the effect of 5 mM salicylate on the efflux of ^{36}Cl was observed is depicted in Fig. 4. Plotted are the results from a pair of muscles isolated from a single frog. Because of the diverse, unpredictable chloride efflux patterns described above, one muscle of a pair in most experiments was untreated so that its efflux provided a base for comparison. In general, paired muscles from the same frog exhibit the same chloride efflux pattern. In the upper portion of Fig. 4, the fractional loss of ^{36}Cl for each muscle is shown as a function of time while in the lower portion the ratio of this quantity for the experimental to control muscle is given. In the experiment illustrated, salicylate increased the rate at which ^{36}Cl left the fibers by a factor of two; the magnitude of the effect in this instance was above the average found with 5 mM salicylate. The stimulation produced

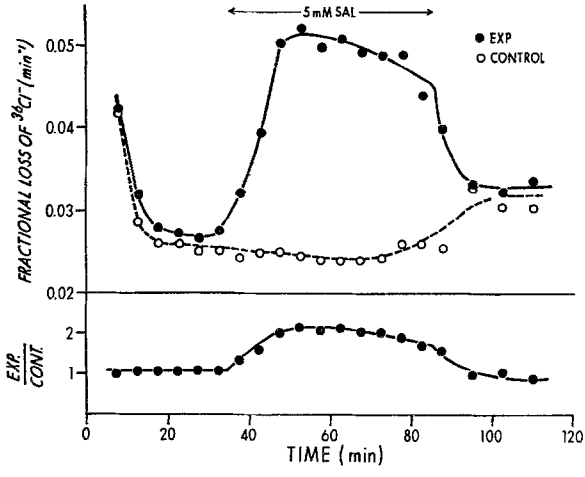

Fig. 4. Effect of 5 mM salicylate on loss of ^{36}Cl. *Upper:* Plot of the fractional loss of $^{36}Cl^-$ from the muscle (•) exposed to salicylate and its mate (○) which was used untreated. *Lower:* Ratio of the rate coefficient from the treated muscle to the rate coefficient from the untreated muscle. (Exps. 12, 17, 68; Muscle nos. 3–4)

Table 1. Chloride efflux in the presence of aromatic anions

Aromatic anion	Rate coefficient ratio: $\frac{k_{Cl}{}^{a}(pH=7.2;\ [AA]^{b}=x\ mM)}{k_{Cl}(pH=7.2;\ [AA]=0\ mM)}$	No. of pairs of muscles used
2 mM Salicylate	1.31 ± 0.09 (mean ± SEM)	4
5 mM Salicylate	1.49 ± 0.07 (mean ± SEM)	14
10 mM Salicylate	1.77 ± 0.06 (mean ± SEM)	22
2 mM Acetylsalicylate	1.18 ± 0.05 (mean ± SEM)	4
5 mM Acetylsalicylate	1.32 ± 0.10 (mean ± SEM)	4
10 mM Acetylsalicylate	1.69 ± 0.18 (mean ± SEM)	4
10 mM Benzoate	1.41 ± 0.09 (mean ± SEM)	4

[a] k_{Cl} = Fractional loss of Cl^- (min^{-1}).
[b] [AA] = Aromatic acid concentration.

by different concentrations of salicylate and the other aromatic anions tested are collected in Table 1. Since the chloride efflux generally reached a maximum 15 to 20 min after exposure to salicylate was started, the averages for this period have been listed in the tables. The results indicate that the chloride efflux progressively increased as the salicylate concentration was increased. With concentrations of salicylate in the range of 5 to 10 mM the stimulated chloride efflux was fairly well maintained for the usual 30- to 60-min exposure period although occasionally there was a slight decline after the initial peak response.

Other aromatic acids were also used to determine how specific salicylate is in stimulating chloride efflux. From Table 1, it is clear that both benzoate and acetylsalicylate produced similar effects to those of salicylate. Both of these aromatic acids are nearly as effective as salicylate in stimulating chloride efflux.

Chloride Efflux in the Presence of Salicylate at Low pH

It is well known that the efflux of chloride from frog muscle is reduced when the extracellular pH is reduced (Brooks & Hutter, 1962; Hutter & Warner, 1967*a*, *b*; Moore, 1969). We were interested, therefore, to determine whether acid solutions altered the effects produced by salicylate.

