Sodium, Potassium, and Chloride Fluxes in Intercostal Muscle from Normal Goats and Goats with Hereditary Myotonia

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ABSTRACT In isolated bundles of external intercostal muscle from normal goats and goats with hereditary myotonia the following were determined: concentrations and unidirectional fluxes of Na^+ , K^+ , and Cl^- , extracellular volume, water content, fiber geometry, and core-conductor constants. No significant difference between the two groups of preparations was found with respect to distribution of fiber size, intracellular concentrations of Na^+ or Cl^- , fiber water, resting membrane potential, or overshoot of action potential. The intracellular Cl^- concentration in both groups of preparations was 4 to 7 times that expected if Cl^- were distributed passively between intracellular and extracellular water. The membrane permeability to K (P_K) calculated from efflux data was (a) at 38°C , $0.365 \times 10^{-6} \text{ cm sec}^{-1}$ for normal and 0.492×10^{-6} for myotonic muscle, and (b) at 25°C , 0.219×10^{-6} for normal and 0.199×10^{-6} for myotonic muscle. From Cl^- washout curves of normal muscle usually only three exponential functions could be extracted, but in every experiment with myotonic muscle there was an additional, intermediate component. From these data P_{Cl} could be calculated; it was $0.413 \times 10^{-6} \text{ cm sec}^{-1}$ for myotonic fibers and was $\geq 0.815 \times 10^{-6} \text{ cm sec}^{-1}$ for normal fibers. The resting membrane resistance of myotonic fibers was 4 to 6 times greater than that of normal fibers.

INTRODUCTION

Isolated external intercostal muscle fibers from goats with hereditary myotonia have a higher resting membrane resistance than similar preparations from normal goats (6, 7). This difference implies a decreased permeability of the resting myotonic muscle membrane to some species of ion; on electrophysiological grounds, this ion appears to be chloride (6, 7). On the other hand, chronically denervated skeletal muscle, which shares with muscle from myotonic goats some properties, including an elevated resting membrane resistance (see reference 19), has a decreased resting membrane permeability to potassium ions (25, 28). In contrast, despite a tendency to fire repetitively

(8, 27), skeletal muscle from mice with hereditary muscular dystrophy has an increased K^+ efflux, which is thought to reflect an increased resting membrane permeability to K^+ (11, 37).

This study was designed to relate the membrane resistances of normal and myotonic muscles to their resting membrane permeabilities to Na^+ , K^+ , and Cl^- . The data were collected from isolated muscle bundles prepared in a standard manner, and most of the parameters were measured from samples of the same biopsy. The core-conductor properties of the muscle fibers were also studied, and previous findings (6) were confirmed.

METHODS

Isolated external intercostal muscle preparations from 13 goats with hereditary myotonia (details regarding goats and maintenance will be published (7)) and 8 normal goats, of both sexes, were studied. Each intercostal space was used only once. Since 6 to 10 intercostal spaces in each goat are suitable for biopsy, several samples from the same animal were studied in different weeks. The biopsy procedure and fine dissection techniques will be described (7). The fine dissection was performed in a water-jacketed chamber that maintained the temperature of a perfusing, gassed, goat physiological salt solution between 34 and 38°C. For present purposes, the dissection was modified so that more than one preparation could be obtained from a single sample.

After initial orientation of the sample, three or more ties, spaced several millimeters apart, were placed through the superior tendon sheet in a straight line perpendicular to the orientation of the muscle fibers. The terminations of the muscle fibers included in the original ties were found in the opposite tendon sheet, by turning the sample over and exerting tension upon the original ties. Additional ties were placed near these terminations. By exerting tension between ties and dissecting away unwanted muscle tissue, one can isolate a sheet of muscle fibers running from tendon to tendon. With further dissection along natural cleavage planes, this sheet could be divided into from one to four preparations less than 1 mm in thickness, varying from 8.5 to 133 mg (usually between 35 and 50 mg) wet weight. A preparation was judged suitable for study if few cut fibers and no opacity of surface fibers were visible under the dissecting microscope at the end of the dissecting period, which took from 3.5 to 4 hr.

A sample was usually divided into three preparations; one for tracer washout studies, another for measurement of extracellular volume, and the third for electrophysiological measurements. Either or both of the first two were used for determination of ionic content at completion of washout or extracellular volume studies. Upon completion of measurements, either the electrical preparations or "waste muscle" obtained during the fine dissection, or both, were fixed in 10% (v/v) formalin for studies of cell geometry.

Concentrations of Na^+ and K^+ were measured with a Baird atomic KY-1 flame photometer having an internal standard. Concentrations of Cl^- were measured with a Buchler-Cotlove chloridometer. ^{24}Na (Oak Ridge) and ^{42}K (Iso Serve Inc.) were counted in a N. Woods well scintillation counter with a Nuclear Chicago scaler.

^{36}Cl and ^{14}C were counted in stainless steel planchets 1 in. in diameter, after evaporation to dryness, with a Nuclear Chicago low background gas-flow counter. Care was taken to maintain a constant salt content in all planchets and to keep temperatures below 50°C when drying planchets containing ^{36}Cl . All counts had an error of 5% or less.

The goat solution, based on goat serum, had the following composition: NaCl 148 mM, KCl 4.5 mM, CaCl_2 2.0 mM, MgCl_2 1.0 mM, NaH_2PO_4 0.44 mM, NaH_2CO_3 12 mM, and glucose 100 mg %. The pH of the solution when equilibrated with 95% O_2 and 5% CO_2 , at room temperature, was 7.0. Whenever possible, isotope was added in trace amounts; when this was not possible, appropriate manipulations of initial ion content of goat solution were done in order to maintain a constant final concentration of Na^+ , K^+ , and Cl^- .

