

# Properties of Chloride Transport in Barnacle Muscle Fibers

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**ABSTRACT** Unidirectional chloride-36 fluxes were measured in internally dialyzed barnacle giant muscle fibers. About 50–60% of the Cl efflux was irreversibly blocked by the amino-group reactive agent, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (SITS), when it was applied either intra- or extracellularly. Similarly, Cl influx was also blocked by SITS. No significant effect on  $[Cl]_i$  of SITS was noted in intact muscle fibers. However, the rate of net Cl efflux from muscle fibers which were Cl-loaded by overnight storage at 6°C could be slowed by SITS treatment. Two classes of anions were defined based upon their effects on Cl efflux. Methanesulfonate and nitrate inhibited Cl efflux either when they replaced external chloride or when they were added to a constant external chloride concentration. The other group of anions (propionate, formate) stimulated both Cl efflux and influx and such stimulation could be blocked by SITS. Propionate influx was not nearly as large as the stimulated Cl efflux and was unaffected by SITS. Neither the effects of SITS nor those of the anion substitutes could be simply accounted for by changes in the membrane resting potential or conductance. These results suggest a mediated transport system for chloride across the barnacle sarcolemma.

## INTRODUCTION

Chloride movements across the sarcolemma of the barnacle giant muscle fiber have been studied by both isotopic flux methods (DiPolo, 1972; DiPolo and Latorre, 1972) and electrophysiological methods (Hagiwara et al., 1968). The electrophysiological results indicated that the rate of chloride movement across the membrane was only about one-seventh that of potassium at a normal external pH of 7.7. However, the isotopic flux studies of DiPolo and Latorre (1972) show that Cl fluxes are at least as large as those of potassium. Furthermore, DiPolo (1972) has pointed out that the ratio of the chloride unidirectional fluxes is much closer to unity than expected if the two fluxes were passive and independent of one another. These observations could be explained by either a large Cl/Cl exchange flux or by an active chloride efflux. Intracellular chloride concentration of these muscle fibers has been measured by several workers (Hagiwara et al., 1964; McLaughlin and Hinke, 1966; Gayton and Hinke, 1968; 1971; Bittar, 1971). These results range from a low of 6.6 mM (McLaughlin and Hinke, 1966) to a high of 75 mM (Gayton and Hinke, 1968) although the bulk of the measurements are in the 30–35 mM range. These latter values give an

equilibrium potential for chloride that is very near the resting membrane potential suggesting that no net active transport takes place across the barnacle sarcolemma.

The apparent electrical insignificance of chloride coupled with its large isotopic flux suggests that a large portion of the movements of chloride are by electrically "silent" pathways. This electrically "silent" pathway is analogous to that found in the red blood cell where isotopic fluxes are much greater than net (electrical) fluxes (Knauf et al., 1977). The study of the transmembrane anion exchange mechanism of the red blood cell has been greatly facilitated by use of the disulfonic acid derivatives which irreversibly inhibit this exchange flux as well as the net flux (Knauf and Rothstein, 1971; Knauf et al., 1977). More recently one of these derivatives, SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) has been shown to inhibit chloride transmembrane movements in turtle bladder (Ehrenspeck and Brodsky, 1976), squid axon (Russell and Boron, 1976) and *Aplysia* neurons (Russell, 1978) showing that this agent may have wide applicability in the study of chloride transport.

We have used the internal dialysis technique (Brinley and Mullins, 1967) as well as studies on intact muscle fibers to examine some properties of chloride fluxes across the barnacle sarcolemma. The purpose of the present study was to distinguish between Cl/Cl exchange and active chloride efflux as explanations of the excess isotopic flux relative to that predicted from electrical conductance measurements. We conclude that the membrane of the barnacle giant muscle fiber does possess a mechanism which promotes Cl/Cl exchange, but that this system can also mediate net movements of chloride in response to passive electrochemical gradients.

A preliminary account of some of these results has been communicated to the Biophysical Society (Russell and Brodwick, 1976).

#### MATERIALS AND METHODS

##### *Materials*

Barnacles were obtained from Puget Sound, Washington. They were maintained in an aerated aquarium (Pacific Bio-Marine Laboratories Inc., Venice, Calif.) at 12°C until used. Only fibers from the *depressor scutorum rostralis* or *lateralis* groups were used as they were more nearly cylindrical in shape. After dissection the fibers were stored in artificial barnacle seawater (BSW; Table I) at 6°C until used. All experiments were completed within 60 h of dissection. The muscle fibers were soaked in 0 Ca-BSW (Table I) for 30 min before being cut from the shell. This prevented a contracture from occurring.

SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) was purchased from ICN Nutritional Biochemicals (Cleveland, Ohio) as the disodium salt.

##### *Solutions*

Compositions of the external solutions used in this study are given in Table I. The pH of these solutions was 7.7-7.8 at 20°C. The osmolalities were routinely measured using a dew point depression osmometer (Wescor Inc., Logan, Utah, model 5100) and were 1070 mosmol/kg.

The composition of the internal dialysis stock solutions is given in Table II. The pH of these solutions was adjusted to 7.3 by the addition of NaOH, raising the sodium

concentration by about 2 mM. Intermediate concentrations of chloride could be easily made by mixing the stock solutions in the appropriate proportions. Stock solutions were stored at 0°F until used.

#### *Dialysis Capillaries*

Cellulose acetate tubing (FRL, Inc., Dedham, Mass.) was used to make the dialysis capillaries. This tubing had an outer diameter of 195  $\mu\text{m}$  and an inner diameter of 145  $\mu\text{m}$ . A 13-cm length of tubing was rendered porous in a central region by hydrolysis in 0.1 N KOH for 16–19 h at room temperature. The length of the porous region for efflux tubes was 12 mm and for influx tubes it was 25 mm. After hydrolysis, the tubing was glued to a Lucite T-block which directed the dialysis fluid flow through the hollow center of the dialysis tube. The tube was stiffened by the axial placement of a 3-mil tungsten wire in order to aid the insertion into the muscle fiber. The tungsten wire was removed after the proper positioning of the dialysis tube within the muscle fiber.

A current-passing dialysis tube was made by threading a 25- $\mu\text{m}$  90% platinum - 10% iridium (Medwire Corp., Mt. Vernon, N. Y.) wire from the T-block past the porous region of the cellulose acetate tube and cementing it into place. The wire was then platinized and used to pass current across the barnacle sarcolemma.

TABLE I  
EXTERNAL SOLUTIONS

	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>++</sup>	Mg <sup>++</sup>	Cl <sup>-</sup>	*X <sup>-</sup>	Tris	Sucrose
	<i>mM/liter</i>							
BSW	494	10	11	32	590	—	10	—
0 Cl-BSW	494	10	11	32	—	590	10	—
50 propionate BSW	494	10	11	32	540	50	10	—
$\frac{3}{4}$ Cl - $\frac{1}{4}$ X <sup>-</sup> -BSW + Sucrose	494	10	11	32	442	148	10	408
$\frac{3}{4}$ Cl - $\frac{1}{4}$ X <sup>-</sup> -BSW + 265 mM NaX	759	10	11	32	442	265	10	—
pH 4.2 BSW‡	494	10	11	32	590	—	—	—
0 Ca BSW	494	10	—	43	590	—	10	—

<sup>36</sup>Cl-labelled artificial seawaters were made by adding 60 mM of the NaCl as Na<sup>36</sup>Cl. This resulted in a specific activity of ~17  $\mu\text{Ci}/\text{mM}$  of Cl. pH = 7.8.

\* X<sup>-</sup> = methanesulfonate, nitrate, propionate, formate.

‡ This solution was buffered with 10 mM K phthalate (Hagiwara et al., 1968).

#### *Experimental Procedures*

The procedures and the chamber have been described earlier (Russell and Blaustein, 1975). The central slot, in which external fluid bathed the dialyzed portion of the fiber, was 12 mm wide. In the present experiments the end-cannula solution contained 275 mM Tris-EGTA, 75 mM MgSO<sub>2</sub>, pH 7.3. The cannulated ends of the muscle fiber were usually allowed to hang in the air. Membrane potential was measured with a 100- $\mu\text{m}$  diameter glass capillary containing 0.5 M KCl. This electrode was inserted longitudinally into the dialyzed region of the fiber and connected to a W-P Instruments, Inc. (New Haven, Conn.) model VF-1 voltage follower by a calomel half-cell. The reference electrode was a calomel half-cell inserted in one guard outlet tube.

The Pt-Ir wire of the current-passing dialysis tube was connected to a stimulator (W-P Instruments, Inc.). The return pathway for the current was via a Ag-AgCl wire dipped into the bath above the muscle fiber. Both membrane potential and current were recorded on a Houston Instrument Div., Bausch & Lomb, Inc. (Austin, Texas) two-channel Omniscribe penwriter.