Fig. 5 illustrates two experiments which show the response of chloride efflux to application of 10 mM salicylate when the external pH is lowered to 5.2. For the two pairs of muscles, the fractional loss of ^{36}Cl is plotted as a function of time. In part *A* of the figure, an experiment is illustrated in

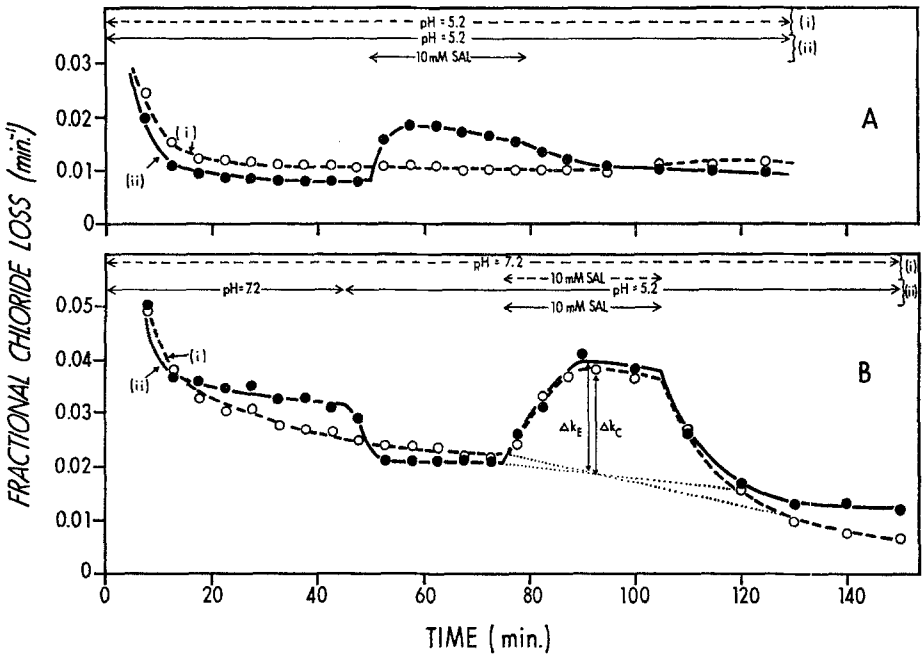

Fig. 5. Effect of low pH on salicylate-stimulated efflux of $^{36}Cl^-$. (*A*) A pair of muscles (*i* and *ii*) were kept in pH 5.2 throughout the experimental period. Fifty minutes after the beginning of the run muscle *ii* was exposed to 10 mM salicylate for 30 min. No drug was added to the solution washing muscle *i* which was used as a control (Exps. 4,7, 70; Muscle nos. 3–4). (*B*) In this experiment another pair (*i* and *ii*) were exposed to standard high-K^+ solution at pH 7.2 for 45 min at which time the pH of the solution bathing muscle *ii* was changed to 5.2 and the fractional loss of $^{36}Cl^-$ decreased to a new steady level. Seventy-five minutes after starting the washout both muscles *i* (pH 7.2) and *ii* (pH 5.2) were exposed to 10 mM salicylate for 30 min. (Exps. 11, 24, 70; Muscle nos. 7–8). Meaning of the symbols used: Δk_E and Δk_C = increments produced by 10 mM salicylate in $^{36}Cl^-$ efflux rate coefficients for the experimental and control muscles respectively

which both muscles were kept in acid solutions throughout the entire experimental period. The chloride efflux in the control muscle (*i*) was reasonably constant; it appeared to be a segment of a broad minimum. In the experimental muscle (*ii*), 10 mM salicylate produced a substantial increase in the efflux of ^{36}Cl. At the peak of the response the efflux was more than double what it was prior to the exposure of salicylate. Recovery was fairly complete after removal of salicylate. In part *B* of Fig. 5 the chloride efflux of the control muscle (*i*), which was kept at a constant pH of 7.2 throughout the experiment, slowly declined with time; during the period when 10 mM salicylate was added, the efflux doubled. In the experimental muscle (*ii*), when the pH was reduced from 7.2 to 5.2 there was an abrupt reduction in the chloride efflux. After 10 min it became relatively