Resting and action potentials, membrane constants, and membrane excitability characteristics were obtained from individual fibers with KCl -filled glass micropipettes in standard fashion. Stimulation and recording were performed through the same electrode using a bridge circuit; details will be described (7). Membrane resistance was calculated from electrotonic potentials produced by passing constant current pulses of 50 to 200 msec duration both inwardly and outwardly in each fiber studied in this way. The resultant electrotonic potentials averaged ± 2.40 mv (range 0.39 to 9.86 mv). In different fibers the change of potential for a given current was similar and there was little rectification. In a few fibers in which enough observations were made, the relation between current and voltage was linear within 10 to 20%. Calculations of membrane resistance were based upon fiber dimensions determined from histological sections (see below).

Preparations and Washout

Each preparation was tied to a stainless steel spinal needle which served as a carrier and allowed continuous stirring and aeration of solutions. Each needle was fitted with two separate, adjustable, Teflon projections. The preparation was tied to these projections with 36 gauge stainless steel wire hooked through the tendon sheet at either end of the muscle bundle. The preparations were thus exposed to the surrounding medium on all sides, were electrically insulated from the holder, and were maintained at an arbitrarily judged resting tension. The needles with their attached preparations were then transferred to test tubes containing 2 ml of solution and kept at 38°C for 30 min before further experimental manipulations.

Preparations were loaded in radioactive goat solution (always at 38°C). After a preliminary rinse in nonradioactive solution (total time 0.09 to 0.11 min), the washout was conducted in a series of test tubes containing nonradioactive solution at $38 \pm 1^{\circ}\text{C}$ or $25 \pm 1^{\circ}\text{C}$ in a manner previously described (26, 36), except that the radioactivity of the entire test tube was counted and the time intervals of collection were not constant. Upon completion of washout the tissue was blotted and placed in a weighing bottle (containing 3 ml of demineralized, distilled water and preweighed) for determination of wet weight. After weighing, the bottle was shaken overnight. Aliquots of elution fluid from this bottle were taken for determination of the concentrations of Na^+ , K^+ , and Cl^- and of the radioactivity remaining in the tissue. From

this residual radioactivity and the radioactivity present in each washout test tube, the time course of decline of radioactivity from the tissue could be reconstructed (36).

Extracellular Volume and Water Content

Extracellular volume was estimated by placing preparations in goat solution containing D-mannitol-1-¹⁴C, inulin-carboxyl-¹⁴C, or sucrose-¹⁴C (New England Nuclear Corp.) for 240 min at 38°C. After loading, the tissue was handled as described above with a preliminary rinse, and then blotted, weighed, and extracted overnight in demineralized water. An aliquot of elution fluid was taken to determine radioactivity and Na⁺, K⁺, and Cl⁻ concentrations. As described by Weingrad and Shanes (36), 7 mg % penicillin and 15 mg % streptomycin were added to both loading and elution fluids when sucrose was used; in this case, the elution fluid was not suitable for measurement of Na⁺ or K⁺.

Water content was measured in a separate set of experiments, from the same goats used for space and kinetic studies, by weighing preparations (prepared in the same standard fashion and kept in goat solution, at 38°C, for the same periods of time as those used for other studies) before and after drying overnight at 105 to 110°C.

Cell Geometry

Electrical preparations were mounted on small pieces of cardboard and kept at constant length during fixation in 10 % formalin. The cardboard also served as orientation for obtaining true cross-sections during sectioning (oriented sections). Muscle bundles cut away from the preparations during fine dissection were not maintained at constant tension during fixation and were oriented for sectioning by stainless steel insect pins inserted parallel to the fibers (nonoriented sections). Fixed preparations were sectioned by frozen section techniques.

Histological sections of tissues fixed in 10 % formalin and stained with hematoxylin and eosin were projected to a magnification of 800. The ratio of area (measured by planimeter) to perimeter (measured by map reader) of outlines of the projected cell images were taken as the ratio of volume to surface area (V/S) as described by Creese (9). These values were used in calculations of flux and electrical membrane constants.

Calculations

The method of least squares was used to fit a straight line to points on each washout curve that were judged, by eye, to represent the terminal (slow component) phase of washout. The slope of this line was used to calculate a rate constant and time constant of washout. These values, taken directly from the experimental data, were used without correction for the influence of one phase of washout upon the other (16, 21, 22); this introduces some error in absolute values but should not influence the comparison of experimental groups. In the case of Cl⁻ washouts, exponential functions (other than the slowest component) were estimated by means of successive extrapolations to zero time and subtractions of each slower phase from those remaining. These estimates were done by eye.

Intracellular concentrations were calculated from the measured ionic content, the water content, and the extracellular volume (as described by Creese (9), but assuming a specific gravity of 1.0000 for both groups of preparations). Efflux was computed by $M_e = k V/S (X)_i$, where M_e is efflux in $M \times 10^{-12} \text{ cm}^{-2} \text{ sec}^{-1}$, k is rate constant of efflux for that ion species in sec^{-1} , V/S is volume to surface area in cm, and $(X)_i$ is the intracellular concentration (Eq cm^{-3}) of the ion under consideration. The time constant, τ , was derived from the measured rate constant by $\tau = \frac{1}{k}$ for K^+ or by $\tau = \frac{t_{1/2}}{0.693}$ in the case of Cl^- , where $t_{1/2}$ = time for original content to decline to one-half of original value. Influx was calculated by a modification of a method used by Winegrad and Shanes (36). A "space" is defined as (13, 14)

$$\frac{\text{Total cpm in specimen}}{\text{Wet weight (g)} \times \text{cpm per ml of loading solution}}$$

and is an expression of relative specific activity. Results are expressed as mean $\pm$ standard error of the mean unless otherwise stated and Student's t test was used to estimate statistical significance.

RESULTS

1. *Electrical Measurements*

One intention of the present study was to obtain electrical values from fibers which were treated in the same manner, for the same time (4 to 8 hr after removal from the goat), at the same temperature, and in the same solutions as were the preparations used for radioactive ion washouts at the time of measurements. The differences between myotonic and normal preparations reported here are less than those seen in fresher preparations (one-half to one hr after removal from the goat, 7). Repetitive firing upon insertion of the microelectrode into the myotonic fiber was sometimes absent, and resting and action membrane potentials were lower. These differences may be related to the length of time out of the animal.