*Efflux Experiments*

Efflux was determined by adding radioisotope to the dialysis fluid taking into account the carrier chloride being added, then collecting and counting the external, superfusing fluid. The dialysis fluid was delivered at a constant rate of 2.2  $\mu\text{l}/\text{min}$  from a motor driven syringe pump (Harvard Apparatus Co., Millis, Mass., model 1100). Two peristaltic pumps (LKB Instruments, Inc., Rockville, Md., model 10200 Perpex) delivered the external fluid to the central slot through openings at either end of the floor of the slot. The fluid then left the slot via an opening in the middle and top of the slot. This fluid was pumped by a third peristaltic pump directly into the scintillation vials. The flow rate around the barnacle muscle fiber was 2.4 ml/min. The volume of the central slot was about 0.2 ml, and a solution change could be completed within 2 min as indicated by an isotope-dilution analysis. Two guard syringes withdrew fluid from either end of the dialyzed region of the fiber at a rate of 0.1 ml/min. This procedure is necessary because, within these lateral regions, control of the internal environment is poor due to diffusion between the dialyzed center region and undialyzed end regions.

TABLE II  
INTERNAL STOCK SOLUTIONS\*

	Na <sup>+</sup>	K <sup>+</sup>	Mg <sup>++</sup>	Cl <sup>-</sup>	Isethionate <sup>-</sup>	Sucrose	Hepes	EGTA	Phenol Red
	<i>mM/liter</i>								
0 Cl	18	200	4	—	200	465	10	0.5	0.5
180 Cl	18	200	4	180	20	465	10	0.5	0.5

<sup>36</sup>Cl-labelled dialysis fluids were made by adding 50 mM of the KCl as K<sup>36</sup>Cl to the 180 Cl stock solution. This resulted in a specific activity of 43  $\mu\text{Ci}/\text{mM}$  of Cl. Hypertonic dialysis fluids were made by adding 340 mM/liter of sucrose to these stock solutions.

\* Intermediate concentrations of Cl were obtained by mixing these two solutions in appropriate proportions.

*Influx Experiments*

The same chamber was used as for efflux experiments; however, several procedural modifications were made. First, the dialyzed region was made much wider (25 mm) than the region exposed to isotope (12 mm; see Russell, 1976) in order to prevent loss of isotope into the undialyzed lateral reaches of the muscle fiber. The guard syringes were disconnected, the guard regions of the chamber being filled with a sealing mixture of vaseline and mineral oil. The radioisotope-containing external fluid was constantly recirculated through the bath at a rate of 2.4 ml/min. Influx samples were collected from the tip of the dialysis tubing directly into scintillation vials by washing the tip with 1 ml of distilled water.

All flux calculations (both influx and efflux) assume the muscle fiber is a smooth right cylinder. To the extent that this is not true (Hoyle et al., 1973) due to surface infoldings, the fluxes reported will be too large.

*Counting Procedures*

Samples were collected directly into scintillation vials and 10 ml of a toluene-Triton X-100 cocktail (Nadarajah et al., 1969) were added. The samples were counted in a Packard Instrument Co., Inc. (Downers Grove, Ill.) Tricarb liquid scintillation counter until at least 1,000 counts above background were obtained.

SITS caused significant quenching of  $^{36}\text{Cl}$ ,  $^{22}\text{Na}$ ,  $^{42}\text{K}$ , and  $^{14}\text{C}$ . The appropriate corrections for this quenching were made.

#### *Isotopes*

$^{36}\text{Cl}$  was obtained from New England Nuclear (Boston, Mass.) as an aqueous solution of the sodium or potassium salt. The solution was evaporated to dryness and then placed in a muffle furnace at  $450^\circ\text{C}$  for 2–3 h in order to ash possible organic contaminants. The appropriate amount of crystalline  $^{36}\text{Cl}$  salt was then added directly to the solution taking into account the carrier salt being added.

$^{22}\text{NaCl}$ ,  $^{14}\text{C}$ -Na propionate, and  $^{42}\text{KCl}$  were added directly to the experimental fluids as the solution provided by New England Nuclear. This caused no change in the cation concentration due to the high specific activity.

#### *Analysis of Chloride Concentration*

The intracellular chloride concentration of single muscle fibers was determined as follows. Fibers which had been carefully separated were incubated for a minimum of 45 min in solutions containing [ $^{14}\text{C}$ ]sorbitol which served as a marker of the extracellular space. The fibers were then cut from their shell and lightly blotted with Whatman, Inc. (Clifton, N.J.) No. 40 filter paper. After blotting, they were transferred to tared vials with air-tight screw caps. After wet weights were obtained, the caps were removed and the vials placed in an oven at  $85^\circ\text{C}$  where the fibers were dried at least 16 h. Then the fibers were reweighed and digested in hot 0.6 N NaOH. Proteins were precipitated with a solution containing 4%  $\text{ZnSO}_4$  in 0.4 N  $\text{HNO}_3$ . Samples of the supernate (1.0 ml) were then placed in chloride titration vials and treated overnight at room temperature with 0.1 ml 0.25 M  $\text{NaBO}_3$  in 3.5 N NaOH to oxidize sulfhydryl groups (Cotlove, 1964). The next day the samples were coulometrically titrated using a Buchler Instruments Div., Searle Diagnostics Inc. (Fort Lee, N.J.) model 4-2500 digital chloridometer. Samples of the supernate were also counted in the scintillation counter to obtain a value for the [ $^{14}\text{C}$ ]sorbitol space. The intracellular chloride concentration was calculated as the difference between the total fiber chloride and the chloride in the [ $^{14}\text{C}$ ]sorbitol space.

#### *$^{36}\text{Cl}$ Efflux from Intact Fibers*

Two groups of muscles were prepared by carefully separating the individual fibers from one another but leaving them attached to the shell. Following the dissection and removal of the damaged fibers, the remaining fibers (30–40 per group) were soaked in  $^{36}\text{Cl}$ -BSW for 3 h. One group was treated with 0.5 mM SITS during this period while the control group was treated with normal  $^{36}\text{Cl}$ -BSW. During the last 45 min of this soaking period [ $^{14}\text{C}$ ]sorbitol and 5 mM cold sorbitol were added to the fluid bathing each group. At zero time both groups were washed with  $^{36}\text{Cl}$ -free BSW containing [ $^{14}\text{C}$ ]sorbitol. The fluid bathing the experimental group always contained SITS. Thereafter, the [ $^{14}\text{C}$ ]sorbitol solution was changed three times between each sampling period. Every 30–40 min five fibers were removed one at a time, their length and diameter were measured and blotted, and each fiber was placed in a tared vial. At the same time, samples of the soaking solutions were also taken. The individual muscles were then processed as previously described under "Analysis of Chloride Concentration." After the precipitation step with  $\text{ZnSO}_4$ , an aliquot of the muscle digest supernate was removed, added to 15 ml of scintillation cocktail, and counted for  $^{36}\text{Cl}$  and  $^{14}\text{C}$  content by the channels ratio method. Samples of the bathing fluids were carried through the same steps. The remaining supernate was then further processed and analyzed for its total chloride content. From these procedures the following pertinent information was derived: the rate constant for  $^{36}\text{Cl}$  efflux ( $k_{\text{Cl}}$ ), the cellular chloride content ( $\text{Cl}_c$ ), and the cellular

surface area (SA). By means of these data, an estimate of chloride efflux was made according to the following formula:

$$\text{Cl efflux} = k_{\text{Cl}} \cdot \text{Cl}_c / \text{SA}.$$

## RESULTS

### *Effect of SITS on Resting Cl Fluxes*

It is well established that the plasma membrane of the red blood cell possesses a mechanism capable of mediating anion exchange. An important contribution to characterizing this system was the demonstration that certain amino group-

TABLE III  
EFFECT OF SITS ON CHLORIDE EFFLUX

Fiber	Control		0.5 mM SITS	
	V <sub>m</sub> -mV	Efflux pmol/cm <sup>2</sup> ·s	V <sub>m</sub> -mV	Efflux pmol/cm <sup>2</sup> ·s
2265B	51	66	46	32
3055A	51	86	49	28
3125A	51	108	54	30
3125B	37	69	38	38
4115	50	62	52	25
1286	44	40	40	16
3246B	58	48	58	11
4076A	54	25	49	10
4086	56	23	57	11
7276	51	25	54	18
7266	54	35	55	17
10146	46	58	46	21
10286	60	40	61	20
12296	58	26	59	14
2077	53	59	53	31
2227	60	60	61	25
4187	55	61	56	24
8207B	47	36	48	15
8277A	49	33	50	18
8277B	63	48	64	19
9207B	57	33	42	16
9217A	51	35	40	15
9277A	58	48	63	10
9277B	49	33	44	20
11217A	57	43	55	19
11227A	49	54	25	35
11237A	44	46	46	18
11307B	51	36	52	23
12207A	56	31	54	16
12207B	59	22	59	12
12217A	39	65	38	24
12217B	32	96	34	58
$\bar{X}$	50	48.4	50.1	21.5
SEM	2.0	3.7	1.6	1.8

reactive agents could completely inhibit this process (Knauf and Rothstein, 1971). It was therefore of interest to us to determine whether such an agent would affect unidirectional chloride fluxes across the barnacle sarcolemma.

