

## Sarcoplasmic Reticulum

### IX. *The permeability of sarcoplasmic reticulum membranes*

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**ABSTRACT** Fragmented sarcoplasmic reticulum (FSR) membranes isolated from rabbit skeletal muscle are impermeable to inulin-<sup>14</sup>C (mol wt 5,000), and dextran-<sup>14</sup>C (mol wt 15,000–90,000) at pH 7.0–9.0, yielding an excluded space of 4–5  $\mu$ l/mg microsomal protein. In the same pH range urea and sucrose readily penetrate the FSR membrane. EDTA or EGTA (1 mM) increased the permeability of microsomes to inulin-<sup>14</sup>C or dextran-<sup>14</sup>C at pH 8–9, parallel with the lowering of the FSR-bound Ca<sup>++</sup> content from initial levels of 20 nmoles/mg protein to 1–3 nmoles/mg protein. EGTA was as effective as EDTA, although causing little change in the Mg<sup>++</sup> content of FSR. The permeability increase caused by chelating agents results from the combined effects of high pH and cation depletion. As inulin began to penetrate the membrane there was an abrupt fall in the rate of Ca<sup>++</sup> uptake and a simultaneous rise in ATPase activity. At 40°C inulin penetration occurred at pH 7.0 with 1 mM EDTA and at pH 9.0 without EDTA, suggesting increased permeability of FSR membranes. This accords with the higher rate of Ca<sup>++</sup> release from FSR at temperatures over 30°C. The penetration of microsomal membranes by anions is markedly influenced by charge effects. At low ionic strength and alkaline pH acetate and Cl are partially excluded from microsomes when applied in concentrations not exceeding 1 mM, presumably due to the Donnan effect. Penetration of microsomal water space by acetate and Cl occurs at ionic strengths sufficiently high to minimize charge repulsions.

#### INTRODUCTION

Vesicular fragments of sarcoplasmic reticulum<sup>1</sup> isolated from skeletal muscle actively accumulate Ca<sup>++</sup> by an ATP-dependent transport system which is

<sup>1</sup> The following abbreviations were used: EGTA, ethyleneglycol-bis-( $\beta$ -aminoethyl ether)-*N,N'*-tetraacetic acid; FSR, fragmented sarcoplasmic reticulum.

tightly bound to the membrane (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961). The  $\text{Ca}^{++}$  accumulation continues until the intravesicular  $\text{Ca}^{++}$  concentration reaches a level at which the Ca-transport-related ATPase activity is inhibited and a steady state, with slow exchange of intravesicular Ca, is set up (Weber, 1966).

While the maximum rate of  $\text{Ca}^{++}$  uptake measured in vitro (2–3  $\mu\text{moles}$  of  $\text{Ca}^{++}/\text{mg}$  protein/min; Hasselbach and Makinose, 1963) is close to the physiological requirement defined by the rate of relaxation of different types of muscles, the in vitro rate of  $\text{Ca}^{++}$  release from microsomes which were previously loaded with  $\text{Ca}^{++}$  is only 0.03–0.06  $\mu\text{mole}$  of  $\text{Ca}^{++}/\text{mg}$  protein/min (Weber et al., 1966). This value is about 1000–10,000 times less than the expected rate of  $\text{Ca}^{++}$  release from sarcoplasmic reticulum in stimulated muscle, implying a major change in the  $\text{Ca}^{++}$  permeability of microsomal membrane on stimulation.

The low passive permeability of fragmented sarcoplasmic reticulum membranes for  $\text{Ca}^{++}$  implied by the slow rate of  $\text{Ca}^{++}$  release is in contrast to the apparently ready penetration of anions (orthophosphate, pyrophosphate, fluoride, oxalate) into the vesicle interior which is inferred from their ability to increase the rate and extent of  $\text{Ca}^{++}$  accumulation by serving as precipitating agents for the transported calcium (Hasselbach, 1964). No direct experimental information is, however, available concerning the permeability of microsomal membranes to  $\text{Ca}^{++}$  or anions and the data on the permeability of EGTA are fragmentary (Weber et al., 1966).

If we consider the wealth of information on the mechanism of  $\text{Ca}^{++}$  transport and the potential physiological significance of the permeability changes underlying  $\text{Ca}^{++}$  release from sarcoplasmic reticulum, it appears worthwhile to investigate systematically the permeability of microsomal membranes to Ca, various anions, and model substances (urea, sucrose, inulin, dextran), under different experimental conditions and to relate the measured permeability changes to the rate of  $\text{Ca}^{++}$  uptake and release.