The results of electrical measurements from muscle fibers of 18 preparations from 5 normal goats and of 24 preparations from 6 goats with hereditary myotonia are summarized in Table I. No striking difference was observed in resting membrane potential between myotonic and normal fibers, nor did temperature influence resting membrane potential. Among all 1301 fibers measured the mean resting membrane potential was 77.6 mv. This includes most fibers (except for the superficial one or two layers) impaled, regardless of potential. Some of these fibers were not excitable or had nonpropagated action potentials, but the data satisfy the usual criteria of satisfactory impalement. We consider this a reasonable estimate of the mean resting membrane potential that was influencing the movements of ions. By being more selective

we would have obtained higher mean resting potentials, but this was not our purpose.

Preparations from goats with myotonia differed from those from normal goats in the following respects: 4 to 5 times greater resting membrane resistance at both 25 and 38°C; longer utilization times for rheobasic stimulation; longer membrane time constants; repetitive firing at currents slightly above rheobase; and overshoot 7 to 8 mv smaller.

2. Extracellular Volume and Water Content

The estimates of extracellular volume obtained after a 240 min equilibration at 38°C with trace amounts of labeled compounds are shown in Table II. There was no significant difference in extracellular volume between normal

TABLE I
ELECTRICAL VALUES DETERMINED IN SINGLE MUSCLE FIBERS
FROM NORMAL AND MYOTONIC GOATS AT 38 AND 25°C

	38°C			25°C		
	Resting potential	Action potential	Membrane resistance	Resting potential	Action potential	Membrane resistance
	mv	mv	$\Omega \cdot \text{cm}^2$	mv	mv	$\Omega \cdot \text{cm}^2$
Myotonic	76 <i>n</i> = 383	92 <i>n</i> = 130	2840 <i>n</i> = 78	78.3 <i>n</i> = 133	97 <i>n</i> = 25	3790 <i>n</i> = 36
Normal	78 <i>n</i> = 595	100 <i>n</i> = 274	727 <i>n</i> = 76	79.2 <i>n</i> = 190	104 <i>n</i> = 81	686 <i>n</i> = 13

n = No. of fibers measured.

and myotonic preparations when compared for the same test substance, but the estimate of extracellular volume varies with the substance used, mannitol providing a smaller volume (29.4 ± 1.1 ml per 100 g wet weight) than sucrose (35.5 ± 3.1) or inulin (37.1 ± 2.1). Neither was a significant difference between normal and myotonic preparations found with respect to water content (Table II); the combined value for both groups was 78.97 ± 0.47 ml of water per 100 g wet weight.

Washout curves with inulin and mannitol were performed. The curves reflected at least two exponential functions, namely, a rapid phase with a time constant of about 2.6 min, and a slower phase with time constants of 38 min and 216 min for inulin and mannitol respectively. Similar curves have been described for sucrose (33, 36) with a time constant of 2.9 and 54 min for fast and slow components, respectively. The slower components for mannitol and inulin (estimated directly from the curves) represent 10 and 30% of the total radioactivity for mannitol and inulin respectively whereas similar values for sucrose of about 25% have been found (33, 36). These results, as well as up-

take experiments in other tissues (4, 30), indicate that mannitol, inulin, and sucrose do not equilibrate with a homogeneous extracellular space. Since mannitol had the smallest slowly exchanging component when extrapolated to zero time, the smallest total space, and the slowest rate of exchange of the slowest component, we regard it as our best estimate of extracellular volume. Except when otherwise noted, intracellular concentrations are based upon the extracellular volume estimated from the combined mannitol space. It should be borne in mind that antibiotics were not used in elution fluids for mannitol or inulin.

TABLE II
EXTRACELLULAR VOLUME AND WATER CONTENT AT
38°C OF ISOLATED EXTERNAL INTERCOSTAL MUSCLE
BUNDLES FROM NORMAL AND MYOTONIC GOATS

	Extracellular volume			Water content
	Mannitol	Inulin	Sucrose	
	<i>ml/100 g wet weight</i>	<i>ml/100 g wet weight</i>	<i>ml/100 g wet weight</i>	<i>ml/100 g wet weight</i>
Myotonic	30.53 ±1.96 <i>n</i> = 8	36.14 ±1.95 <i>n</i> = 8	32.61 ±2.73 <i>n</i> = 4	79.38 ±0.68 <i>n</i> = 8
Normal	28.57 ±1.30 <i>n</i> = 11	39.63 ±6.58 <i>n</i> = 3	38.46 ±5.68 <i>n</i> = 4	78.68 ±0.67 <i>n</i> = 11
Combined Myotonic and normal	29.39 ±1.10 <i>n</i> = 19	37.09 ±2.13 <i>n</i> = 11	35.54 ±3.09 <i>n</i> = 8	78.97 ±0.48 <i>n</i> = 19

For combined results: $P < 0.01$ for mannitol vs. inulin.
 $P < 0.10$ but >0.05 for mannitol vs. sucrose ($t = 1.87$).

n = No. of preparations measured.

Our estimates of extracellular volume are higher than those found in other isolated tissues irrespective of the substance used (10, 22, 25), and so are our values for water content (9, 10). These differences may reflect the fact that our preparations were altered by the fine dissection and were out of the animal for long periods of time before measurement. Regardless of the substance used, the extracellular volume was not great enough to account for the high Na^+ and Cl^- content of the whole tissue (see below), as had been suggested by Creese (10) for human intercostal preparations.

3. Na^+ , K^+ , and Cl^- Concentrations

As a check on our methods, we analyzed pairs of sartorius muscles from 12 September frogs for Na^+ , K^+ , and Cl^- content, with the same techniques that we used for goat muscle. The values determined (Na^+ 25.45 ± 1.12 , K^+ 85.78

± 2.04 , and Cl^- 23.31 ± 0.87 mEq/kg wet weight) approximate those of Kimizuka and Koketsu (24) (Na^+ 26.40, K^+ 80.20 mEq/kg wet weight).