Table 2*A*. Comparison of stimulating chloride efflux with 10 mM salicylate at pH = 5.2 and pH = 7.2

Parameter		Mean ± SEM	No. of pairs (p) or muscles (m)
Resting fractional loss of $^{36}Cl^-$ at pH's 5.2 and 7.2 (min^{-1})	(*a*) k_{Cl}(pH = 5.2)	0.0230 ± 0.0022	12 (m)
	(*b*) k_{Cl}(pH = 7.2)	0.0383 ± 0.0038	12 (m)
	Ratio *a*/*b*	0.61 ± 0.03	12 (p)
Increase of fractional loss due to salicylate at pH's 5.2 and 7.2 (min^{-1})	(*c*) Δk_{Cl}(pH = 5.2)	0.0184 ± 0.0007	4 (m)
	(*d*) Δk_{Cl}(pH = 7.2)	0.0189 ± 0.0041	4 (m)
	Ratio *c*/*d*	1.01 ± 0.10	4 (p)

Table 2*B*. Effect of 10 mM salicylate on chloride efflux at pH = 5.2

Parameter	Mean ± SEM	No. of muscles
Fractional loss of $^{36}Cl^-$ in the absence of salicylate $-k_{Cl}$(pH = 5.2) (min^{-1})	0.0176 ± 0.0021	8
Increment in k_{Cl}(pH = 5.2) in the presence of 10 mM salicylate Δk_{Cl}(pH = 5.2; [S] = 10 mM) (min^{-1})	0.0147 ± 0.0014	8
$\frac{\Delta k_{Cl}(pH = 5.2;\ [S] = 10\ mM)}{k_{Cl}(pH = 5.2)}$	0.89 ± 0.08	8

stable and upon addition of 10 mM salicylate the efflux in the acid solution was considerably increased; the absolute magnitude of the increment was nearly equal to the increment produced by salicylate in the control muscle which was kept at a pH of 7.2. Upon removal of salicylate from the bathing solutions, the recovery of efflux was prompt and distinct in both muscles.

There are two noteworthy features which are apparent upon examination of these two experiments. First, the response of the muscle to salicylate at low pH is not significantly altered by increasing the preincubation period in acid solution. Second, the percentage increase in efflux induced by salicylate seems to depend on neither the absolute value of the rate coefficient (the average rate of loss in the two pairs differs by more than a factor of 2), nor the nature of the resting efflux pattern (one pair had a slowly declining resting efflux while the other went through a minimum in the unstimulated, basal efflux).

The protocol scheme depicted in part *B* of Fig. 5 was adopted for comparing the increment of efflux produced by salicylate in the standard solution with that in acid solution. Table 2 summarizes the results and

comparisons made. It is clear that reducing pH from 7.2 to 5.2 reduces resting chloride efflux by about 39% while having no effect on the salicylate-stimulated efflux. This suggests that the aromatic acids are stimulating a component of the resting efflux which is pH insensitive.

Effects of Nitrate on the Resting and Salicylate-Stimulated Chloride Efflux

A well-known property of the chloride permeability system in frog twitch muscle membranes is that exposure to any one of several foreign anions of the lyotropic series, such as nitrate, markedly reduces chloride efflux. Fig. 6 illustrates the effects produced by both partial and complete substitution of nitrate for extracellular chloride on chloride efflux and how