The observations indicate that the permeability of microsomal membranes to EDTA, inulin, and carboxydextran is a dual function of pH and membrane-bound divalent cation content. Under conditions permitting penetration of EDTA or inulin into the microsomes there is an abrupt fall in the rate of  $\text{Ca}^{++}$  uptake and a simultaneous rise in the ATPase activity, which can be attributed to increased membrane permeability to  $\text{Ca}^{++}$ . Changes in the permeability of the microsomal membrane to inulin were also found at elevated temperature (40°C) and after treatment of microsomes with salyrgan, trypsin, and phospholipase C, which are known to inhibit the transport of Ca.

In addition to the factors regulating the penetration of neutral substances, charge effects appear to enter significantly into the definition of the permeability of small ions through sarcoplasmic reticulum membranes. In contrast to

mitochondria, sarcoplasmic reticulum membranes are freely permeable to acetate, chloride, and citrate under conditions in which charge effects are minimized.

#### EXPERIMENTAL PROCEDURES

Preparation of rabbit skeletal muscle microsomes, measurement of  $\text{Ca}^{++}$  transport and ATPase activity were carried out essentially as described earlier (Martonosi et al., 1968).

*Permeability Measurements* For the determination of the exclusion volume, intermicrosomal space, and the impermeable water space with respect to a particular permeability marker, microsomes (protein concentration 15–25 mg per ml) were incubated at stated temperatures and pH values with inulin carboxyl- $^{14}\text{C}$  (mol wt 5000–5500), dextran-carboxyl- $^{14}\text{C}$  (mol wt 15,000–17,000 and 60,000–90,000), EDTA- $^{14}\text{C}$ , urea- $^{14}\text{C}$ , sucrose- $^{14}\text{C}$ , acetate- $^{14}\text{C}$ ,  $^{36}\text{Cl}$ , and citrate- $^{14}\text{C}$  in concentrations indicated in the figure legends for 10–30 min. Total volume was usually 0.8 ml. Microsome-free control samples with identical concentrations of radioactive material were included in each series. After centrifugation at 41,000 *g* for 1 hr in preweighed tubes of 2 ml capacity the supernatant was carefully decanted, the fluid adhering to the wall of the tube was removed, and the weight of the pellet was determined on an analytical balance to the nearest 0.1 mg. One pellet of the duplicate samples used for each assay was dried overnight at 70°C; during this time constant weight had been reached. Since cellulose nitrate tubes lose approximately 3–4 mg weight under these conditions, only tubes previously exposed to 70°C overnight were used for the dry weight determination.

The total wet weight of the pellet minus the dry weight yields the volume of *total pellet water*. The values for total pellet water volume range from 7 to 10  $\mu\text{l}/\text{mg}$  protein. This volume includes the intermicrosomal water space, the water content of the microsomal membrane, and the intramicrosomal water.

The *intermicrosomal water space* is essentially the space between the microsomal particles. It can be determined by measuring the radioactivity of inulin- $^{14}\text{C}$ , or carboxy-dextran- $^{14}\text{C}$  included in the pellet under conditions in which the penetration of these polysaccharides into the intramicrosomal space and the membrane water space is minimized (freshly prepared microsomes at neutral pH). For the determination of radioactivity, one pellet of the duplicate samples run for each assay was suspended in 10 mM histidine buffer to 0.8 ml volume and the radioactivity of an aliquot was measured in a Packard Tricarb 3320 liquid scintillation counter according to the method of Loftfield and Eigner (1960). A quench correction of 2.5% was applied to all measurements using resuspended sediments. The volume equivalent of the measured radioactivity was derived from radioactivity measurements on microsome-free control samples. The total pellet weight minus the intermicrosomal water space yields the *excluded volume* which in microsomes that are impermeable to the substance used (usually inulin) is equal to the sum of the intramicrosomal water space, the water space of the microsomal membrane, and the space occupied by other membrane constituents.

In a few cases the excluded volume was also determined independently from the increased radioactivity of the supernatants of microsomal suspensions obtained after ultracentrifugation, relative to the radioactivity of microsome-free control samples. For example, if one-half the total volume of a microsomal suspension is not accessible to inulin- $^{14}\text{C}$ , the radioactivity of the supernatant per unit volume will be twice as high as the radioactivity per unit volume of a microsome-free control sample containing identical amount of inulin- $^{14}\text{C}$  per ml. The value for the excluded volume of fresh microsomal preparations at pH 7.0 with respect to inulin or carboxydextran is about 4.5–6.0  $\mu\text{l}/\text{mg}$  protein. In a microsomal suspension containing 20 mg protein per ml the excluded volume is about 100  $\mu\text{l}/\text{ml}$  corresponding to a 10% increase in the radioactivity of the supernatant of microsomal suspensions relative to the microsome-free control. There was reasonable agreement between excluded volumes determined from pellet weights and from radioactivity measurements on the supernatants. The excluded volume of microsomes which are penetrated by the permeability marker is less than 4.5–6.0  $\mu\text{l}/\text{mg}$  protein and in the case of complete penetration of microsomal water space may be as low as 1.0–1.5  $\mu\text{l}/\text{mg}$  protein corresponding to the space occupied by membrane proteins and phospholipids.