Table III contains our results from goat external intercostal muscles and results from human intercostal and rat diaphragm preparations from the lit-

TABLE III
CONCENTRATIONS OF Na^+ , K^+ , AND Cl^- IN ISOLATED
EXTERNAL INTERCOSTAL MUSCLE BUNDLES FROM NORMAL AND MYOTONIC
GOATS (COMBINED VALUES FROM PREPARATIONS AT 38 AND
25°C) COMPARED WITH VALUES IN OTHER ISOLATED MAMMALIAN
SKELETAL MUSCLE PREPARATIONS

	Total content			"Intracellular" concentrations					
	I			II			III		
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
	mEq/kg wet weight			mEq/liter fiber water			mEq/liter fiber water		
Myotonic	101.01 ± 3.94 $n = 50$	79.19 ± 1.99 $n = 50$	68.18 ± 2.09 $n = 50$	107.5	159.7	41.6	100.2	180.8	25.6
Normal	91.55 ± 3.42 $n = 31$	68.93 ± 1.58 $n = 31$	70.02 ± 2.15 $n = 31$	90.9	133.5	49.7	78.1	156.9	29.9
<i>P</i> for normal vs. myotonic	0.10 > <i>P</i> > 0.05	<0.01	>0.50						
Isolated rat diaphragm (9)	—	—	—	—	—	—	60	149	—
Isolated human intercostal (10)	117.2	62.3	—	—	—	—	—	—	—

n = No. of preparations measured.

erature. Since extracellular volume and water content of normal and myotonic preparations were indistinguishable (Table II), we used the common values for calculation of intracellular concentrations. The extracellular space measured by mannitol (29.39 ml/100 g wet weight) and a water content of 78.97 ml/100 g wet weight were used in column II of Table III and in all subsequent calculations. Since mannitol space may represent only a lower limit of extra-

cellular space, we have included in column III of Table III intracellular concentrations based on a common combined inulin and sucrose space to indicate the possible range of intracellular concentrations. It is clear that goat external intercostal muscle has higher Na^+ and Cl^- content than frog sartorius (see above) but is much like human external intercostal in this respect. The calculated intracellular Na^+ concentration is higher than that of rat diaphragm.

The most striking group difference is in K^+ content of normal and myotonic muscle ($P < 0.01$). The intracellular concentration (based on mannitol) is about 25 mEq/liter of fiber water higher in myotonic than in normal muscle. Initial K^+ contents have not been determined and it is possible that the K^+ content at the time of biopsy was the same in both groups of preparations. Although there is a higher mean Na^+ concentration in myotonic muscle, the difference from normal muscle is not statistically significant. Since intracellular Cl^- concentrations are about equal, it would appear that, at the times of our measurements, there is an excess of some other anion in myotonic muscle. If this anion (or anions) and the Na^+ and K^+ are free and osmotically active, the osmolarity of myotonic fibers should be 86 milliosmols greater than in normal muscle. Indirect evidence that osmotic factors may play a role in the pathophysiology of myotonia stems from observations by Hegyeli and Szent Györgyi (17) (confirmed in our laboratory) that the overt manifestations of myotonia in the intact animal disappear during water deprivation.

4. Fiber Geometry

The frequency distributions of volume to surface area (V/S) measurements of 845 muscle cells (489 myotonic and 356 normal) in cross-sections of muscle bundles from 11 myotonic and 7 normal goats are shown in Fig. 1. These data are from nonoriented sections. Although they consistently gave ratios about 1.45 times those obtained from oriented preparations, the number of fibers measured is large enough to indicate that the populations of fibers in the two groups of animals are similar. Plotted in a different way the data have a bell-shaped distribution, and they show no tendency toward bimodal distribution in either group of animals. Such a comparison is not available in the literature at the present time. The weights of myotonic goats ranged from 16 to 24 kg with an average of 19.8 kg, whereas those of the normal goats ranged from 22 to 63 kg with an average of 38.5 kg. Thus, our myotonic animals have a larger fiber size relative to their body weight than do normal goats. In absolute terms, however, myotonic fibers are slightly smaller than normal fibers (see below).

For calculations of ionic flux and core-conductor properties 215 normal and 178 myotonic cells were measured from oriented sections. The dimensions calculated from these sections were in closer agreement with diameter measurements of unfixed fibers, made by micrometer (at a magnification of 80) at the time of electrical measurements, than were the dimensions calculated from

unoriented sections. The mean V/S from oriented sections was 8.09μ for myotonic and 8.43μ for normal muscle fibers.

5. K^+ Washout and Uptake

Two comparisons of normal and myotonic preparations were performed; those loaded for 200 min with ^{42}K at $38^\circ C$ and washed out at $38^\circ C$ and, those loaded for 200 min with ^{42}K at $38^\circ C$ and washed out at $25^\circ C$. There was little difference in relative specific activity at the beginning of the washouts as expressed by the total ^{42}K space either between the two temperatures or between normal and myotonic preparations. Fig. 2 A and B show the average time course of decline of radioactivity for each group at each temperature. After the first 30

FIGURE 1. Cumulative frequency distributions of an estimate of fiber diameter in muscle cells of nonoriented, formalin-fixed bundles from normal and myotonic goats. V/S , volume to surface ratio. Number of fibers studied, myotonic 489; normal 356.

min of washout there was no deviation from single exponential kinetics in any of the K^+ washouts performed. Initial deviation from the final rate of exit has been discussed by Sjodin and Henderson (34) and is, in part, a function of the extracellular space. In our data the zero time intercept of the slow component is not a function of temperature, but at $38^\circ C$ the time constant of efflux is longer in preparations from normal goats than in those from goats with hereditary myotonia whereas at $25^\circ C$ this relationship is reversed.

