Cat Heart Muscle *in Vitro*

III. *The extracellular space*

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ABSTRACT The "osmotic gradient" method, an intracellular microelectrode technique for determining whether an uncharged, water-soluble molecule enters cells or remains extracellular, is described. Using this method, a series of carbohydrates of graded molecular size were examined. In cat papillary muscles mannitol, molecular radius 4.0 Å, remained extracellular while arabinose, molecular radius 3.5 Å, entered the cells. Measurement of the simultaneous uptake of H^3-mannitol and C^{14}-inulin showed that mannitol equilibrates with 40 per cent of total water in 1 hour, after which the mannitol space does not further increase. By contrast, inulin, molecular radius ~15 Å, equilibrates with 24 per cent of total water in 1 hour; thereafter the inulin space continues to increase very slowly. The intracellular K concentrations are significantly higher and the intracellular Na and Cl concentrations significantly lower when mannitol rather than inulin is used to measure the extracellular space. The intracellular Cl concentration determined with Cl^{36} or Br^{82} is significantly higher than that calculated from the membrane potential assuming a passive Cl distribution. In addition, it is shown that choline enters and is probably metabolized by the cells of papillary muscle.

INTRODUCTION

The extracellular space of heart muscle is generally considered equivalent to the interfiber space. Although this compartment may contain connective tissue and vascular elements, it is regarded as possessing no detailed structure. In the interpretation of *in vitro* experiments it is usually assumed to be continuous with the bathing medium and bounded by a well defined cell membrane or sarcolemma. The work to be reported in this paper leads to the conclusion that this rather simple model of the extracellular space is inadequate.

A new criterion of extracellularity, based on the intracellular microelectrode technique, was used for determining whether a molecule enters cells at an appreciable rate or is effectively excluded from the cytoplasm by the cell membrane. The results of this method, to be referred to as the osmotic gradi-

ent method, led to an analysis of the simultaneous uptake of mannitol and inulin by the extracellular space of quiescent cat papillary muscle. This analysis indicates that this space is not a homogeneous compartment. A portion of the extracellular compartment was observed to equilibrate very slowly with inulin. It is suggested that this portion of the extracellular space, although separated from the cytoplasm by the cell membrane, may be located within rather than between the muscle fibers. In addition, it will be shown that the magnitude of the calculated intracellular ion concentrations depends critically on the size of the molecule used to measure the extracellular space. Abstracts of this work have been previously published (1–3).

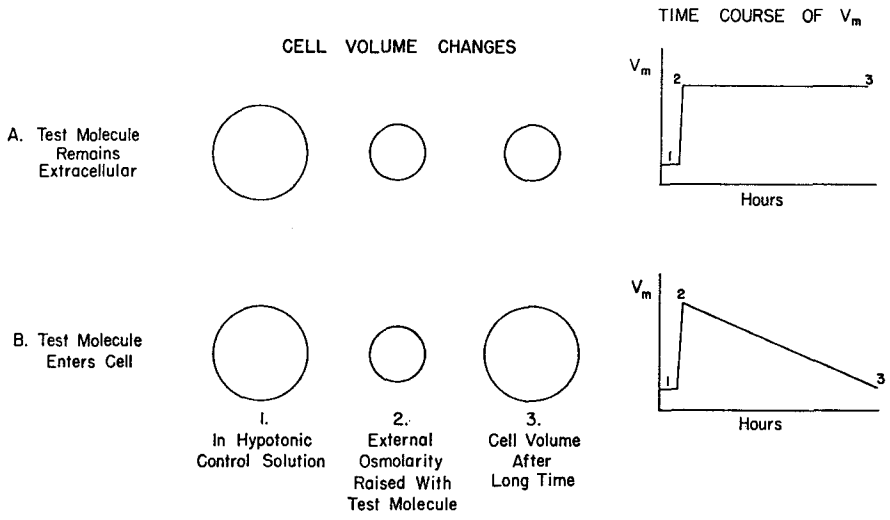

FIGURE 1. The osmotic gradient method.