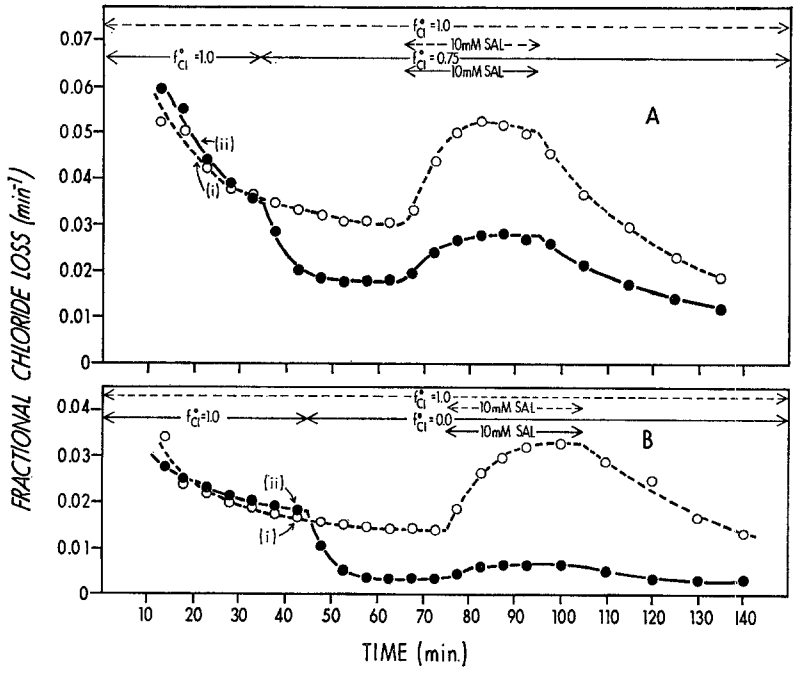

Fig. 6. Effect of partial and total substitution of Cl^- by NO_3^- in the external solution on salicylate-stimulated Cl^- efflux. (*A*) Two paired muscles were washed in the standard high-K^+ solution during the first 35 min; at this time the Cl^- content of the solution bathing muscle *ii* (experimental) was reduced to 75% ($f_{Cl}^0 = 0.75$) using NO_3^- to replace Cl^- on a mM for mM basis. The solution bathing the control muscle *i* was unchanged. After the expected fall in Cl^- efflux from muscle *ii* reached a steady level, both muscles were exposed to 10 mM salicylate. (*B*) The protocol of this experiment is similar to (*A*) except for the fact that here the experimental muscle *ii* was exposed to 10 mM salicylate in the complete absence of Cl^- in the external medium. (Exps. 9, 3, 70; Muscle nos. 7–8)

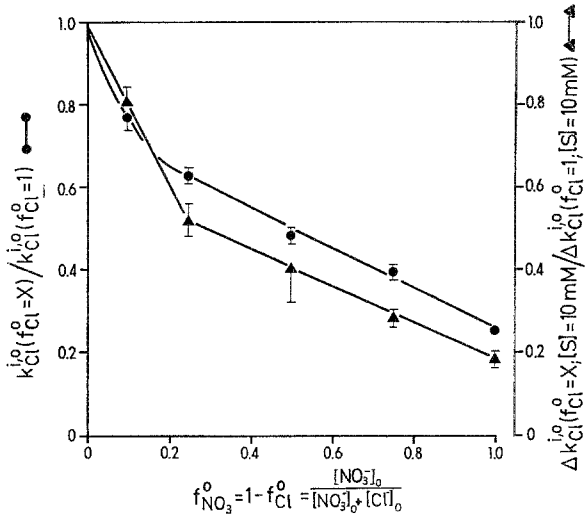

Fig. 7. Effect of nitrate for chloride replacement on resting and salicylate-stimulated chloride efflux. *See* text

these substitutions changed the effect usually produced by 10 mM salicylate. The fractional loss of chloride from two muscle pairs are plotted as a function of time. After following chloride efflux in the standard solution for 35 to 45 min, the external solution for one muscle of each pair was altered so that some or all of the chloride was replaced by nitrate. For the test muscle, the fractional concentration of nitrate in the external solution was kept constant for the remainder of the experiment. After an additional 30-min period, 10 mM salicylate was added to both the muscle kept in the control solution and the muscle immersed in the test solution. In the final sequence of each experiment, the recovery of chloride efflux was followed upon removal of salicylate. In each case, there was a rapid and complete return of chloride efflux to levels consistent with the trends present prior to exposure to salicylate. It is apparent that the salicylate-stimulated efflux is reduced in the presence of external nitrate. It is noteworthy that there is a definite, though small, salicylate-stimulated chloride efflux for the case in which nitrate completely replaced chloride in the external fluid.