**EFFLUX** SITS, an irreversible amino group-reactive agent, was applied in the external fluid usually as a 0.5 mM solution, but once as a 0.05 mM solution. In the case of Cl efflux, the result was always ( $n = 32$ ) a substantial reduction (Table III). Fig. 1 shows that the effect was prompt, having a time constant of about 12 min and usually being complete within 30 min of its application. Membrane hyperpolarization immediately after SITS application such as that noted in Fig. 1 was observed in 27 of the 32 experiments, but was reversible when SITS was removed from the BSW. The effect on efflux, however, was irreversible over the time-course of the present experiments.

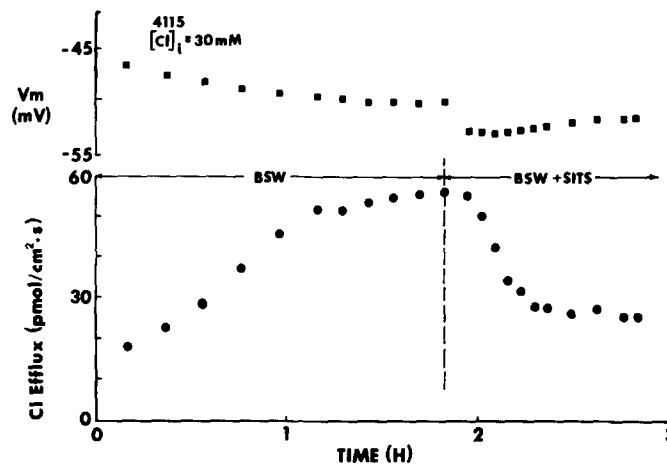


FIGURE 1. Effect of 0.5 mM SITS on  $^{36}Cl$  efflux from a dialyzed barnacle muscle fiber. At zero time dialysis with the  $^{36}Cl$ -containing fluid was begun. Temperature = 20°C. Fiber diameter = 1,325  $\mu m$ .

SITS was applied intracellularly via the dialysis fluid in two fibers. In one (not shown), it was applied as a 0.5 mM solution, and resulted in a 24% inhibition of Cl efflux and a slight membrane depolarization. The other fiber was treated with 5 mM intracellular SITS and the results can be seen in Fig. 2. The time to maximal effect on efflux was considerably longer ( $\sim 90$  min) than was the case for extracellular treatment. This undoubtedly reflects an intracellular diffusion delay inasmuch as it usually took 60–80 min for Cl efflux to reach a steady level after introduction of the isotope into the dialysis fluid. The subsequent addition of extracellular SITS resulted in only a slight further decline in Cl efflux. Because SITS cannot cross cell membranes (Maddy, 1964), this result means that the mechanism responsible for 50–60% of chloride efflux must possess similar sites on both sides of the sarcolemma. When applied internally, 5 mM SITS resulted in considerable depolarization, yet still caused about the same fall

in Cl efflux as when applied externally in which case only slight changes in membrane potential occurred (usually hyperpolarizations).

Two other putative anion flux inhibitors, sulfanilate (Ho and Guidotti, 1975) and pyridoxal phosphate (Cabantchik et al., 1975) were tested for their effects on Cl efflux. Pyridoxal phosphate caused a slowly reversible inhibition of ~70% when applied in a concentration of 15 mM. However, unlike the case for red blood cells, sulfanilate was without effect even at a concentration of 100 mM.

**INFLUX** The effect of 0.5 mM SITS applied externally on Cl influx was tested in six fibers. Table IV shows that in every instance a marked inhibition was observed. As seen in Fig. 3 the onset of the effect had a latency of 15–30 min and a time constant of ~29 min. This slower time-course for the SITS effect on the influx probably reflects the diffusion time for  $^{36}\text{Cl}$  from the sarcolemma to the dialysis tube.

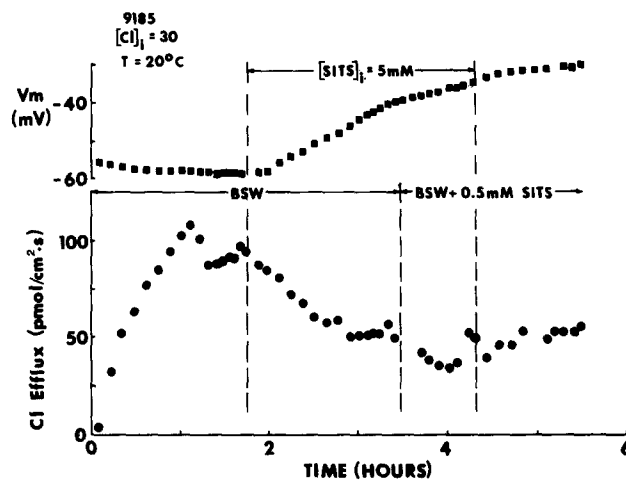


FIGURE 2. Effect of SITS applied internally via the dialysis fluid on  $^{36}\text{Cl}$  efflux. Addition of SITS to the external fluid had little additional effect. Temperature =  $20^{\circ}\text{C}$ . Fiber diameter =  $1,225\ \mu\text{m}$ .

#### *Effect of SITS on Intracellular Chloride Concentration*

Further evidence that SITS blocked an exchange process comes from the results of experiments on intracellular chloride concentration in the presence or absence of SITS. The effect on the intracellular chloride concentration of 1–2 h treatment with 0.5 mM SITS was tested in muscle fibers from three different animals. The results may be seen in Table V. Although the average intracellular chloride concentration was slightly higher in SITS-treated fibers for all three groups, the difference is not statistically significant. There was, however, a small but statistically significant difference in total tissue water. These results suggest some cellular swelling has occurred in SITS-treated fibers with a net gain of cellular chloride. Nevertheless, the differences are too small to permit a clear conclusion.

#### *Effect of SITS on Net Chloride Fluxes*

Another approach was to soak two fiber groups in BSW at  $6^{\circ}\text{C}$  overnight. We have found that such treatment causes  $[\text{Cl}]_i$  to increase somewhat but that



recovery of untreated fibers takes place within 90–120 min after rewarming to 20°C. In the experiment illustrated in Fig. 4, we treated one group with SITS for the last 45 min at 6°C. Upon rewarming the fibers to 20°C we found a faster rate of decline of  $[Cl]_i$  in control fibers than in 0.5 mM SITS-treated fibers although both groups had essentially the same intracellular chloride levels at the end of 3 h. Again, although the data from individual fibers show scatter, the trend of the means is quite clear. The scatter of these data is the inevitable result of the method which requires numerous procedural steps and the subtraction of the extracellular space contribution. These data suggest that the

TABLE IV  
EFFECTS OF SITS ON  $^{36}Cl$  INFLUX

Fiber	Control		0.5 mM SITS	
	$V_m$	Influx	$V_m$	Influx
	$-mV$	$\mu mol/cm^2 \cdot s$	$-mV$	$\mu mol/cm^2 \cdot s$
10037B	52	117	—	—
10117B	42	84	—	—
10127B	43	129	—	—
10137B	51	131	—	—
11237B	53	78	—	—
12017B	50	56	—	—
12057B	47	56	—	—
$n=7$	$\bar{X}$	48.3	93.0	
	SEM	1.8	13.3	
11157B	45	65	47	43
11167B	46	54	47	29
11177B	47	93	46	60
11217B	52	88	53	60
11227B	51	112	49	59
12067B	49	81	46	38
12077B	57	58	52	29
$n=7$	$\bar{X}$	49.6	78.7	48.6
	SEM	1.7	8.6	1.2
				5.8

apparent exchange process blocked by SITS can mediate a net flow of chloride inasmuch as SITS slows the rate of fall of the intracellular chloride concentration. This implies that the exchange process is not an obligatory one which requires the simultaneous movement of two chloride ions in opposite directions.

#### $^{36}Cl$ Efflux from Intact Fibers: Effect of SITS

$^{36}Cl$  efflux was studied from intact fibers which had been soaked in  $^{36}Cl$ -containing BSW for 3 h. The fibers were treated and prepared as described in Methods under the heading " $^{36}Cl$  Efflux from Intact Fibers." The results of the  $^{36}Cl$  washout are shown in Fig. 5 where it may be seen that treatment with 0.5 mM SITS reduced the rate at which the isotope left the fibers. In addition, it should be noted that less  $^{36}Cl$  entered the SITS-treated fibers during the loading

period. Thus, these data are qualitatively in agreement with the results obtained from dialyzed fibers.