METHODS

Principle of the Osmotic Gradient Method

Fig. 1 illustrates the use of the osmotic gradient method to determine whether an uncharged, water-soluble carbohydrate molecule remains extracellular or enters the cells. The tissue is preincubated in a hypotonic high K solution in which Cl is replaced by an impermeant anion, thereby causing the cell to lose its diffusible Cl. The osmolarity of the bathing medium is then raised by addition of the carbohydrate molecule to be tested, all other constituents of the medium being held constant. This increase in extracellular osmolarity causes a rapid movement of water out of the cells, which shrink as shown schematically in the figure. The cellular water loss results in a rise in the intracellular K concentration, $[K]_i$. Since the experiments are carried out at constant external K concentration, $[K]_o$, the increase in the ratio $[K]_i/[K]_o$ causes a rise in V_m (Fig. 1) provided $[K]_o$ is sufficiently high for the membrane to behave, to a first approximation, like a K electrode. When the test molecule pene-

trates very slowly, this elevation in V_m persists for several hours. If the test molecule enters the cell more rapidly, V_m, after an initial rise, falls toward the control (hypotonic) level. The rate of fall depends on the rate of entry, if the perfusion is efficient.

A variant of this method may be used to test for anion permeability. In this case the tissue is preincubated in a hypotonic high K solution in which the anion to be tested replaces Cl. The osmolarity of the bathing medium is then raised by addition of a carbohydrate molecule known to be impermeant. The resultant rise in intracellular concentrations disturbs the relation $[K]_i[X]_i = [K]_o[X]_o$ in which X denotes a passively distributed anionic species. If X has entered the cell during the preincubation period, the increase in the product $[K]_i[X]_i$ will give rise to the net ion movements described by Boyle and Conway (4): KX will diffuse out until $[K]_i$ returns to its control level. There will therefore be no *sustained* elevation in V_m. If, on the other hand, X has not penetrated into the cell during the preincubation period, there will be no mobile intracellular anion to accompany an outward movement of cellular K when the external osmolarity is raised. The result is a sustained rise in $[K]_i$ and hence in V_m. In this latter connection it is of interest that Adrian (5) and Hodgkin and Horowicz (6) were able to raise $[K]_i$ and V_m in SO_4 solutions whose osmolarity was varied with sucrose.

Experimental

Cats were anesthetized with pentobarbital and papillary muscles were dissected as previously described (7). For the purpose of measuring V_m, the microelectrode technique and the chamber illustrated in the preceding paper were used (8). In some experiments muscles were perfused in the apparatus of Page and Solomon (7). Other experiments were carried out in the special chamber described below. All observations were made at 26.5–28°C. Muscles were quiescent in all experiments except those with Br^{82}, in which they were stimulated to contract semiisometrically at a rate of 20/minute.

Fig. 2 shows the apparatus designed for equilibration experiments in 1 ml of bathing medium. This small volume, which was in all cases at least 300 times greater than the volume of the extracellular space, made possible high specific activities for radioactive tracers used in experiments involving simultaneous assays of two or three labeled substances. Experiments with labeled mannitol to check on the possibility of evaporation of water showed no detectable increase in concentration of tracer in 1 hour.

In addition to the experiments with the osmotic gradient method a series of determinations of intracellular concentrations and extracellular spaces were carried out either in the perfusion apparatus of Page and Solomon (7) or in the apparatus for incubation in small volumes. These experiments, the results of which will be described in detail in the next section, are summarized for convenience in Table I. In all cases muscles were preincubated in non-radioactive bathing solution for 1 hour. Special precautions were taken to avoid handling, squeezing, or desiccating the tissue, or exposing it to anoxic conditions, since such procedures were found to produce inordinately large extracellular spaces. The mean muscle radius, measured with a measuring telescope in two perpendicular planes at multiple points on the axis of the cylinder, was 0.55 ± 0.02 mm.