A summary of all our findings dealing with the effects of nitrate substitution is given in Fig. 7. In most instances, the protocols followed the description given above. Some additional experiments with slightly different protocols were performed for the case of complete nitrate substitution. All such experiments including the ones on paired muscles are combined in Fig. 7. From this figure, it is clear that both the resting chloride efflux

(filled circles) and the salicylate-stimulated chloride efflux (filled triangles) are progressively diminished as the fractional concentration of nitrate in the external solution increases. With the exception of low nitrate concentrations, the salicylate-stimulated efflux appears more susceptible to external nitrate than the resting efflux. It will be shown below that there is no measurable membrane conductance change which can be ascribed to the salicylate-stimulated chloride efflux. This finding suggests that salicylate is stimulating a chloride exchange-diffusion type of mechanism. The fact that complete nitrate replacement for external chloride did not entirely abolish the salicylate-stimulated chloride efflux implies that the exchange diffusion mechanism is not perfectly selective and that it can carry out a nitrate for chloride exchange.

Effect of Salicylate on Potassium Efflux

A study of the effects of salicylate on potassium efflux was also made. The aim was to determine whether or not the action of salicylate was fairly specific and confined largely to altering the chloride permeability. Futher-

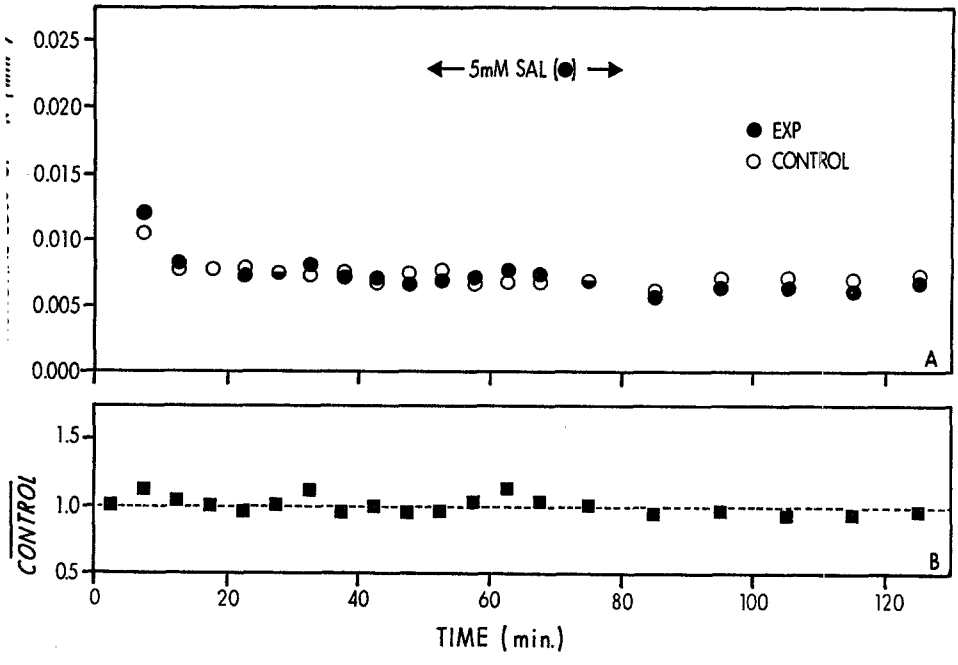

Fig. 8. Effect of salicylate on $^{42}K^+$ efflux. *Upper:* Fractional loss of $^{42}K^+$ in a pair of muscles, one of them exposed to 5 mM salicylate for 30 min and the other used as a control. *Lower:* Ratio of the experimental to control values in the upper part. The dotted line denotes ratio = 1

Table 3. Potassium efflux in the absence and presence of salicylate

Rate coefficient ratio	Mean ± SEM	No. of muscles
$\frac{k_K{}^a([S]^b = 5\ mM)}{k_K([S] = 0\ mM)}$	0.99 ± 0.03	6
$\frac{k_K([S] = 10\ mM)}{k_K([S] = 0\ mM)}$	1.03 ± 0.06	6

[a] k_K = Fractional loss of K^+ (min^{-1}).
[b] [S] = Salicylate concentration.

more, since our purpose was to determine the effect of salicylate on the chloride conductance of the membrane it was important to ascertain whether or not salicylate altered the potassium permeability.