A more quantitative comparison of  $^{36}\text{Cl}$  efflux between intact and dialyzed fibers was possible because, in addition to  $^{36}\text{Cl}$  data, we also measured the extracellular space, total intracellular chloride content, and cellular volume and surface area. These data are summarized in Table VI. Notice that the calculated

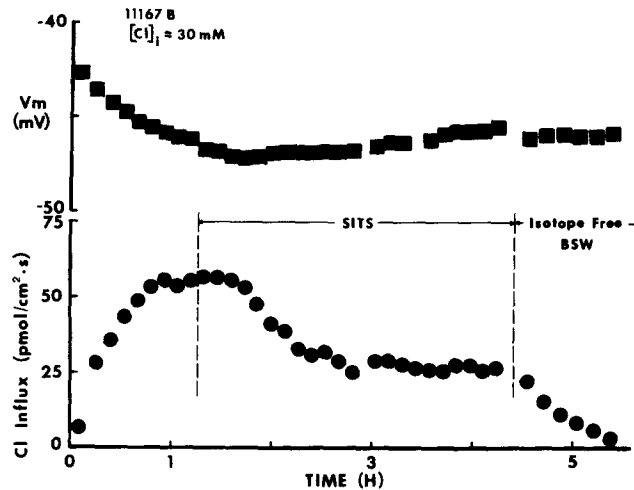


FIGURE 3. Effect of SITS applied externally on  $^{36}\text{Cl}$  influx. Notice that membrane potential ( $V_m$ ) hyperpolarized just as was noted in many efflux experiments. Temperature =  $20^\circ\text{C}$ . Fiber diameter =  $1,125\ \mu\text{m}$ .

TABLE V  
EFFECT OF SITS ON INTRACELLULAR CHLORIDE  
CONCENTRATION

	<i>n</i>	$[\text{Cl}]_i$ <i>mM</i>	Total tissue water	Extracellular space %
Control	58	32.8 $\pm 1.0$	74.2 $\pm 0.1$	9.3 $\pm 0.2$
0.5 mM SITS	53	34.8 $\pm 0.9$	75.6 $\pm 0.1$	9.0 $\pm 0.2$

These results were accumulated from the fibers of three different animals done at separate times. Each experiment had one group of fibers serving as control and another that was treated with SITS.

unidirectional efflux of chloride from control and SITS-treated intact fibers, namely 54 and  $29.3\ \text{pmol}/\text{cm}^2\cdot\text{s}$ , did not differ greatly from comparable data obtained from dialyzed muscle fibers (see Table III). Such close agreement may be somewhat fortuitous given the number of procedures that are required to obtain the intact fiber data, but we believe it shows that the experimental conditions of the dialyzed muscle fibers closely resembles those obtaining in intact fibers. This agreement is important in that we had no data on what the

predominant intracellular anion in the sarcoplasm might be. Our choice of isethionate was therefore arbitrary, and given the effects of substitute anions presented extracellularly (see below), we were concerned about complicating effects of internal anions. These results suggest isethionate does not significantly affect unidirectional Cl efflux. Recently, we have substituted glutamate for isethionate with no noticeable effect on Cl fluxes.

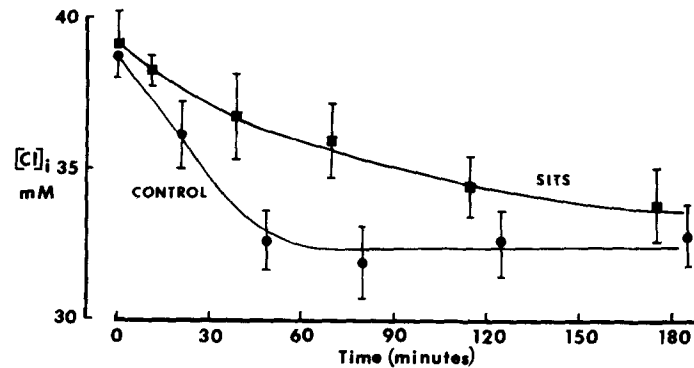


FIGURE 4. Effect of SITS treatment on intracellular chloride concentration. Intact fibers were incubated overnight at 6°C in BSW. 45 min before warming, 0.5 mM SITS was added to the BSW bathing one group. At zero time both groups were placed in solutions at 20°C. Each point is the mean  $\pm$  SEM of 9–10 fibers except for the zero time points which represent 5 fibers each. The curves are drawn to fit by eye.

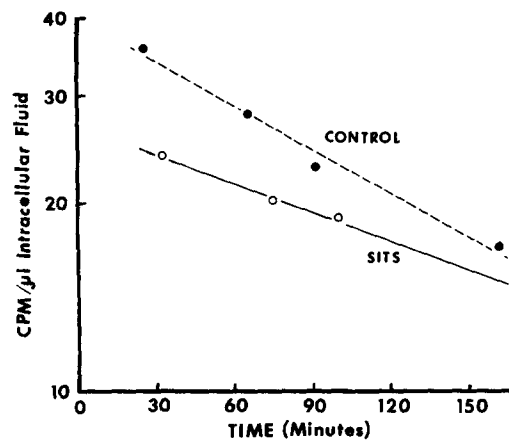


FIGURE 5. A semilogarithmic plot of counts remaining in intact muscle fibers during a washout of  $^{36}\text{Cl}$ . Fibers were initially soaked in  $^{36}\text{Cl}$ -BSW for 192 min. One group was exposed to SITS throughout this period. At zero time, fibers still attached to the shell were transferred to the appropriate  $^{36}\text{Cl}$ -free BSW. Each point represents the average of five fibers. The regression lines drawn through the points have correlation coefficients greater than 0.99. The calculated time constants are: control, 183 min; SITS-treated, 281 min. Temperature = 20°C.

*Effect of SITS on <sup>22</sup>Na and <sup>42</sup>K Efflux*

Although an early report (Knauf and Rothstein, 1971) indicated that SITS had no effect on cation movements in red blood cells, more recent evidence shows that sodium permeability is somewhat increased by SITS (Castranova and Miles, 1976). On the other hand, no effect of SITS on sodium-dependent short circuit current was noted in turtle bladder (Ehrenspeck and Brodsky, 1976). In view of these divergent results we tested the effect of SITS on <sup>22</sup>Na and <sup>42</sup>K efflux from the barnacle muscle fiber.

Fig. 6 shows that SITS had no effect on sodium efflux either before or during the Na-K ATPase inhibition by ouabain. Ouabain, on the other hand, caused

TABLE VI  
<sup>36</sup>Cl EFFLUX FROM INTACT FIBERS

	Control	0.5 mM SITS
[Cl] <sub>i</sub> , mM	39.5 (1.8)	35.5 (1.8)
Intracellular fluid space, μl	21.3 (0.8)	18.9 (0.8)
Diameter of fibers, mM	1250 (45)	1225 (47)
Length of fibers, cm	3.5 (0.1)	3.4 (0.1)
Rate constant of <sup>36</sup> Cl efflux, s <sup>-1</sup>	8.8 × 10 <sup>-5</sup>	6.0 × 10 <sup>-5</sup>
Chloride content of fibers, mol	8.4 × 10 <sup>-7</sup> (0.5)	6.4 × 10 <sup>-7</sup> (0.2)
Surface area, cm <sup>2</sup>	1.3744	1.3085
Cl efflux, pmol/cm <sup>2</sup> · s	54.0	29.3

Mean ± (SEM).

considerable inhibition of Na efflux as previously reported (Brinley, 1968; 1969; DiPolo and Latorre, 1972). Thus, the SITS-sensitive Cl fluxes are not coupled to sodium efflux nor does SITS, applied externally, inhibit the sodium pump or prevent ouabain from doing so.

The effects of 0.5 mM SITS on K efflux was measured simultaneously with its effect on Cl efflux in three fibers. Fig. 7 illustrates the lack of effect of SITS on K efflux while in this case it inhibited Cl efflux by ~40%. SITS therefore seems to be without effects on cation effluxes while significantly blocking those of chloride.

*Effects of Varying the External Chloride Concentration*

In view of the inhibition by SITS of both chloride efflux and influx, a Cl/Cl exchange diffusion process seemed a likely explanation for a large portion of the transmembrane chloride fluxes in the barnacle muscle fiber. In order to determine whether this system had an obligatory requirement for *trans*-side chloride, we performed experiments in which we substituted other anions for extracellular chloride.

METHANESULFONATE OR NITRATE AS SUBSTITUTE ANIONS DiPolo (1972) re-

ported and we have confirmed that substituting either methanesulfonate or nitrate for extracellular chloride results in a 40–60% decline of Cl efflux from dialyzed barnacle muscle fibers. Although this is what one would expect from an obligatorily linked exchange diffusion system, the results of several kinds of experiments lead us to the conclusion that these substitute anions have direct effects on the chloride transport mechanism, i.e., independent of changes in external chloride concentration.