Total water, Na, and K were determined as previously described (7), except that an electrobalance (Cahn, model M-10) was used to weigh the tissue. Extracellular spaces were measured by assay of an 0.5 ml aliquot of the 0.1 N HNO_3 used to extract the muscle. Radioactivity was assayed in a liquid scintillation counter (Packard, tricarb model 314-DC) using the scintillation fluid described by Herberg (9). This procedure gave a reproducibility of 1.1 ± 0.6 per cent for ten consecutive paired samples (99 per cent confidence limits, Student *t* test). For simultaneous measurement of H^3 and C^{14} on the same 0.5 ml HNO_3 extract the "discriminator ratio" method of Okita *et al.* (10) was employed. This method gave recoveries of 98 to 102 per cent for

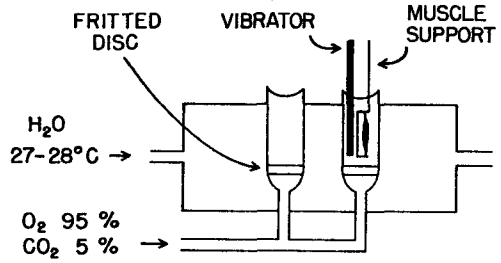

FIGURE 2. Apparatus for perfusion of papillary muscles in 1 ml of fluid. The experimental vessels were 2 ml funnels (Misco No. 7494), the floor of which consisted of a fritted glass disc of medium porosity. The muscle was held in the bath between two perforated horizontal projections on a vertical silver rod to which it was tied with silk. The muscle holder could be transferred at will from the dissection chamber into the incubation vessel and from one incubation vessel to another. The solution was oxygenated with water vapor–saturated gas from below through the fritted glass disc. A small piece of stainless steel mesh over this disc served to prevent bubbles from striking the muscle directly. The vessel was stirred with a vibrator mounted on the same block with the muscle holder. The chamber was provided with a lucite water jacket through which water from a constant temperature bath was continuously circulated.

each isotope. For the determination of Cl^{36} simultaneously with C^{14} and H^3 the procedure was to reduce the voltage of the counter to a setting on which the radioactivity from C^{14} and H^3 did not register, while the more energetic β-rays of Cl^{36} could still be assayed. At the higher voltage used to count C^{14} and H^3 a small constant fraction (3.5 per cent) of counts due to Cl^{36} was subtracted from the total count. The γ-ray of Br^{82} was counted by inserting the dried muscles into a well-counter. The muscles were stored in the refrigerator until the Br^{82} had decayed and then they were analyzed for C^{14}.

SOLUTIONS AND REAGENTS The solutions used were solutions A and D given in Table I of the preceding paper (8). For the osmotic gradient method the hypotonic reference medium was a 50 mM K SO_4–Ringer's solution having a sucrose concentration 50 mM lower than that of the corresponding isotonic solution (solution I). Sources of reagents were as follows: Sucrose, Merck; mannitol and raffinose, Eastman Organic Chemicals; L-arabinose, California Corporation for Biochemical Research; l-erythritol, Nutritional Biochemicals Corporation; mannitol-1,6-C^{14}, Nuclear

TABLE I

EXPERIMENTS WITH RADIOACTIVE TRACERS

Measurement	No. of experiments	Incubation time	Radioactive tracers: H^3-mannitol	C^{14}-mannitol	H^3-inulin	C^{14}-inulin	Others	Bathing solution
		min.						
1. Uptake rate								
Mannitol and inulin influx	6–8 for each interval	1–20	x			x		Normal Ringer
Mannitol space*	8	60		x				Normal Ringer
2. Cell volume and intracellular cation concentration								
Mannitol space, $[K]_i$, $[Na]_i$*	12	120		x				Normal Ringer
Mannitol space, $[K]_i$, $[Na]_i$*	12	120		x				Cl Ringer + 50 mM K
Mannitol and inulin space	8	60		x	x			SO_4 Ringer + 5.32 mM K
Mannitol and inulin space	9	60		x	x			SO_4 Ringer + 50 mM K
3. Miscellaneous								
$[Cl]_i$, mannitol, inulin space	14	60		x	x		Cl^{36}	Normal Ringer
$[Br]_i$, inulin space*	11	120 or 180				x	Br^{82}	Normal Ringer
Choline and mannitol space	7	60 or 180	x				C^{14}-choline	Normal Ringer + 3 mM choline Cl

x denotes extracellular tracer used in the experiment.