Fig. 8 illustrates the results of a potassium efflux experiment on a pair of muscles in which one muscle was exposed to 5 mM salicylate for 30 min in the middle of an experimental sequence. In part *B*, the ratio of the potassium efflux in the control muscle to the efflux in the experimental muscle is given for each collection interval. It is clear that salicylate produced no detectable effect on the potassium efflux. Table 3 summarizes the results from a number of experiments of this type for two concentrations of salicylate. No significant effect on the potassium efflux was produced by either 5 mM or 10 mM of salicylate. In the few experiments performed with acetylsalicylate and benzoate in concentrations up to 10 mM, there was also no significant alteration of the potassium efflux.

Effect of Salicylate on Membrane Resistance

Since more than one-half of the total membrane conductance is caused by chloride ion movement for the solutions chosen (Hodgkin & Horowicz, 1959) and since 5 mM salicylate produces a 50% increase in chloride efflux (*see* Table 1), one would expect at least a 25% reduction in the total membrane resistance. With this in mind, the cable properties of fibers in sartorius muscles were measured in the absence and presence of salicylate.

The findings obtained are given in Table 4. In part *A* the results of measuring the membrane resistance twice on the same fiber for 20 fibers are given. Within a standard error of 1%, the membrane resistance did not change during a 20-min interval. On any given fiber the measurement of membrane resistance had a standard deviation of about 4% as compared to a duplicate measurement on the same fiber.

Table 4. Summary of measurements on cable constants

A. Sequential determination of cable constants in standard solutions				*B.* Effect of 5 mM salicylate on cable constants				Row reference
	Number of fibers		20		Number of fibers		9	1
V_m (mV)	First measurement	-18.2 ± 1.4	(± 0.32)	V_m (mV)	Control measurement	-17.4 ± 1.6	(± 0.5)	2
	Second measurement	-16.9 ± 1.6	(± 0.32)		Salicylate measurement	-17.0 ± 3.3	(± 1.1)	3
λ (mm)	First measurement	0.860 ± 0.113	(± 0.025)	λ (mm)	Control measurement	0.865 ± 0.094	(± 0.031)	4
	Second measurement	0.886 ± 0.118	(± 0.026)		Salicylate measurement	0.867 ± 0.093	(± 0.031)	5
r_m ($10^3/\Omega$cm)	First measurement	9.19 ± 3.10	(± 0.69)	r_m (10^3 Ωcm)	Control measurement	11.04 ± 2.22	(± 0.74)	6
	Second measurement	9.19 ± 3.31	(± 0.74)		Salicylate measurement	11.16 ± 1.77	(± 0.58)	7
$\frac{r'_m}{r_m}$	Ratio of second to first measurement	0.99 ± 0.04	(± 0.01)	$\frac{r_m(s)}{r_m(c)}$	Ratio of salicylate to control measurement	0.98 ± 0.07	(± 0.02)	8
	Time between measurements	21.5 ± 2.8	(± 0.6)		Time between measurements	17.9 ± 1.3	(± 0.4)	9

Data is presented as means $\pm$ standard deviations. Figures in parentheses are the standard errors of the mean.
Abbreviations used: V_m = resting transmembrane potential; λ = space constant; r_m = transmembrane resistance for a unit length of fiber.

A summary of duplicate measurements of membrane resistance on nine fibers before and during exposure to 5 mM salicylate is given in part *B* of Table 4. From row (8), which gives the ratio of the membrane resistance in the presence of 5 mM salicylate to the membrane resistance in the absence of salicylate, it is apparent that, within a standard error of 2%, there was no measurable effect of salicylate on membrane resistance. The measurements in salicylate were made 15 to 20 min after starting the exposure to salicylate, at a time when the salicylate effect on chloride efflux was usually fully developed. As mentioned above, during a similar interval under control conditions no change in membrane resistance occurred. Furthermore, there was no change in membrane potential on applying 5 mM salicylate to the muscle fibers (*see* rows (2) and (3) of part *B*, Table 4).