The computed values for efflux of K^+ and for the time constant of efflux (τ) for each experiment are shown in Table IV. Preparations with the same date are from the same biopsy and those with different dates are from the same goat but on different days and consequently different biopsies. Each preparation was assigned equal significance in arriving at the mean values in the table and in statistical testing. Washouts after 10 min loading are included in the table

but are not part of means expressed in Fig. 2. As a consequence of the higher intracellular K^+ concentration in myotonic preparations, the K^+ efflux in myotonic preparations was indistinguishable from that in normal preparations at 25°C even though the time constant of efflux was longer in the myotonic group. If the paired data are considered (values at 38° and 25°C from the same animal, but different biopsies), a Q_{10} of efflux may be calculated for each group. The Q_{10} is 1.85 ± 0.03 for myotonic preparations and $1.43 \pm$

FIGURE 2. Average time course of washout of ^{42}K in isolated external intercostal muscle from normal and myotonic goats. (A) loaded for 200 min at 38°C and washed out at 38°C. (B) loaded for 200 min at 38°C and washed out at 25°C. (C) one preparation loaded for 10 min at 25°C and washed out at 25°C.

0.14 for normal preparations ($P = 0.05$). On this basis K^+ efflux may be more sensitive to temperature in myotonic than in normal fibers.

Estimates of ^{42}K influx were made in one myotonic and one normal goat. Two preparations from each goat were exposed to ^{42}K for 10 min at 25°C and washed out at 25°C. A washout curve from one myotonic preparation is shown in Fig. 2 C. The zero time intercept of the slow component was taken as the intracellular ^{42}K (36) and used to estimate the influx. The average influx from the two myotonic preparations was 5.44×10^{-12} Eq $cm^{-2} sec^{-1}$ (4.30 and 6.57) and from the two normal preparations was 3.61×10^{-12} Eq $cm^{-2} sec^{-1}$ (3.50 and 3.72); this gives a grand average of 4.52×10^{-12} Eq $cm^{-2} sec^{-1}$, the same as the average efflux (4.75×10^{-12} Eq $cm^{-2} sec^{-1}$) at 25°C obtained from

TABLE IV
K⁺ EFFLUX IN ISOLATED EXTERNAL INTERCOSTAL MUSCLE BUNDLES
FROM NORMAL AND MYOTONIC GOATS, LOADED WITH ⁴²K AT 38°C FOR
200 MIN AND WASHED OUT AT 38°C (A) OR 25°C (B)

Myotonic						Normal					
Goat identification No. and date of preparation		τ^* Slow com- ponent	V/S	Efflux	Goat identification No. and date of preparation		τ^* Slow com- ponent	V/S	Efflux		
		<i>min</i>	μ	$Eq\ cm^{-2}$ $s^{-1} \times 10^{-12}$			<i>min</i>	μ	$Eq\ cm^{-2}$ $s^{-1} \times 10^{-12}$		
A 38°C	2-36M	5/6/64	183		11.66	2-48N	12/3/63	244		8.54	
		5/6/64	188	8.01	11.34		12/18/63	171	9.36	12.17	
	2-27M	11/12/63	211	8.15	10.28	2-45N	11/8/64	228	9.62	9.45	
	2-19M	11/14/63	198	7.61	10.23	2-46N	12/4/63	260	6.91	5.91	
	2-43M	11/13/63	209	9.57	12.19	2-49N	12/5/63	261	6.55	5.58	
	Mean		197.8		11.70	Mean		232.8		8.33	
	SEM		±5.46		±0.38	SEM		±16.44		±1.21	
	2-36M	4/8/64	512	8.01	4.16	2-48N	1/23/64	407	9.36	5.12	
		5/6/64	365		5.84	2-45N	2/19/64	417	9.62	5.09	
			1/21/64	568		3.82	2-46N	3/4/64	343	6.91	4.48
B 25°C	2-27M	5/5/64	403	8.15	5.38		4/1/64	334		4.36	
		5/5/64	374		5.79	2-49N	6/2/64	394	6.55	3.70	
			3/18/64	457		4.14		6/2/64	353		4.12
	2-37M	6/3/64	384	7.11	4.93	2-14N	4/22/64	423	9.70	5.10	
		6/3/64	391		4.85						
	2-19M	3/5/64	406	7.61	4.99						
	2-43M	2/18/64	467	9.57	5.45						
	Mean		432.7		4.94	Mean		381.6		4.57	
SEM		±21.08		±0.22	SEM		±14.17		±0.21		

* τ , slow component = time constant of efflux (min).

τ , P for myotonic vs. normal at 38°C = (0.10) 0.05 ($t = 1.99$), at 25°C = (0.10) 0.05 ($t = 2.01$).
Flux, P for myotonic vs. normal at 38°C = (0.05) 0.02 ($t = 2.65$) at 25°C = >0.20.

Table IV. This agreement may be fortuitous. We suspect that, as in most isolated tissues, the efflux of K⁺ is greater than the influx and that there is a slow net loss of intracellular K⁺ in both groups of preparations.

6. Cl⁻ Washout

The average concentrations of total Cl⁻ in the preparations, determined by total ³⁶Cl content after equilibration, were 75.76 mEq/kg and 62.76 mEq/kg

wet weight for myotonic and normal preparations, respectively. These values are within 10% of the values for total Cl^- determined by the chloridometer (Table III) and indicate that probably all of the Cl^- in the preparations was exchangeable. The small differences might be due to unavoidable variations in the time of the preliminary rinse. Such errors should not influence the shape of the washout curve.

Fig. 3 A shows the average time course of decline of ^{36}Cl from preparations loaded for 200 min at 38°C and washed out at 25°C . These curves represent 5 myotonic goats (7 preparations) and 5 normal goats (6 preparations). The

FIGURE 3. Average time course of decline of ^{36}Cl in isolated external intercostal muscle bundles from normal and myotonic goats. (A) loaded 200 min at 38°C , washed out at 25°C . (B) Typical discontinuous washout.

averaged curves resemble the individual curves. A qualitative difference between the washout curves of normal and myotonic preparations is present, and suggests that a component of ^{36}Cl washout is present in myotonic preparations that cannot be detected in most normal preparations.