* Experiment carried out in perfusion system of Page and Solomon (7). All other experiments utilized 1 ml perfusion chamber shown in Fig. 2.

Research Chemicals, Inc; inulin-C^{14} carboxylic acid, mannitol-1-H^3, and inulin-methyl-H^3, New England Nuclear Corp.; choline chloride–methyl-C^{14}, Nichem, Inc.

RESULTS AND DISCUSSION

The Osmotic Gradient Method

The application of the osmotic gradient method to cat papillary muscle necessitates adjustment of the experimental conditions to meet two somewhat contradictory requirements. On the one hand, the rise in V_m produced by raising the osmolarity with the test molecule must be sufficiently large to be readily discernible. On the other hand, large fluctuations in cell volume must be avoided, since excessively swollen or shrunken cells are difficult to penetrate with microelectrodes. Under these conditions, one may inadvertently enter cells several cell layers below the surface; such cells may be incompletely depolarized in high K solutions giving a spuriously high potential difference. Using SO_4 as the impermeant anion, the muscle was perfused for at least 1 hour with a solution 50 mM hypotonic to normal cat Ringer's solution. The solution was then abruptly changed to an isotonic one, the osmotic deficit being made up by the test molecule. Satisfactory experiments were also possible after changing to a medium which was 50 mM hypertonic. Similar procedures in Cl-Ringer in which the osmolarity was raised with raffinose yielded no sustained change in V_m, confirming that the cell membrane must be much more permeable to Cl than to SO_4. In SO_4 solutions V_m was stable up to 5 hours, so that the changes observed could safely be attributed to the imposed osmotic gradients.

The osmotic gradient method was devised to find the smallest impermeant molecule suitable for extracellular space measurements. For molecules which enter the cell, the method does not differentiate between diffusion through pores and carrier-mediated transport. In the estimation of pore dimensions it is of interest to know, not only the smallest molecule which is prevented from entering, but also the largest molecule which can permeate by diffusion. Accordingly, for molecules that enter cells, it is expedient to block carrier-mediated pathways as effectively as possible. For the rat heart, Fisher and Zachariah (11) have demonstrated a carrier-mediated pathway for arabinose which is stimulated by insulin and protein and competitively inhibited by glucose. Zachariah (12) has further shown that a 1 hour preincubation period suffices for the disappearance of residual intrinsic insulin-like activity. In order to minimize carrier-mediated arabinose transport, the present experiments were therefore carried out after 1 hour of preincubation in presence of glucose and in absence of protein and insulin.

It is apparent from the left side of Fig. 3 that an increase of 100 mM in the concentration of sucrose or mannitol gives rise to a sustained elevation in V_m.

A similarly sustained but somewhat smaller rise was found if the external osmolarity was raised to the isotonic level by increasing the sucrose concentration by 50 mM. By contrast, the curves for arabinose, shown on the right side of Fig. 3, demonstrate the typical time-dependent elevation in V_m expected for a molecule which enters the cell at an appreciable rate. Five ex-