These results suggest that salicylate stimulates a chloride exchange diffusion mechanism since there was no measurable effect on the membrane resistance when the chloride fluxes were appreciably increased and the potassium fluxes were unaffected.

Discussion

Before discussing the effects produced by aromatic anions, consideration will be given to the resting chloride efflux. The most surprising feature of the resting chloride efflux was the occurrence of minima and maxima in the efflux rate coefficient for constant external conditions in some muscles. At the end of the experiment, these muscles, like those which did not have this complex chloride efflux pattern, showed no signs of either damage or contracture. Muscles exposed to solutions with added KCl for long periods are known to recover their normal membrane potential and excitability (Hodgkin & Horowicz, 1959; Adrian, 1960). One other point to be noted is that in the many potassium efflux experiments which were performed, no such 'oscillatory' behavior was ever observed. This rules out any explanation which involves a generalized increase of membrane permeability for the muscles exhibiting the 'oscillatory' pattern in the efflux of ^{36}Cl.

The fact that some muscles did show this 'oscillatory' behavior implies a fair degree of synchrony among the individual fibers. If each fiber was behaving in this 'oscillatory' fashion with a phase independent of the others then at any moment the number of fibers at a minimum in their efflux should be about equal to the fibers at their maximum. In this case, the total fractional rate of efflux would have been either constant or, possibly, declining monotonically since the second minimum was generally lower than the first (*see* Fig. 3).

The fact that the flux of chloride in muscles taken from different frogs could be in fairly good synchrony implies that some procedure during preparation of the muscles provided the synchronizing event. The most likely possibility is the immersion of muscles in high KCl solutions. Our usual practice was to dissect the muscles first and then immerse all of them into the standard equilibrating solution simultaneously. An extensive study of this 'oscillatory' behavior proved difficult owing to its erratic occurrence. However, evidence was obtained suggesting that the phase of the 'oscillations', when they occur, are set by the time when the muscles are placed in high potassium solutions.

The nitrate sensitivity of the resting chloride efflux for the present experiments is comparable to that reported by others. From Fig. 7, the rate coefficient for the resting loss of chloride in nitrate solutions is about 25% of the loss in chloride solutions. Harris (1958) reports that the chloride efflux from KCl-loaded muscles is greater in chloride solutions than in nitrate solutions by a factor ranging from 2.3 to 5.0. Adrian (1961) finds a reduction of about 50% when nitrate substitutes for chloride in normal Ringer's fluid. Moore's (1969) results indicate that complete nitrate substitution for external chloride reduces the chloride efflux by about 85% in depolarized semitendinosus fibers.

Acidification, on the other hand, seems to have produced less of a reduction in the resting chloride efflux in the experiments reported here than found by others. Hutter and Warner (1967*b*) report that the rate coefficient for chloride efflux at pH 5.0 is about 78% less than that found at pH 9.8. The results of Moore (1969) indicate that the chloride efflux is reduced by about 67% when going from a pH of 7.2 to 6.2. On the other hand, Table 2 indicates that the chloride efflux in the sartorii we have studied drops by only 39% when going from a pH of 7.2 to 5.2. One possible explanation for this discrepancy is that the relation between chloride efflux and pH is shifted to a higher pH for the muscles in this study.

Turning to the effects produced by aromatic anions, the addition of these to sartorius muscle clearly increases the efflux of chloride. This is in marked contrast to the reduction of anion permeability produced by these agents in red cells (Parpart, 1940; Passow, 1969; Passow & Schnell, 1969; Wieth, 1970*b*; Gunn & Tosteson, 1971). At the relatively low concentrations used in this study there was no detectable effect on potassium movements when salicylate, benzoate, and acetylsalicylate were added to the bathing medium. This again contrasts with the increased cation permeability produced by these and other aromatic compounds in red cells (Passow, 1969; Passow & Schnell, 1969; Wieth, 1970*a*; Gunn & Tosteson, 1971).

Although the effects observed on erythrocytes may be present in sartorius muscles at the high concentrations of these aromatic compounds used by investigators working with red cells, they do not seem to be the principal effects at the concentrations employed in this study.