For example, substitution of methanesulfonate for chloride in stepwise increments revealed that chloride efflux was unaffected by chloride replacement until nearly half the external chloride was replaced by methanesulfonate and the maximal effect occurred when only 75% of the chloride had been replaced (Fig. 8). Such results would not be expected from a simple 1:1 obligatory

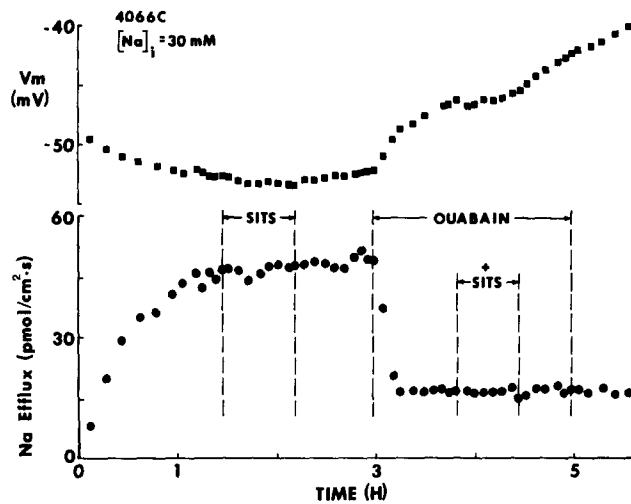


FIGURE 6. Effect of 0.5 mM SITS and  $3 \times 10^{-5}$  M ouabain on  $^{22}\text{Na}$  efflux from dialyzed barnacle muscle fiber. Temperature = 20°C. Fiber diameter = 1,000  $\mu\text{m}$ .

exchange diffusion scheme if the reduction of the external chloride concentration were the only important factor.

Inasmuch as the degree of inhibition of Cl efflux resulting from the complete replacement of chloride by methanesulfonate or nitrate was similar to that found after SITS treatment, we combined the two treatments in six fibers. Fig. 9 illustrates that after SITS treatment, replacement of extracellular chloride with methanesulfonate actually stimulated Cl efflux from an average of  $21.5 \pm 3.9$  to  $33.2 \pm 6.3$  pmol/cm<sup>2</sup>·s (means  $\pm$  SEM). This effect was usually (four out of six times) reversible. In one case, the SITS was added to O Cl-BSW ( $\text{NO}_3$ ) with the result that Cl efflux increased slightly; thus, the order of treatments does not seem to be crucial. These results suggest that extracellular chloride may actually be somewhat inhibitory to Cl efflux that occurs by non-SITS-sensitive pathways.

**PROPIONATE OR FORMATE AS SUBSTITUTE ANIONS** The results of experiments using other anion substitutes further reinforced our suspicion of direct effects

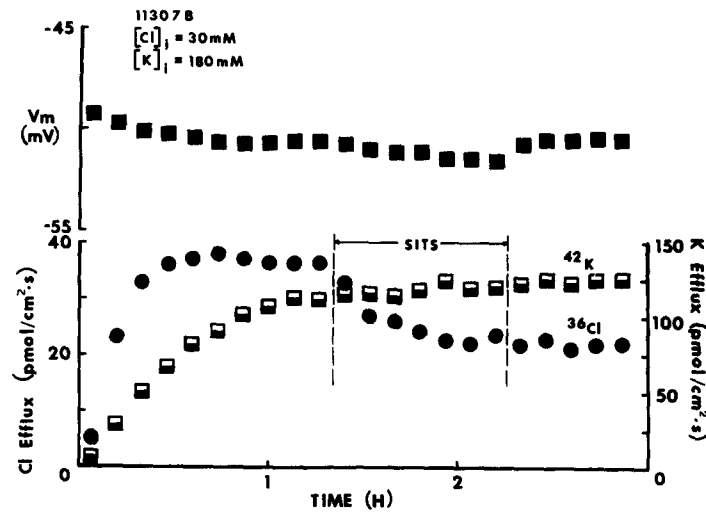


FIGURE 7. Effect of 0.5 mM SITS applied externally on  $^{42}\text{K}$  and  $^{36}\text{Cl}$  efflux from a dialyzed barnacle muscle fiber.  $^{42}\text{K}$  was counted by Cerenkov radiation, then allowed to decay for 2 wk (27 half-lives). Scintillation cocktail was then added and the samples were recounted for  $^{36}\text{Cl}$  activity. SITS quenched the  $^{42}\text{K}$  signal by 58% and the appropriate correction was made in data illustrated above. Temperature = 20°C. Fiber diameter = 1,125  $\mu\text{m}$ .

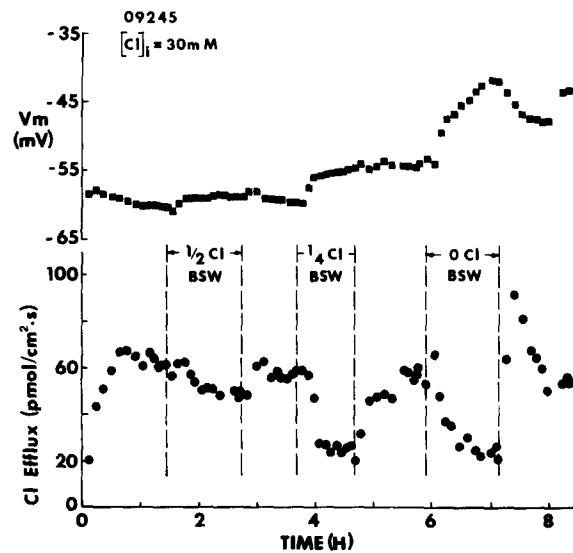


FIGURE 8. Effect of fractional replacement of external chloride with methanesulfonate on  $^{36}\text{Cl}$  efflux. Notice that replacement of half the external chloride had only a slight effect but replacement of 75% of the chloride had the same large effect as total replacement. External fluid was BSW unless otherwise indicated. Temperature = 20°C. Fiber diameter = 1,225  $\mu\text{m}$ .

by substitute anions. As illustrated in Fig. 10, when propionate was substituted for external chloride a large, reversible stimulation of Cl efflux resulted. In fact, in seven fibers, substitution of as little as 50 mM propionate caused Cl efflux to increase by threefold, from 31 to 127 pmol/cm<sup>2</sup>·s. Similar results were obtained using formate as a substitute anion. The propionate stimulation could always ( $n = 4$ ) be inhibited by SITS (Fig. 10). Interestingly enough, application of 15 mM propionate internally via the dialysis fluid at a constant [Cl]<sub>i</sub> of 30 mM was completely without effect on Cl efflux. DiPolo (1972) reported that substitution of propionate for chloride caused Cl efflux to fall. We have no explanation for this difference except to note that the pH of DiPolo's dialysis fluid was somewhat more acid than that used in the present work.

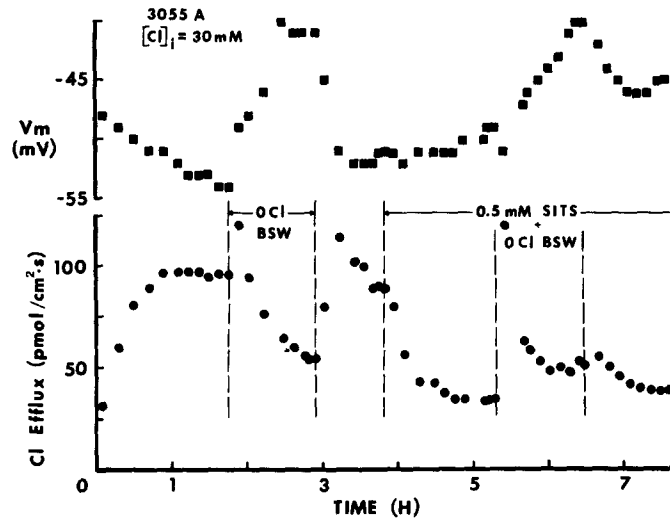


FIGURE 9. Effect of replacing all the external chloride with methanesulfonate on <sup>36</sup>Cl efflux before and after treatment with 0.5 mM SITS. Transient stimulation of chloride efflux after methanesulfonate substitution was characteristic of about half the experiments. Notice that SITS had no significant effect on the membrane potential change that resulted from methanesulfonate substitution. Temperature = 20°C. Fiber diameter = 1,200 μm.

**SUCROSE OR SORBITOL AS SODIUM CHLORIDE REPLACEMENTS** In view of the differing effects of various anion substitutes, the effects of nonionic substitutes, sucrose and sorbitol were studied. Fig. 11 illustrates the effect of iso-osmotically replacing half the NaCl with sucrose. Instead of a decline of Cl efflux as expected from an obligatory exchange diffusion model, an increase is actually observed. Thus, external chloride (or perhaps sodium, but see below) ions appear to inhibit Cl efflux. That most of this extra Cl efflux was mediated by the same pathway as that responsible for 60% of the efflux in the presence of normal extracellular chloride can be inferred from the fact that SITS inhibited almost all the extra efflux (Fig. 11).