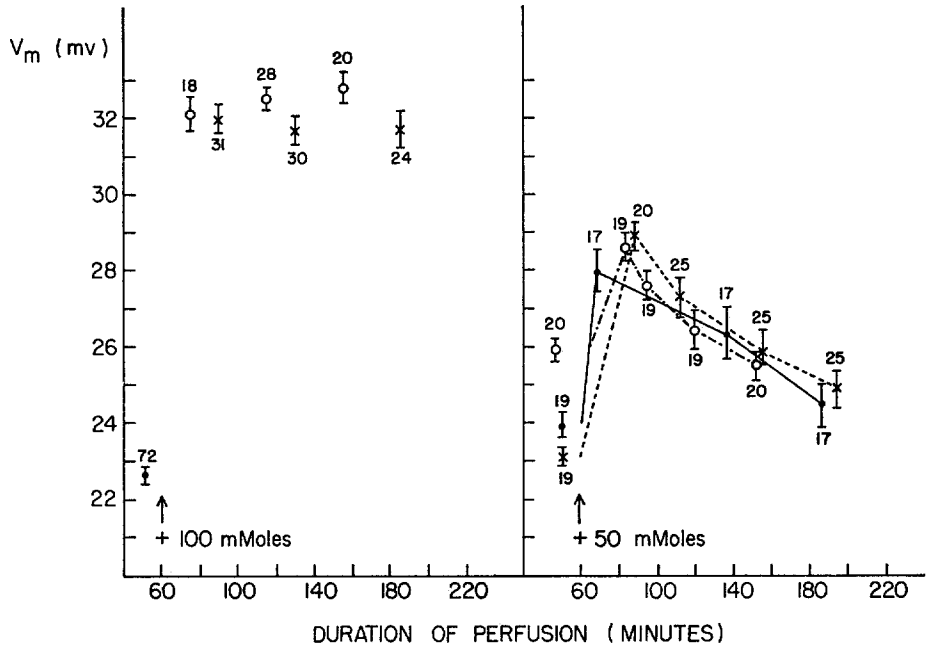

FIGURE 3. The osmotic gradient method. Left, experiments with sucrose (circles) and mannitol (crosses), each point representing pooled data from three animals. The molarity of the reference solution, hypotonic to normal Ringer's by 50 mmoles solute/liter, was raised by 100 mM at the point indicated by the arrow. Right, experiments with arabinose, plotted separately for each animal. 50 mM added to same hypotonic solution at the arrow. Dispersions are standard errors of the mean. Points are plotted at the midpoint of each interval. Figures above points give number of measurements.

periments with erythritol also indicated that this substance had entered the cells.

The size of the carbohydrate molecules tested may be estimated on molecular models (13). A comparison of membrane permeability with molecular radius shows that sucrose (molecular model radius 4.5 Å) and mannitol (4.0 Å) appear to remain extracellular, while arabinose (3.5 Å) and erythritol (3.2 Å) penetrate into the cells.

Comparison of Mannitol and Inulin Uptakes

The results of the osmotic gradient method demonstrate that the smallest test molecule to remain extracellular was mannitol. In Fig. 4 the influx of

mannitol is compared with that of the much larger inulin molecule (molecular radius $\sim$ 15 Å). The experiments were done by incubating the muscle for a given period, cutting off the tissue damaged by the silk ties, blotting, and weighing as described in the section on methods. The points prior to 1 hour were obtained with simultaneous determinations of H^3-mannitol and C^{14}-inulin spaces in the same muscle, those for the later points in separate experiments for the two carbohydrates. Fig. 4 shows that mannitol has effectively equilibrated with 40 per cent of total tissue water by 1 hour. Thereafter the mannitol space does not increase significantly ($P < 0.01$). On the other hand, over the period between 1 and 3 hours of incubation, the inulin space appears

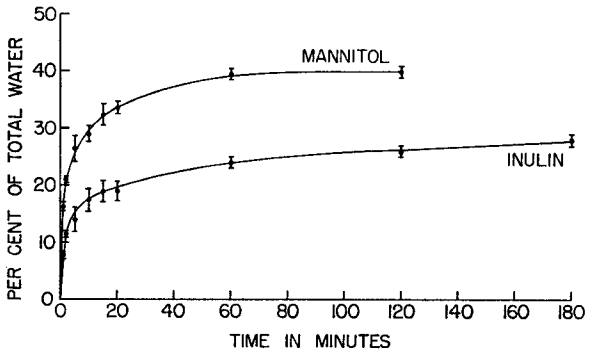

FIGURE 4. Uptake of mannitol and inulin by papillary muscles. The last three points for the inulin uptake are taken from (7).

to be increasing at a very slow rate, the difference between the 1 hour and 3 hour inulin spaces being significant at the level $P < 0.05$.