The fact that the increased chloride efflux produced by salicylates is not accompanied by an increased membrane conductance suggests that the aromatic compounds stimulate an exchange-diffusion type of mechanism for chloride. This notion is supported by the finding that complete nitrate for chloride substitution reduces the salicylate-stimulated chloride efflux to less than one-fifth of its value in chloride solutions (*see* Fig. 7). A total elimination of chloride efflux through an exchange-diffusion system by a foreign anion such as nitrate would be expected only if the system were completely selective for chloride with no affinity for nitrate. If a finite affinity for nitrate is present then an exchange of external nitrate for internal chloride can occur. How the aromatic anions stimulate exchange diffusion of chloride remains unsettled.

Finally, a few comments can be made with regard to the residual chloride efflux in acid solutions and its possible relation to the salicylate-stimulated chloride efflux. Since the salicylate-stimulated chloride efflux is pH insensitive, it is tempting to suppose that the acid-insensitive chloride-efflux mechanism present in the standard physiological solutions is being stimulated by the aromatic anions. If this were the case, then the unstimulated chloride efflux in acid solutions would largely be an exchange-diffusion type of flux. However attractive, such an inference is probably not entirely correct. Although the measurements of Hutter and Warner (1967*a*) indicate a considerable reduction in chloride conductance on acidification to a pH in the range of 5.0 to 6.0, other evidence suggests that some chloride conductance remains at these acid pH's. For example, Hutter and Warner (1967*a*) show an experiment (*see* their Fig. 12) in which the application of chloride-Ringer's after equilibration in a methylsulfate-Ringer's produces a hyperpolarization of about 8.5 mV when all solutions were kept at a constant pH of 5.0. Applying the constant field equations (Goldman, 1943; Hodgkin & Katz, 1949) to this result gives a P_{Cl}/P_K ratio of about 0.47. Similar results have been obtained with depolarized single fibers (Horowicz & Caputo, *unpublished*). For example, application of chloride solutions to muscles equilibrated in high potassium methylsulfate solutions at pH = 5.7 result in hyperpolarizations consistent with a P_{Cl}/P_K ratio of about 1.2.

Ratios of this magnitude can be obtained from the measured potassium and chloride fluxes given in this report. The average resting rate coefficient for chloride efflux in 118 muscles at pH = 7.2 was 0.03 min^{-1} while the

average resting rate coefficient for potassium efflux in 20 muscles was 0.0105 min^{-1}. These values can be converted into fluxes and permeabilities using estimates of fiber diameter, internal concentrations, and internal potential. A value of 80 μ can be taken as fairly representative for the average diameter of fibers in sartorius muscle (e.g., *see* Shanes, 1958). This corresponds to an average volume to surface ratio of 20 μ. From an internal potential of −17 mV and the external potassium and chloride concentrations, the internal concentrations can be calculated on the assumption that both these ions are in equilibrium. The calculated efflux per unit surface area is 92×10^{-12} mole cm^{-2} sec^{-1} for chloride ions and 55.7×10^{-12} mole cm^{-2} sec^{-1} for potassium ions. If chloride is taken as obeying the independence relations and allowance is made for the nonindependence of potassium movements (Horowicz, Gage & Eisenberg, 1968) the calculated conductance for chloride and potassium are 356 μmho cm^{-2} and 426 μmho cm^{-2}, respectively. These values give a P_{Cl}/P_K ratio of 0.46 assuming: (a) the potassium conductance does not vary with pH; and (b) the chloride conductance at pH 5.2 is 0.61 of its value at pH 7.2. The apparent P_{Cl}/P_K ratio will be some multiple of 0.46, if there is interaction in chloride movements. For example, if the rate-limiting process for translocation is second order in chloride concentration, then the apparent P_{Cl}/P_K ratio will be 0.92. From these calculations it is clear that the permeability ratios derived from the flux data at pH = 5.2 are in the range of the permeability ratios derived from the electrochemical data. This implies that a significant part of the measured resting chloride efflux in acid solutions is not exchange diffusion. Nevertheless, a small fraction due to exchange diffusion could be present and it is possible that the aromatic anions are stimulating this exchange diffusion.

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