*Effects of Anion Addition at a Constant External Chloride Concentration*

A test for direct actions of substitute anions requires that they be added without changing the external chloride concentration, a protocol that increases osmolality and ionic strength. Control for osmolality changes was accomplished by first raising the internal and external osmolalities using sucrose. Then the sodium salt of the test anion could be iso-osmotically substituted for the external sucrose. When first the internal, then the external osmolalities were increased, there was a substantial fall of Cl efflux in the experiment of Fig. 12. Such a pronounced effect of changing the osmolality was not common (e.g., Figs. 13 and 14) although some decrease was usually observed. In the experiment of Fig. 12, the control solution contained sucrose in addition to 148 mM methanesulfo-

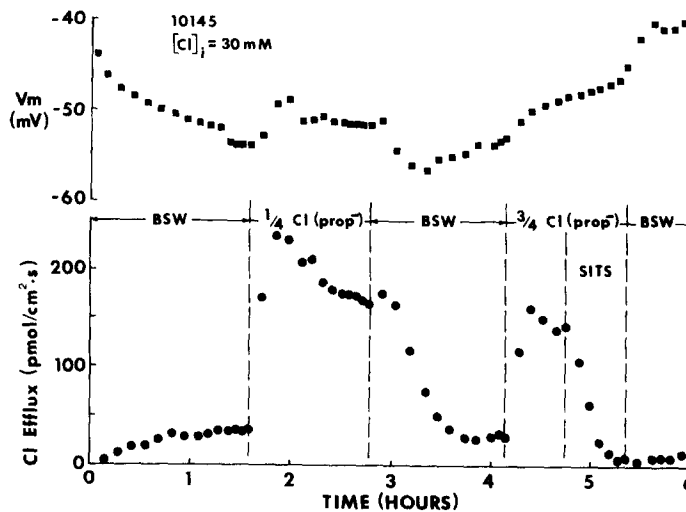


FIGURE 10. Stimulation of  $^{36}\text{Cl}$  efflux by partial replacement of external chloride with propionate and inhibition of this stimulation by SITS. Replacement of as little as 25% of the external chloride (148 mM) resulted in a threefold increase in  $^{36}\text{Cl}$  efflux. Treatment with 0.5 mM SITS abolished all the extra as well as about 50% of the control efflux. Temperature = 20°C. Fiber diameter = 1,250  $\mu\text{m}$ .

nate. This concentration corresponds to the amount of methanesulfonate it would take to replace one-quarter of the normal extracellular chloride. It will be recalled from Fig. 8 that about one-half the chloride had to be replaced by methanesulfonate in order for a decline of Cl efflux to become evident. When an amount of methanesulfonate equivalent to almost one-half the normal chloride concentration (i.e., 265 mM) was isosmotically substituted for sucrose, Cl efflux was reduced almost 50%. This effect was fully reversible. Thus, the fall of Cl efflux attendant to the addition of methanesulfonate must reflect a direct inhibitory action of this anion on the flux. It is also possible that the inhibition might be the result of an increased ionic strength inasmuch as one interpretation of the results in Fig. 11 is that a decrease in ionic strength causes a stimulation of Cl efflux.

The effect of a stimulating anion, propionate, was similarly tested. Fig. 13



shows that simply adding 100 mM sodium propionate (not reducing the chloride concentration) resulted in a fourfold increment of Cl efflux. As was the case for methanesulfonate treatment, the effect was fully reversible. Notice that this treatment also involved an increase of external sodium concentration as well as an increased ionic strength. Because the result was a stimulation of Cl efflux, it seems unlikely that the inhibition observed after sodium methanesulfonate or sodium nitrate addition was due to the increased sodium ion concentration or increased ionic strength.

Finally, the effects of external chloride on chloride efflux were tested utilizing the hypertonic internal and external fluids. Fig. 14 shows that Cl efflux varied

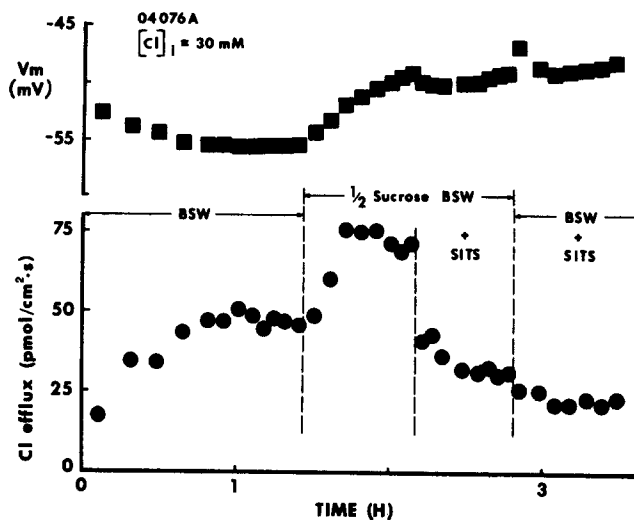


FIGURE 11. Effect on  $^{36}\text{Cl}$  efflux of replacing half the external NaCl with an isotonic equivalent of sucrose. At 1.5 h the NaCl concentration of the external fluid was reduced to 247 mM by substituting 406 mM sucrose maintaining osmolality at about 1,070 mosmol/kg. Temperature = 20.4°C. Fiber diameter = 1,125  $\mu\text{m}$ .

inversely with the external sodium chloride concentration; raising the concentration from 270 mM to 540 mM resulted in a fall of Cl efflux whereas lowering it to 96 mM caused an increase. These results are in good agreement with those illustrated in Fig. 11 which indicate that reducing the external sodium chloride concentration to 295 mM also increased Cl efflux under conditions of normal osmolality. These data suggest that extracellular chloride can act to inhibit chloride efflux, a conclusion suggested from the combined effects of SITS and OCl-BSW treatment (see Fig. 9).

Taken altogether the preceding results suggest the existence of a site or sites located on the outside of the sarcolemma with which anions can interact to modify chloride efflux.

#### *Chloride-Propionate Exchange?*

Inasmuch as it seems likely that a mechanism exists in the sarcolemma of the barnacle muscle fiber which can mediate Cl/Cl exchange, we were interested in

testing whether propionate might be stimulating chloride efflux by serving as a more effective counter-ion for exchange. If so, one might expect the addition of external propionate to reduce Cl influx and for the propionate influx to be inhibited by SITS.

These predictions were tested by simultaneously measuring  $^{36}\text{Cl}$  and [ $^{14}\text{C}$ ]propionate influx as seen in Fig. 15. It can be immediately seen that neither of the above predictions were borne out. In two fibers, external propionate stimulated Cl influx from 57 to 245 pmol/cm<sup>2</sup>·s. Treatment with 0.5 mM SITS

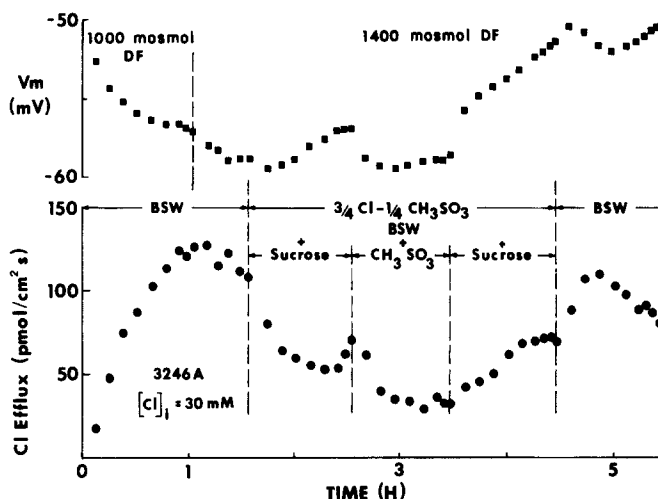


FIGURE 12. Effect of adding 265 mM Na methanesulfonate externally on  $^{36}\text{Cl}$  efflux. Experiment was begun in normal internal and external solutions. At 1 h the osmolality of the dialysis fluid (DF) was raised to 1,400 mosmol by addition of sucrose. At 1.5 h the external fluid was changed to a  $3/4 \text{ Cl} - 1/4 \text{ CH}_3\text{SO}_2$  BSW made hypertonic (1,500 mosmol) by addition of sucrose. At 2.5 h the extra sucrose was replaced by 265 mM Na  $\text{CH}_3\text{SO}_3$ . The hypertonic  $3/4 \text{ Cl} - 1/4 \text{ CH}_3\text{SO}_3$  BSW was returned at 3.5 h. Notice that, in contrast to the effect noted where methanesulfonate is substituted for chloride,  $V_m$  hyperpolarizes when methanesulfonate is added to the external fluid. This may reflect a significant methanesulfonate permeability via the membrane electrical pathway. Temperature = 20°C. Fiber diameter = 1,325  $\mu\text{m}$ .

reduced the Cl efflux to  $\sim 58$  pmol/cm<sup>2</sup>·s while only reducing propionate influx from 45.5 to 34 pmol/cm<sup>2</sup>·s. Thus, a small proportion of propionate influx may exchange for intracellular chloride, but clearly the large effect of propionate on Cl fluxes is not by means of a counter-transport exchange mechanism.