These differences in the behavior of mannitol and inulin at long equilibration times suggested that mannitol might be gaining access to regions of the extracellular space small enought to impede the diffusion of inulin to a significant extent. The portions of the mannitol space into which inulin diffuses rapidly and slowly will be referred to, for convenience, as the "fast" and "slow" spaces, respectively.[1]

[1] Although it would be of interest to derive the relative sizes of the fast and slow compartments from Fig. 4, such an analysis does not appear to provide unequivocal results. Assuming 40 per cent of total water to represent complete equilibration of the mannitol space, a semilogarithmic plot of $(1 - \alpha)$ against time, in which α is the fractional equilibration of the mannitol space at time t, may be readily resolved into two exponentials with half-times of 11.5 minutes and 0.48 minute. (A similar plot of the inulin uptake cannot be made without an assumption as to the size of this space at equilibrium.) Huxley (24) has shown that the usual procedure of equating the intercepts on the ordinate of such a plot to the sizes of the respective compartments may lead to errors of several hundred per cent.

The analysis is further complicated by the fact that the appropriate solution of the diffusion equation for a cylindrical body [Hill (25), equation 47] is itself an infinite series of exponential terms, even if the cylinder is a kinetically homogeneous compartment. From the Hill equation, the ratio of rate

The size of the mannitol space at 1 and 2 hours did not change when the chemical concentration of mannitol was varied from 0.5 to 2.7 mM. This observation implies that if mannitol is adsorbed to fixed sites, these sites must be saturated by amounts of mannitol too small to affect the present results. It was found incidentally that the space at 1 hour was the same whether measured with mannitol-1,6-C^{14} or with mannitol-1-H^3.

The results lead to certain inferences about the characteristics of the extracellular compartment in this tissue. The dimensions of the slow space have a lower limit defined by the fact that the inulin molecule, diameter ~ 30 Å, can apparently enter it, though with marked restriction on its movement. This lower limit will have to be raised somewhat if, in addition to steric considerations, the diffusion of inulin is impeded in the presence of certain charged substances by virtue of its polyelectrolyte character, as described by Ogston and Phelps (14). The measurements obtained with the osmotic gradient method may be taken as evidence that all of the volume of tissue water equilibrating with mannitol is indeed extracellular; *i.e.*, that the slow compartment is not an artifact reflecting a slow leak of mannitol into the cells. The use of this method makes it possible to define a cell operationally as the compartment penetrated by the microelectrode, independently of the definition of the cell as that compartment not equilibrating with a radioactive extracellular tracer. Moreover, the osmotic gradient method indicates that the membrane separating the cytoplasm from both the fast and slow extracellular spaces should not have water-filled pores appreciably larger than the 8 Å diameter of the mannitol molecule. This conclusion ought to hold even if the slow space should actually be enclosed within the volume of the muscle cells themselves.

The finding that a portion of the extracellular compartment is only very slowly accessible to inulin suggested a search for extracellular structures which might account for this observation. In this connection, the most relevant studies would seem to be the striking recent electron microscopic discoveries of Simpson and Oertelis (15) in ventricular muscle from sheep hearts. These workers were able to demonstrate infoldings (invaginations) of the sarcolemma, indicating that the intercellular spaces are continuous with an extensive tubular system lined by relatively thick walled membranes. This tubular system appears to run transversely into the interior of the cell at the longitudinal level of the Z bands. It is separated from the true intracellular (cytoplasmic) compartment at all points by a thick walled membrane continuous with the cell membrane. The thick walled transverse tubular system is at points contiguous with a second tubular system lined with thin walled

constants of the first two exponentials arising from geometrical considerations only should be $\nu_1^2/\nu_2^2 = 0.20$, in which ν_1 and ν_2 are Bessel functions of zero order. The ratio of rate constants for the observed mannitol uptake is 0.04, suggesting that the observed exponentials are not explained on geometric grounds only.