Several Cl^- washout curves showed discontinuities of washout in both normal and myotonic preparations; a typical curve is shown in Fig. 3 B. These unusual curves, which did not occur with either Na^+ or K^+ washouts, had several common characteristics. The sudden jump never occurred until at least 50 min of washout were completed. The rate of decline of radioactivity with time in the component before the discontinuity (identified as component d in the following analysis and in Table V) was considerably slower than that observed in the final component (d) of any curve without the discontinuity. The sudden jump was produced by a large amount of radioactivity being present in one

or, at the most, two collection periods and was unrelated to any obvious change in the preparation or to any obvious experimental manipulation. The curves up to the point of rapid egress (disregarding the portions of the curves after the discontinuity) were not distinguishable, with respect to the number of extractable components, from other curves and have been included in the analysis that follows. The final rate of decline of radioactivity in these discontinuous curves did not follow a consistent pattern.

TABLE V
TIME CONSTANTS OF COMPONENTS EXTRACTABLE FROM ^{35}Cl
WASHOUT CURVES IN ISOLATED EXTERNAL INTERCOSTAL MUSCLE
BUNDLES FROM NORMAL AND MYOTONIC GOATS

Preparations were loaded with ^{35}Cl for 200 min at 38°C and washed out at 25°C.

Myotonic					Normal						
Goat identification No. and date of preparation		τ , min				Goat identification No. and date of preparation		τ , min			
		a	b	c	d			a	b	c	d
2-27M	3/30/64	0.51	2.23	10.82	72.03	2-46N	3/27/64	0.58	2.60	—	87.58
	2/14/64	0.50	2.96	11.83	54.82		5/15/64	0.80	2.31	—	64.07
2-19M	4/10/64	0.29	1.73	6.29	51.47	2-49N	2/17/64	0.50	2.95	7.35	94.37
	5/12/64	0.29	1.37	4.85	39.59		3/6/64	1.05	3.47	—	74.04
2-36M	2/21/64	0.58	2.67	12.27	67.51	2-14N	4/3/64	0.72	3.13	—	103.30
2-37M	2/25/64	0.60	2.96	13.98	76.24	2-45N	4/24/64	1.01	4.32	—	68.70
2-43M	5/13/64	0.51	3.10	12.05	56.80						
2-43M	5/1/64*	0.58	3.25	16.23	205.33	2-45N	3/23/64*	0.21	1.51	—	125.26
	5/13/64*	0.87	3.10	17.53	276.40		5/15/64*	1.01	4.40	—	193.70
Mean		0.47	2.43	10.29	59.78	Mean		0.78	3.13	—	82.01
SEM		±0.05	±0.25	±1.28	±4.86	SEM		±0.09	±0.29	—	±6.32

* These preparations had "discontinuous" washout curves and are not included in the means.

Despite the hazards and inadequacies (16, 21, 22, 35), it is convenient to assume, for the purposes of a qualitative argument, that each of the components of the washout curve is related to one discrete compartment of Cl^- in the preparations and that the observed rate constants are related to the rate constants of these compartments. The components extracted from each washout curve, by consecutive extrapolation to zero time, are shown in Table V. The slowest component extracted is labeled "d" and the fastest "a." An intermediate exponential component (c) is seen in 9 out of 9 myotonic preparations but in only 1 of 8 normal preparations. This confirms the impressions obtained

from the curves and establishes orders of magnitude of the various rate constants involved.

7. Na^+ Washout

Because of the very large metabolically linked Na^+ movements relative to the passive fluxes (32), we considered it unlikely that a major difference in the Na^+ flux would be found between the normal and myotonic preparations. One determination of Na^+ efflux was made in preparations from each of 2 myotonic goats and 2 normal goats loaded and washed out at 38°C . Three exponential functions were extractable. The average time constant of the slowest component was 58.1 min in the normal and 49.84 min in the myotonic. From these we calculate values for Na^+ efflux of 32.4 and $30.6 \times 10^{-12} \text{ Eq cm}^{-2} \text{ sec}^{-1}$ at 38°C for normal and myotonic preparations, respectively. Creese found a Na^+ efflux of $28 \times 10^{-12} \text{ Eq cm}^{-2} \text{ sec}^{-1}$ at 38°C in isolated rat diaphragm (9).

DISCUSSION

A. General

In order to compare typical muscle fibers from normal goats and goats with hereditary myotonia, we thought it necessary first to find consistent differences between the two groups of animals, even if only in a qualitative sense. Primary attention has been paid to this orientation in experimental design. Quantitative precision is impossible in a multicellular preparation variable in its thickness (and hence in diffusion characteristics), made up of cells of different size and physiologic status, and with a variable extracellular space difficult to estimate.

Each goat used from the myotonic stock exhibited clinical myotonia, but no effort was made to select animals on the basis of severity of myotonia as exhibited clinically or electrophysiologically. The results from all preparations studied are reported, provided they met our criteria of satisfactory dissection. Any differences between the two groups of animals were not influenced by selection of either the clinical material or the data. Yet, since normal goats were procured from local sources and goats with hereditary myotonia were obtained from Alabama, it is possible that the differences observed are related to some factor inherent in the two strains other than myotonia. Furthermore, the severity of myotonia exhibited electrophysiologically seemed to decrease with long times out of the animal, and the changes underlying this phenomenon may be different in normal muscle.

The preparations studied remained functional as long as 18 hr after removal from the animal, as evidenced by maintenance of twitch, propagated action potentials, resting potentials near -80 mv in the majority of fibers impaled, and intracellular K^+ concentrations comparable to those of other isolated mus-

cle systems. There are discrepancies, however, between measured and calculated potentials. Equilibrium potentials calculated by $E_K = -61.73 \times \log (K^+)_i / (K^+)_o$ are -91.1 and -95.6 mv for myotonic and normal preparations, respectively; but the observed grand average resting potential was -77.6 mv. Similarly, calculated E_{Na} is 11 and 15 mv whereas the observed overshoots are 12 and 19 mv for myotonic and normal preparations, respectively. Among fibers that manifested propagated action potentials or showed overshoots of 8 mv or more, or both, the resting potential observed was -83 and -86 mv for myotonic and normal preparations, respectively. These considerations indicate that there was some injury and depolarization presumably due to the dissection or to hypoxia resulting from excessive thickness of the preparations. This factor undoubtedly has a quantitative influence on the matters to be discussed, but we cannot estimate the degree of inaccuracy introduced. These problems are not unique to our system (5), nor do they detract from our conclusions.