#### *Effect of SITS and Propionate on Membrane Electrical Resistance*

Because SITS blocked both influx and efflux of Cl it was necessary to determine whether this effect was on an electrical permeability pathway. It has been reported that certain disulfonic acid stilbene derivatives can inhibit the electrical flux (i.e., net movement) of chloride across red blood cell membranes (Kaplan et al., 1976; Knauf et al., 1977).

Membrane electrical resistance ( $R_m$ ) was measured in dialyzed muscle fibers by passing constant inward and outward current pulses of  $\sim 1.0$ -s duration from a platinized Pt-Ir wire situated inside the dialysis capillary as described in Methods. In nine dialyzed fibers  $R_m$  averaged  $690 \Omega \cdot \text{cm}^2$  before treatment with SITS. After  $0.5 \text{ mM}$  SITS, the average  $R_m$  actually fell to  $618 \Omega \cdot \text{cm}^2$ , although the difference is not statistically significant and the general trend for untreated fibers is for  $R_m$  to decline with time.

According to Hagiwara et al. (1968) chloride movements contribute only about one-sixth of the total membrane conductance when the external pH is 7.7. Inasmuch as SITS blocks a little more than half the isotopically measured

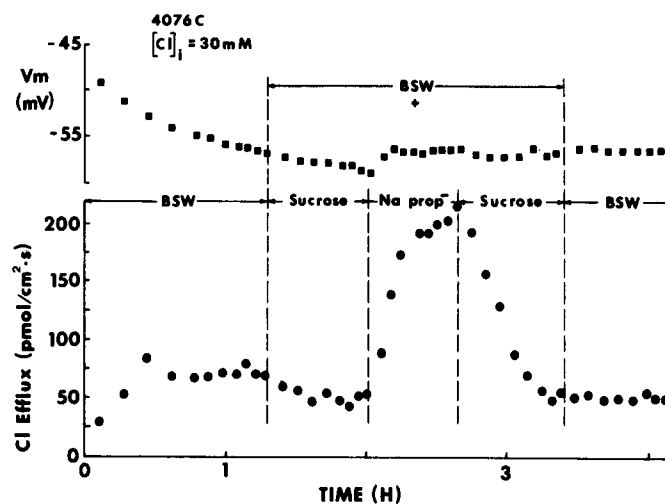


FIGURE 13. Effect of hypertonic addition of external sodium propionate on  $^{36}\text{Cl}$  efflux. Dialysis fluid osmolality throughout this experiment was  $1,115 \text{ mosmol/kg}$ . Hypertonic BSW ( $1,250 \text{ mosmol/kg}$ ) was made by addition of sucrose. At 2 h the sucrose was replaced iso-osmotically with  $100 \text{ mM}$  Na propionate resulting in a reversible fourfold stimulation of  $^{36}\text{Cl}$  efflux. Temperature =  $20^\circ\text{C}$ . Fiber diameter =  $1,250 \mu\text{m}$ .

Cl efflux, a quantitatively similar inhibition of chloride conductance would mean an increase of  $R_m$  of only about one-twelfth, a change that would be lost in the error of our measurement. However, at very acid external pH values ( $< 4.5$ ) the barnacle sarcolemma increases its conductance significantly by increasing the chloride conductance. Hagiwara et al. (1968) reported that under such acidic conditions chloride conductance comprises about five-sixths of the total membrane conductance. It is also known that under these acidic conditions Cl efflux is increased four- to fivefold (DiPolo, 1972), and SITS reduces the acid-stimulated Cl efflux by at least one-half.<sup>1</sup> If this SITS-inhibited flux is mediated by an electrical conductance pathway, it should be quite apparent when measuring total membrane resistance. The resistance of four fibers was tested

<sup>1</sup> Russell, J. M., and M. S. Brodwick. Unpublished observations.

at pH 7.6 and 4.2 before and after treatment with 0.5 mM SITS. As shown in Table VII, SITS did not prevent the decline of membrane resistance caused by the acidic external pH.

Inasmuch as the propionate-stimulated  $^{36}\text{Cl}$  efflux was not by means of a Cl-propionate exchange, the possibility existed that an increase in chloride conductance might be occurring. This seemed unlikely because an increase in chloride conductance should result in membrane hyperpolarization inasmuch as the chloride equilibrium potential in the dialyzed fibers was  $-75\text{ mV}$ . In fact, membrane depolarization usually accompanied propionate treatment (e.g.,

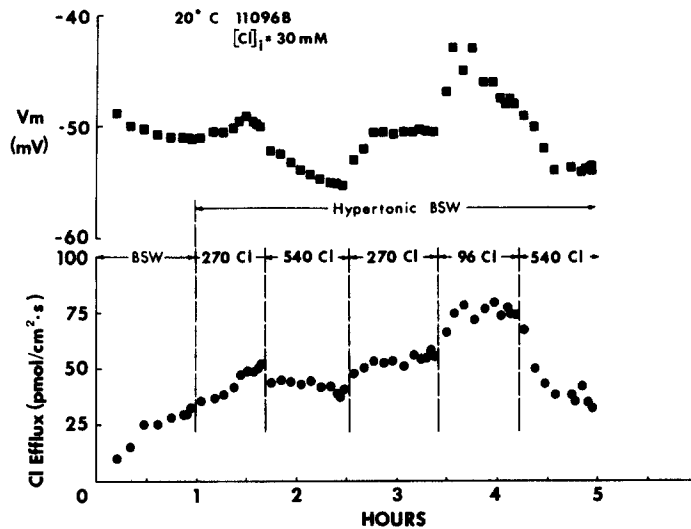


FIGURE 14. Effect on  $^{36}\text{Cl}$  efflux of varying the external chloride concentration by substituting sucrose for NaCl. At 1 h external solutions were introduced whose osmolalities were always  $\sim 1,450$  mosmol/kg. The chloride concentration was varied by adding or subtracting NaCl while making up the osmotic difference by subtracting or adding sucrose. Notice that the 540 Cl in hypertonic BSW has about the same chloride concentration as normal BSW and that the  $^{36}\text{Cl}$  efflux is not greatly different in the two solutions. The dialysis fluid throughout this experiment contained sufficient extra sucrose to increase its osmolality to 1,400 mosmol/kg. Temperature =  $20.2^\circ\text{C}$ . Fiber diameter =  $1,450\ \mu\text{m}$ .

Figs. 10, 13). When the effects of propionate on membrane resistance were directly measured in three dialyzed fibers it was found that the total membrane resistance increased slightly in all cases (Table VII). Thus, all the fluxes we have studied which can be inhibited by SITS appear to be unrelated to electrical conductance pathways.

#### *Membrane Potential Effects*

In the course of these studies, it was noted that Cl efflux seemed relatively impervious to changes in the membrane resting potential and that changes in potential caused by the various treatments could not be used to predict the effect the treatment might have on Cl efflux. For instance, methanesulfonate

and nitrate both inhibited Cl efflux but had opposite effects on membrane resting potentials. Complete replacement of extracellular chloride with methanesulfonate resulted in an average membrane depolarization of  $11.3 \pm 0.6$  mV ( $n = 13$ ) whereas replacement with nitrate resulted in an average membrane hyperpolarization of  $5.1 \pm 0.4$  mV ( $n = 6$ ). Moreover, these membrane potential effects were not abolished by SITS treatment. Finally, we have noticed that there is absolutely no relationship between the steady-state resting potential and Cl efflux into normal BSW (Fig. 16). These data are

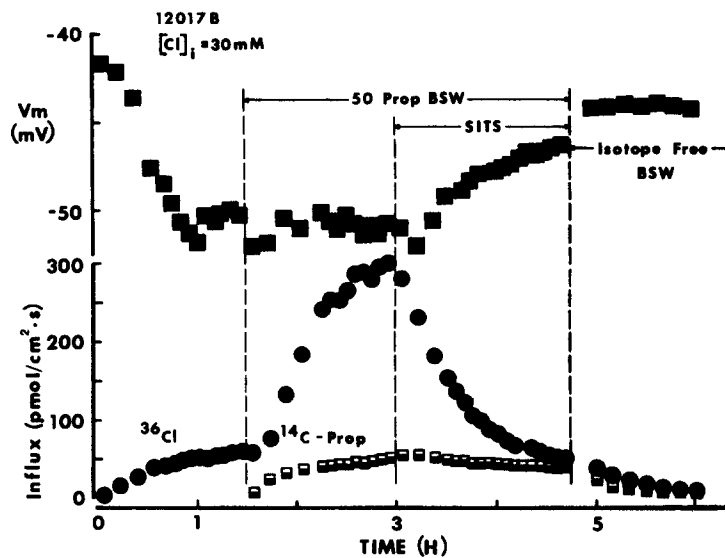


FIGURE 15. Effect of 50 mM propionate on  $^{36}\text{Cl}$  influx and of 0.5 mM SITS on  $[^{14}\text{C}]$ propionate influx and  $^{36}\text{Cl}$  influx. After a control period of 89 min in which only  $^{36}\text{Cl}$  influx was measured, the external fluid was changed to one containing  $^{36}\text{Cl}$  in the same specific activity but in which 50 mM NaCl was replaced by 50 mM Na propionate with  $[^{14}\text{C}]$ propionate. Then at 178 min, enough SITS was added to the external fluid to make a final concentration of 0.5 mM. At 290 min the fiber was washed with cold BSW and influx samples collected for another 76 min at which time there were no residual counts left in the muscle. Temperature =  $20^\circ\text{C}$ . Fiber diameter =  $1,500 \mu\text{m}$ .

consistent with the notion that a large fraction of Cl fluxes are by means of some electrically silent pathway. However, it should be pointed out that these results do not rule out possible offsetting effects of membrane potential on the activation of the Cl-translocation process as opposed to effects on the actual translocation process itself.