membranes, presumably the sarcoplasmic reticulum described by Porter and Palade (16) in rat myocardium. Simpson and Oertelis were not able to demonstrate continuity between the thick walled and thin walled systems. They review the literature bearing on the suggestion of A. F. Huxley (17) that a tubular system continuous with the sarcolemma may function in the transmission of the depolarization at the cell membrane to the myofibrillar contractile elements in the cell interior.

Cell Volumes and Intracellular Concentrations

The difference in cell volumes as calculated using mannitol rather than inulin is strikingly reflected in the calculated intracellular ion concentrations, pre-

TABLE II
CATION AND WATER ANALYSES FROM DATA OBTAINED USING MANNITOL SPACES*

$[K]_o$	Weight H_2O/wet weight	H_2O in mannitol space	$[K]_i$	$[Na]_i$	$(Cl)_i$
mM	*Per cent*	*Per cent of total water*		*mmols/kg cell water*	
5.32	75.8±0.3	40±1	208±6	5±2	17±2
50.0	78.6±0.3	34±1	199±6	0±2‡	

* Mean ± SE.
‡ Includes five negative values.

sented for Cl solutions in Table II. The figures (in millimoles per kilo cell water) of 208 for $[K]_i$ and 5 for $[Na]_i$ derived on the basis of the 2 hour mannitol space are in striking contrast to the corresponding figures of 168 and 49 computed using the 2 hour inulin spaces of Page and Solomon (7).

The figure of 17 mmol/kg cell water for $[Cl]_i$ was obtained from a separate series of experiments on fourteen muscles incubated for 1 hour in normal Ringer's solution containing Cl^{36}, C^{14}-mannitol, and in five muscles, H^3-inulin. The use of Cl^{36} to measure $[Cl]_i$ assumes that this isotope reaches specific activity equilibrium with all fractions of tissue Cl during the experiment. The value of 17 mM, calculated taking the mannitol space as the extracellular space, compares with a figure of 46 ± 2 calculated on the basis of the inulin space in the five muscles in which this space was also measured. The mannitol space of 40 ± 1 per cent of total water may be combined with a Br^{82} space of 46 ± 1 per cent of total water measured in eleven additional muscles incubated for 2 and 3 hours in normal Ringer's solution labeled with Br^{82}. The figure of 18 mmol Br^{82}/kg cell water so obtained is in good agreement with that derived from the experiments with Cl^{36}, suggesting that the two halides are distributed in the same way. The agreement between the

values at 1 hour from Cl^{36} measurements and at 2 and 3 hours from Br^{82} measurements further indicates that the cells were in a steady state with respect to Cl during this period.

The value of 17 mM for $[Cl]_i$ is not consistent with a passive distribution for Cl. In normal Ringer's solution V_m is 80 mv (8), which corresponds to a $[Cl]_i$ of 7.4 mM if this ion is distributed passively. A similar discrepancy was observed in auricles from rat hearts by Lamb (18), who found values of 19.5 and 25 mmol/kg fiber water *in vivo* and *in vitro*, respectively. These findings are compatible with several interpretations. A possibility which cannot be ruled out is an active transport of Cl into the cells. However, an apparently high intracellular Cl concentration might arise without the necessity for active transport, if mannitol should fail to measure all of the extracellular space, or if there should be inhomogeneities in the composition of the extracellular compartment. Inhomogeneities might originate from extracellular regions measured by mannitol but having an ionic composition different from that of the bathing medium, as would occur, for example, around localized fixed charge groups. A similar result would be expected if such fixed charge groups were confined to either one of the extracellular compartments. Another possibility is a lowering of the activity coefficient of a part or all of the intracellular Cl, so that only a fraction of the analytically determined Cl participates in a passive distribution. Finally, any comparison of values derived from chemical analysis with values calculated from V_m must take into account the uncertainty in the relation of V_m as measured with the microelectrode technique to the potential difference across the cell membrane. This uncertainty, which is present in all microelectrode measurements, arises because the liquid junction potential between the 3 M KCl solution in the microelectrode and the cytoplasmic solution has not been reliably estimated. Cole and Moore (19), who recently reinvestigated this problem in the squid giant axon, concluded that the liquid junction correction for the microelectrode-cytoplasmic junction was no more than 4 mv when microelectrodes were filled with 3 M KCl. If the results of Cole and Moore are applicable to the present experiments, the large difference of 21.5 mv between V_m and V_{Cl} cannot be explained solely as a liquid junction potential correction.