TABLE VI
PERMEABILITY TO POTASSIUM (P_K) AND
CHLORIDE (P_{Cl}) IN ISOLATED MUSCLE

	P_K		P_{Cl}
	38°C	25°C	25°C
	<i>cm · sec⁻¹ × 10⁻⁶</i>		
Normal goat intercostal muscle	0.365	0.219	>0.824
Myotonic goat intercostal muscle	0.492	0.199	0.413
Normal rat diaphragm (25)		0.60	
Denervated rat diaphragm (25)		0.31	

We are aware of two studies with intracellular microelectrodes in human myotonia congenita. In one (31) there was no difference in resting membrane potential between normal and myotonic fibers in 5 patients. In the other (29), the records indicate spontaneous repetitive activity in myotonic muscle. In our experience, this is a sign of excessive injury due to impalement or dissection, and we believe this accounts for the low membrane potentials reported. Both results are therefore consistent with our findings, in goats, that there is no significant difference in resting membrane potential between normal and myotonic fibers.

B. Permeability to K^+

The permeability of the muscle membranes to K^+ is expressed by (1, 18)

$$P_K = M_K \frac{RT}{VF} \frac{\frac{VF}{eRT} - 1}{[K]_i \left(\frac{VF}{eRT} - [K]_o \right)}$$

Introducing into this formula our values for flux (Table IV), resting membrane potential (section 1), and internal concentration of K^+ (Table III), we find the values shown in Table VI. A greater decrease in P_K in myotonic preparations than in normal preparations is apparent upon cooling from 38 to 25°C. The ratio of P_K myotonic to P_K normal at 25°C is 0.91, whereas at 38°C the ratio is 1.35. Our values for P_K are considerably less than the value ($1 \text{ to } 2 \times 10^{-6} \text{ cm sec}^{-1}$) obtained for frog sartorius by electrical means, but permeabilities calculated from flux data are usually less than those calculated from electrical data (1, 3, 22). Our values for P_K are somewhat less than those calculated for rat diaphragm (25) from flux data at 25°C.

C. Permeability to Cl^-

Generally, one assumes that the slowest component of washout curves is related to the intracellular space. In our data, however, there is a wide discrepancy between the intracellular Cl^- calculated from the intercept of the slowest component (d) at zero time (without correction factors) and the total radioactivity after equilibration, and that calculated from total Cl^- measured by the chloridometer and estimates of extracellular volume. The washout data lead to 1.27 and 2.0 mEq/kg of fiber (1.68 and 2.3% of total radioactivity) for normal and myotonic preparations, respectively, and this is only $\frac{1}{25}$ of that estimated by the chloridometer and extracellular volume (Table III). Furthermore, in additional experiments the zero time intercept did not vary as a function of the time of exposure to ^{36}Cl . Adrian (2, 3) also found low values of intracellular Cl^- in frog sartorius when he estimated them in long washout experiments and concluded, as we do, that the slowest component (d) cannot be interpreted as washout of the intracellular phase.

The first two components (a and b) isolated from our Cl^- washout curves, from either normal or myotonic preparations, have time constants of 3 min or less. Components with time constants of magnitude equal to or faster than that of sucrose or inulin (2.6 min in our hands) are generally assumed to reflect diffusion from the extracellular space rather than membrane-limited processes. Thus, without other measurements which would lend themselves to multi-compartmental analysis, components a and b in our curves cannot be readily interpreted as reflecting washout from the intracellular space. Yet the time constants of phases a and b in the normal preparations (excluding the one with an intermediate component) are longer (i.e., reflect slower rate of washout) than those in the myotonic preparations, and in the normal component c cannot be isolated. This suggests that component c may be contained in a and b in washouts from normal muscle. In any case in most of our normal preparations components a, b, and d probably do not represent the intracellular space, and hence intracellular washout cannot be differentiated from extracellular washout. Adrian (2, 3) reached the same conclusion from long washout experi-

ments in normal frog sartorius muscle. On the other hand the intermediate component (c) isolated from myotonic preparations and one normal preparation has an average zero time intercept of 17.1% of the total ^{36}Cl . This corresponds to 11.76 mEq/kg of fiber or, when corrected for fiber water, 26.1 mEq/liter of fiber water. This value is closer to that measured by total Cl^- and extracellular volume than is the value calculated from component d. Component c is therefore the most likely component to account for movement from an intracellular compartment. The fact that this intermediate component is present in all myotonic preparations studied and in only one of 8 normal preparations supplies a qualitative ionic confirmation of the previously proposed (6) decreased membrane permeability to Cl^- in myotonic fibers.

The foregoing arguments are strengthened by two further observations. The frequency distribution of cell dimensions in the two sets of preparations (Fig. 1) shows no significant differences between dispersions of fiber size. Thus the qualitative difference between the two Cl^- washout curves cannot be explained on the basis of a differing distribution of fiber size, which if present, could give similar results. If component c is present in normal preparations but not distinguishable from a and b, then its influence on washout later in time (its influence on component d) should be small and one would expect that the estimated component d in normal preparations would have a longer time constant (i.e., reflect slower rate of washout) than that of myotonic preparations, where component c occurs late enough in time to influence component d. This is what was found, the time constants for component d being 82 and 60 min for normal and myotonic preparations, respectively.