#### DISCUSSION

The present results as well as those of DiPolo and Latorre (1972) show that the isotopic fluxes of chloride and potassium are nearly equal, yet Hagiwara et al. (1968) have shown that the electrical conductance of the sarcolemma is six times greater for  $\text{K}^+$  than it is for  $\text{Cl}^-$ . These observations prompted DiPolo (1972) to

suggest two possible explanations: either there was active transport of chloride in the outward direction, or a large portion of transmembrane  $^{36}\text{Cl}$  movements were the result of Cl/Cl exchange. The present results clearly indicate that Cl/Cl exchange is an important mode of chloride transport across the membrane of the barnacle giant muscle fiber.

According to the Ussing flux-ratio equation (Levi and Ussing, 1948) the ratio of chloride influx to efflux ought to be about 2.7. In the present experiments it was about 1.6–1.7. A one-for-one Cl/Cl exchange would tend to make the flux ratio closer to 1.0 than predicted by the Ussing formulation. Treatment with SITS reduced both unidirectional fluxes by  $\sim 30 \text{ pmol/cm}^2\cdot\text{s}$  with the result that the flux ratio became 2.1, somewhat closer to the predicted value.

TABLE VII  
MEMBRANE RESISTANCE OF DIALYZED BARNACLE  
MUSCLE FIBERS

A. Effect of Acid External pH and 0.5 mM SITS				
Experiment	pH 7.6		pH 4.2	
	Control	SITS	Control	SITS
	$\Omega \cdot \text{cm}^2$			
8046B	650	606	302	377
8056	600	626	493	333
11106A	595	492	492	369
11106B	567	567	307	168
$\bar{X} \pm \text{SEM}$	$603 \pm 20$	$573 \pm 34$	$399 \pm 63$	$312 \pm 56$

B. Effect of 50 Propionate BSW		
Experiment	Control	Propionate
	$\Omega \cdot \text{cm}^2$	
8046A	748	819
8076	598	689
11096	682	719
$\bar{X} \pm \text{SEM}$	$676 \pm 53$	$742 \pm 48$

The lack of effect of SITS on membrane electrical conductance, even under conditions where  $\text{Cl}^-$  movements contribute the bulk of membrane current provides further evidence that the fluxes blocked by SITS are predominantly exchange fluxes. The rather marked insensitivity of  $^{36}\text{Cl}$  efflux to membrane potential gives further evidence that most of the chloride efflux is electrically "silent." Such electrical "silence" is exactly what would be expected of a system which simply exchanges an intracellular chloride for an extracellular one.

The fact that SITS inhibited Cl efflux regardless of whether it was applied externally or internally argues that the system responsible for SITS-sensitive Cl fluxes has similar sites on both sides of the membrane. In the case of the red blood cell anion exchange mechanism, it has been shown that some disulfonic acid derivatives can inhibit from both sides of the membrane while others cannot (Kaplan et al., 1976; Barzilay and Cabantchik, 1978).

The effects of SITS on intracellular chloride concentrations of intact fibers are important for our understanding of the SITS-sensitive Cl fluxes. The  $[\text{Cl}]_i$

of fibers which have been allowed to equilibrate at 20°C before being treated with SITS was essentially unaffected. This fits well with the observation of roughly equal inhibition of unidirectional Cl efflux and influx. However, those fibers whose  $[Cl]_i$  was increased by prior equilibration at 6°C showed a significantly slower return to normal  $[Cl]_i$  upon rewarming if they were treated with SITS. Because the resting membrane potential has a strong positive correlation with temperature (DiPolo and Latorre, 1972), this shows that, under conditions of an electrochemical driving force, the SITS-sensitive chloride transport system can mediate a net movement. This result is similar to the situation in the red blood cell (Knauf et al., 1977) in which about 65% of the net flux of chloride could be inhibited by DIDS (4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid).

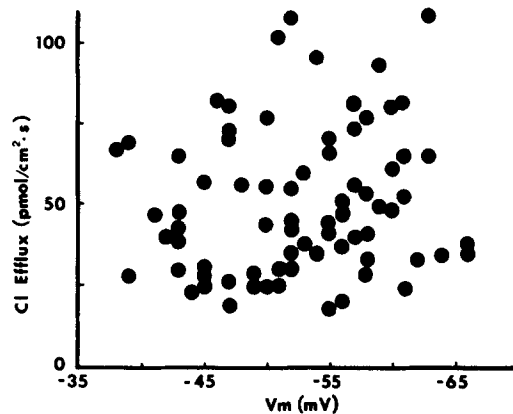


FIGURE 16. Lack of relationship between the steady-state resting potential and  $^{36}Cl$  efflux in 80 dialyzed barnacle muscle fibers. All the efflux data were taken from fibers dialyzed with normal dialysis fluid containing 30 mM chloride and superfused with BSW. None of the fibers had yet been treated in any other way. Thus, all these data points come from fibers dialyzed no longer than about 100 min. The equation for the best-fit straight line through these points is  $y = 0.56 + 20.3x$  with a correlation coefficient of 0.16. Temperature = 20°C.

The effects of certain anion substitutes to reduce Cl efflux when they were substituted for external chloride caused DiPolo (1972) to suggest that Cl/Cl exchange was taking place across the sarcolemma. The present results show that some anion substitutes may cause a reduction of Cl efflux, as reported by DiPolo (1972) while others result in a stimulation. Furthermore, these effects are not due to extracellular chloride removal because they were observed to occur when  $[Cl]_o$  remained constant. Thus, the use of anion substitutes to study chloride movements in cells may be fraught with unforeseen perils. In an effort to avoid these problems, we substituted nonionic substances (sucrose or sorbitol) for extracellular NaCl with the result that such substitution caused Cl efflux to increase. Inasmuch as this increase was blocked by SITS, it presumably reflects a stimulation of the mediated chloride transport mechanism. Obviously, such a result would not be expected of an obligatory exchange diffusion mechanism. An explanation for this surprising result is not immediately forthcoming. It may

reflect either a self-inhibition by chloride on chloride efflux or an inhibitory effect of the sodium ion on chloride efflux.

These results taken altogether suggest that there exists in the sarcolemma a specific mechanism, perhaps carrier-mediated, for moving chloride (and maybe other anions) into and out of the cell. Inasmuch as under control conditions the SITS-sensitive influx is about equal to the SITS-sensitive efflux, the mechanism is electrically silent and appears to be simply exchange diffusion. However, the results of the anion-replacement experiments and the net flux studies show that the two unidirectional fluxes are not rigidly coupled. Thus, the SITS-sensitive chloride fluxes appear to be manifestations of a mediated transport system which can promote net movement of chloride but which under most experimental conditions operates in an apparent Cl/Cl exchange mode. Such a system cannot be properly called an exchange diffusion mechanism because this nomenclature is reserved for a system which is unable to effect a net flux (Levi and Ussing, 1948; LeFevre, 1975).

The identification of a mediated chloride transport system in the barnacle sarcolemma leads us to the question of its function. It has recently been shown that chloride is involved in the regulation of intracellular pH for several preparations (Russell and Boron, 1976; Thomas, 1977; Russell, 1978), and that SITS inhibits intracellular pH regulation in these preparations as well as in barnacle muscle (Boron, 1977). The mechanism involved appears to be an exchange of intracellular chloride for extracellular bicarbonate, the bicarbonate reacting intracellularly to neutralize protons (Russell and Boron, 1976). In view of this proposed mechanism, it is tempting to speculate that the system studied in the present report can engage in chloride-bicarbonate exchange under appropriate conditions. Further experiments will be required to establish a link between the chloride movements that we have been studying and the process of intracellular pH regulation.

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