The results of the osmotic gradient method and of Cl^{36} equilibration, together with the fact that net inward movements of KCl occur on changing to 50 mM K solutions, all indicate that Cl can enter heart muscle cells. Since V_m changes only slightly when Cl is replaced by an impermeant anion, the permeability for Cl may be so low relative to that for K or Na that Cl makes no significant contribution to V_m. Alternatively, Cl may distribute itself passively in accordance with V_m within a time too short to have been detected by the membrane potential measurements of the preceding paper.

Since this formulation of alternatives is based on the assumption that SO_4

behaves like an impermeant anion, this assumption was tested by the substitution of K for Na in SO_4 solutions. In these solutions cell volume should remain constant when the external K is raised isosmotically to 50 mM. This expectation was confirmed; the cell volume of 1.67 ± 0.05 (milligrams cell water per milligrams dry weight) in 5.32 mM K solution remained at 1.72 ± 0.05 in 50 mM K solutions. The corresponding figures using the inulin space were 2.27 ± 0.06 and 2.23 ± 0.06. This constancy of cell volume differs sharply from the cell swelling observed in isosmolar Cl solutions containing 50 mM K. Having shown SO_4 to be an impermeant anion, it is possible to use the conductance measurements of Hutter and Noble (20) to decide between the alternative interpretations of the Cl permeability. The low value for Cl conductance found by these workers, in conjunction with SO_4 substitution experiments (8), suggests that the passive Cl permeability is so small that the contribution of Cl to V_m may be neglected.

When KCl diffuses across the cell membrane of heart muscle, the rate of net movement of salt may be limited by the low permeability to Cl. Moreover, the increase in Na permeability at the beginning of the action potential (21) will be more effective in decreasing the potential difference across the membrane because the Cl permeability is low.

It was initially anticipated that the volume of tissue water equilibrating with choline, a cation which has been frequently used as an "impermeant" substitute for Na, might be useful as a measure of the extracellular space. However, simultaneous equilibration with C^{14}-choline and H^3-mannitol gave a figure for the ratio (choline space)/(mannitol space) of 1.75 after 1 hour and of 2.5 to 3 after 3 hours. The equilibration of choline with a volume of tissue water in excess of 100 per cent at long times suggests that this substance may be utilized in intracellular metabolic reactions. These results resemble those of Renkin (22) in skeletal muscle.

The complexity of the extracellular compartment in papillary muscle may be expected to influence the kinetics of diffusion of ions and other substances. Since ion flux measurements must be carried out on the muscle as a whole, extracellular diffusion will inevitably be superimposed on the fluxes across the cell membrane, which may be of primary interest. Investigation of the kinetics of extracellular diffusion should yield a clearer separation of transmembrane from extracellular ion movements, and may ultimately lead to a more satisfactory tissue model of mammalian heart muscle. From the results of this paper, it is clear that the conventional model of the relationship of heart muscle cell and extracellular space is incomplete, a conclusion also reached for certain other tissues by Bozler (23). The possibility that an extracellular space may exist within what is conventionally considered the boundaries of the cell itself has implications for cardiac electrophysiology which remain to be explored.

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