The inaccessible water space of microsomes is defined as the volume of microsomal water which is not penetrated by the permeability marker; its value in fresh microsomes at pH 7.0 is about 3.5–5.0  $\mu\text{l}/\text{mg}$  protein with respect to the nonpermeant inulin or dextran. The inaccessible water space becomes zero when full penetration of microsomal water space occurs. The observed changes in the inaccessible water space under various experimental conditions are generally not attributable to the swelling or shrinking of the microsomal pellet, as they are not accompanied by corresponding changes in total pellet water.

On fresh microsomes at pH 7.0 the excluded volume minus inulin inaccessible space yields the volume occupied by membrane proteins, phospholipids, and sterols corresponding to 1.0–1.5  $\mu\text{l}/\text{mg}$  protein. Similar value is obtained from dry weight determinations of microsomal material assuming a partial specific volume of 1.0 or from measuring the distribution of substances which fully penetrate all microsomal water spaces such as urea- $^{14}\text{C}$  or sucrose- $^{14}\text{C}$ . In the latter case the total pellet weight minus urea or sucrose permeable water space yields the space occupied by microsomal membrane materials.

*Determination of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{K}^+$*  A Perkin-Elmer Model 303 atomic absorption spectrometer was used for the determination of  $\text{K}^+$ ,  $\text{Mg}^{++}$ , and  $\text{Ca}^{++}$  (Willis, 1963). Protein was removed with 3% TCA. Standard  $\text{K}^+$ ,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$  solutions containing 3% TCA were used for calibration. Glassware and cellulose nitrate tubes used in all experiments were rinsed with 1 mM EDTA followed by repeated distilled water rinses before drying.

An association constant of  $2.5 \times 10^8$  was used for the calculation of free  $\text{Ca}^{++}$  concentration in the presence of EDTA at pH 8.0 (Raafaub, 1956).

*Materials* Only analytical grade reagents were used. Inulin- $^{14}\text{C}$ , carboxydextran- $^{14}\text{C}$ , urea- $^{14}\text{C}$ , sucrose- $^{14}\text{C}$ ,  $^{45}\text{Ca}$ , EDTA- $^{14}\text{C}$ , acetate- $^{14}\text{C}$ ,  $^{36}\text{Cl}$ , and citrate- $^{14}\text{C}$  were obtained from New England Nuclear Corp., Boston, Mass.

## RESULTS

*The Permeability of Microsomal Membrane to Inulin, Dextran, and EDTA*

The exclusion volume of microsomes in 10 mM histidine at pH 7.4 was measured using inulin-carboxyl- $^{14}\text{C}$  (mol wt 5000–5500), dextran carboxyl- $^{14}\text{C}$  (mol wt 15,000–17,000), and dextran carboxyl- $^{14}\text{C}$  (mol wt 60,000–90,000). Under these conditions the water space of microsomes from which inulin or dextran was excluded, amounted to about 4–5  $\mu\text{l}/\text{mg}$  microsomal protein and was the same for each of the polysaccharides (Fig. 1). With aged microsomes

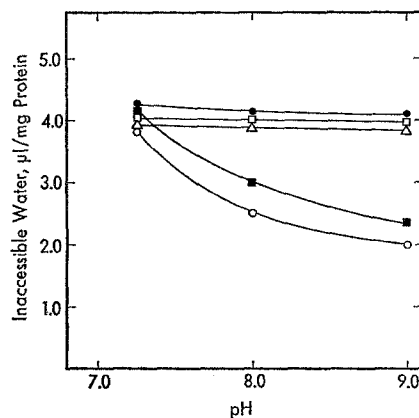


FIGURE 1. Effect of pH and treatment with EDTA on the inaccessible water space of muscle microsomes. Microsomes were suspended in 10 mM histidine-NaOH solutions at a concentration of 16 mg/ml, and inaccessible water was determined as described under Experimental procedures. Inulin-carboxyl- $^{14}\text{C}$ , mol wt 5000–5500 (open triangles); dextran-carboxyl- $^{14}\text{C}$ , mol wt 15,000–17,000 (open squares); dextran-carboxyl- $^{14}\text{C}$ , mol wt 60,000–90,000 (solid circles); 1 mM EDTA- $^{14}\text{C}$  (solid squares); inulin-carboxyl- $^{14}\text{C}$ , mol wt 5000–5500 and 1 mM EDTA (open circles). The mean values for total pellet water in the absence of EDTA at pH 7.0, 8.0, and 9.0 were  $7.0 \pm 0.5$ ,  $7.3 \pm 0.2$ , and  $8.5 \pm 0.5$   $\mu\text{l}/\text{mg}$  protein, respectively. The corresponding values with 1 mM EDTA were  $7.0 \pm 0.50$ ,  $7.75 \pm 0.25$ , and  $8.85 \pm 0.35$ , respectively. These data indicate that the observed changes in inaccessible water space are not due to shrinking of microsomes.