Assuming that the intermediate component (c) of the myotonic curves represents a portion of the Cl^- washout related to intracellular Cl^- , the resting efflux of Cl^- in myotonic preparations may be calculated as $56.69 \pm 7.63 \times 10^{-12}$ Eq $\text{cm}^{-2} \text{sec}^{-1}$. Since the intracellular Cl^- concentration appears to be the same in normal and myotonic preparations (Table III), and since the intermediate phase of washout cannot be detected in normal preparations, the efflux in normal preparations must be greater than in myotonic preparations. We estimate the Cl^- efflux in normal preparations to be two or more times greater than in myotonic preparations since, if it were less, an intermediate component should have been evident in normal washout curves as well as in myotonic washout curves.

The intracellular Cl^- concentration found in our preparations presents major problems to formal analysis. The chloride potential, calculated from $E_{\text{Cl}} = 61.73 \log (\text{Cl})_o/(\text{Cl})_i$, is -35.8 and -31.0 mv for myotonic and normal preparations, respectively. The potentials are far from the observed grand mean of -77.6 mv and even farther from the best resting potentials of -83 to -86 mv. An intracellular concentration of Cl^- of 8 mEq/liter fiber water

or less would be required to satisfy a Donnan or Nernst relationship. For this concentration to exist in our preparations would require a 30% error in Cl^- analysis or an extracellular volume greater than 41 ml/100 g wet weight. Neither of these possibilities seems likely. It could depend upon a nonionic intracellular chloride of about 40 mEq/liter fiber water. In our preparation Cl^- does not appear to be passively distributed between intracellular and extracellular water simply as a function of potential; the portion of total Cl^- which is identifiable as intracellular on the basis of chemical and extracellular volume measurements and wholly (or nearly so) exchangeable on the basis of radioisotope analysis is not fully correlated with the electrical properties of the membrane. Similar results have been found in taenia coli (4, 12) and, more convincingly, in squid axon (23), where analysis of the extruded axoplasm obviates calculations based on measurement of extracellular volume. We would anticipate that, as further isolated tissues other than frog sartorius (1, 3, 15) are examined, similar problems of Cl^- distribution will be encountered.

Since the observed intracellular Cl^- concentrations are high, a formal expression of permeability to chloride cannot be calculated from (1)

$$P_{\text{Cl}} = M_{\text{Cl}} \frac{RT}{VF} \frac{e^{\frac{VF}{RT}} - 1}{[\text{Cl}]_i (e^{\frac{VF}{RT}} - [\text{Cl}]_o)}$$

because of the concentration terms in the equation. Kimizuka and Koketsu (24) derived a modification of the Goldman equation which allows a calculation of P_{Cl} without a concentration term, namely,

$$\frac{P_{\text{Cl}}}{P_{\text{K}}} = \frac{\tau_{\text{K}}}{\tau_{\text{Cl}}} e^{\frac{VF}{RT}}.$$

We use this equation on the assumptions that there is a fraction of the total Cl^- which does interact with the electrical properties of the membrane in predictable fashion, that the rate constant of that fraction is represented by component c of our washout curves, and that we cannot identify this fraction by concentration and space studies. From the data from Tables IV and V, $P_{\text{Cl}}/P_{\text{K}} = 2.07$ for myotonic and ≥ 3.76 for normal preparations at 25°C, and from P_{K} (see above), $P_{\text{Cl}} = 0.413$ and 0.824 (or greater) $\times 10^{-6}$ cm sec $^{-1}$ for myotonic and normal fibers respectively at 25°C (Table VI). These values for P_{Cl} are in agreement with the generally accepted view that the Cl^- conductance in resting skeletal muscle is a major portion of the total resting conductance (20). The finding of a lower value in myotonic muscle is consistent with the hypothesis that the major abnormality in skeletal muscle from goats with hereditary myotonia is a decreased permeability to Cl^- (6, 7).

D. *Myotonic vs. Normal Muscle*

Although our results are not satisfying in a quantitative sense, they offer ample evidence that skeletal muscle of goats with hereditary myotonia differs from that of normal goats in an increased resting membrane resistance (or conversely a decreased resting membrane conductance), a decreased permeability to Cl^- , and probably some decrease in permeability to K^+ . The electrical measurements revealed that the resting membrane conductance of myotonic muscle is 4 times that of normal muscle at 38°C (Table I). This difference represents differences in one or more of several ionic conductances. At 25°C the conductances (permeabilities) of normal and myotonic membranes to potassium are almost the same (Table VI). The differences in conductance to chloride at 25°C calculated in section C (Table VI) are in the same direction as the changes of electrical conductance, but the twofold change in P_{Cl} that we are able to deduce accounts for less than half of the latter. Permeabilities from flux data are usually smaller than those from electrical data; furthermore, the difference in P_{Cl} may in fact be greater than we were able to find. For example, we estimated (section 6) in the washout curve of normal muscle, that a rate constant of Cl^- efflux double that of myotonic muscle would prevent us from detecting component c, to which we attribute the membrane permeability to Cl^- ; but the normal rate constant might be much more than double that of myotonic muscle.

The possible elevation of osmolarity in myotonic muscle suggested in section 3 may be related to the fact that myotonia decreases when the animals are deprived of water.

The difference in P_{K} 's at 25 and 38°C (Table VI) may be related to the increase in myotonia that occurs when the animals are exposed to cold. In a few measurements of Cl^- washout at 38°C , the differences between normal and myotonic muscle observed at 25°C were absent. This finding suggests a temperature sensitivity greater in myotonic muscle than in normal muscle for Cl^- as well as K^+ , but it is equally possible that the differences in Cl^- washout curves at these 2 temperatures depend upon changes in extracellular diffusion rather than upon membrane permeability.

The few quantitative cellular measurements in human congenital myotonia (29, 31) and our current and previous (6) electrical measurements suggest that hereditary myotonia in goats is a suitable experimental model for human congenital myotonia.

It seems clear that repetitive activity in skeletal muscle can result from at least three fundamentally different mechanisms: an increased resting membrane resistance predominantly due to a decreased P_{K} , as in denervated skeletal muscle (19, 25, 28); depolarization, low intracellular K^+ , and probably

increased P_K , as in muscular dystrophy of mice (8, 11, 27, 37); and an increased membrane resistance predominantly due to a decreased P_{Cl} in myotonic muscle of goats.

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