there was a tendency toward greater inulin penetration. Incubation of polysaccharides and microsomes for up to 1 hr at pH 7.4 at room temperature did not change the inaccessible water space. Urea and sucrose penetrated the microsomal water fully, giving an exclusion volume of about 1.5  $\mu\text{l}/\text{mg}$  protein which approximates the volume occupied by membrane proteins and lipids assuming an average specific gravity of 1. Under identical conditions (10 mM histidine, pH 7.4) but using EDTA- $^{14}\text{C}$  at a final concentration of 1 mM, instead of inulin, the inaccessible water space was similar to that obtained with inulin (Fig. 1).

The permeability of dextran- $^{14}\text{C}$  or inulin- $^{14}\text{C}$  was not affected markedly by

increasing the pH of the solution from 7.0 to 9.0 (Fig. 1). In the same pH range the inaccessible space of EDTA used in 1 mM concentration decreased from 4.1 to 2.4  $\mu\text{l}/\text{mg}$  microsomal protein. It appears that the observed decrease in EDTA inaccessible water space at pH 8–9 is influenced by the salt concentration of the medium, as even greater penetration of microsomes by 1 mM EDTA occurs if 0.05–0.1 M KCl is added to neutralize charge effects (see below).

Since the inaccessible space of EDTA used in  $2.5 \times 10^{-5}$  M concentration remained about 5.0  $\mu\text{l}/\text{mg}$  protein even at pH 9.0, the effects observed at 1 mM EDTA concentration were further investigated with regard to the role of bound divalent cations in the regulation of the permeability of microsomal membranes. EDTA was found to increase the permeability of microsomal membranes to inulin as indicated by the decrease of inulin inaccessible space in the presence of 1 mM EDTA from 3.8  $\mu\text{l}/\text{mg}$  protein at pH 7.2 to 2.0  $\mu\text{l}/\text{mg}$  protein at pH 9.0 (Fig. 1). Similar effects were observed with dextran- $^{14}\text{C}$  preparations of 15,000–17,000 and 60,000–90,000 mol wt which yielded 1.85 and 1.65  $\mu\text{l}/\text{mg}$  protein inaccessible space, respectively, at pH 9.0 with 1 mM EDTA. The effect of EDTA on the penetration of inulin into microsomes is most pronounced at pH 9.0 whereas there is no effect at pH 7.0 (Fig. 2).

The EDTA-induced change in the inulin permeability of microsomes does not show a simple correlation with the amount of calcium and magnesium bound to microsomal membrane (Table I). Although EDTA decreased the bound  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  content of microsomes by 83% at pH 7.0 the inulin permeability remained unchanged, whereas at pH 9.0 for the same loss of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  the inulin inaccessible water space decreased from 4.0 to 2.0  $\mu\text{l}/\text{mg}$  protein. Thus the permeability increase appears to be a dual function of  $\text{H}^+$  concentration and bound divalent cation content.

As the affinity of EGTA is about  $10^6$  times greater for  $\text{Ca}^{++}$  than for  $\text{Mg}^{++}$  while the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  affinities of EDTA differ only slightly, the relative importance of calcium and magnesium in the regulation of the permeability of microsomal membranes to inulin can be assessed from the differences in the effects of EDTA and EGTA on the calcium and magnesium content and inulin inaccessible space of microsomes. As shown in Fig. 3 A and B, EDTA and EGTA cause a similar decrease in the inulin inaccessible space of microsomes, which correlates well with the decrease of bound  $\text{Ca}^{++}$  content, while the bound  $\text{Mg}^{++}$  content of microsomes is only moderately affected by EGTA.

In essential agreement with the experiments described in Fig. 3 A and B inclusion of 2 mM  $\text{CaCl}_2$  with 2 mM EDTA markedly diminished the effect of EDTA on the inulin inaccessible water space (Table II). On treatment of microsomes with a solution containing 2 mM  $\text{MgCl}_2$  and 2 mM EDTA, while the bound Mg content of microsomes increased, the effect of  $\text{Mg}^{++}$  on the inulin inaccessible water space was variable.

The reversibility of EDTA-induced increase in the inulin permeability of microsomes was tested in two ways: (a) Microsomes were exposed to 0.5 mM EDTA for 10 min at pH 8.2. Calcium was then added to the microsomal suspensions still containing EDTA to 0.5 mM final concentration and the inulin

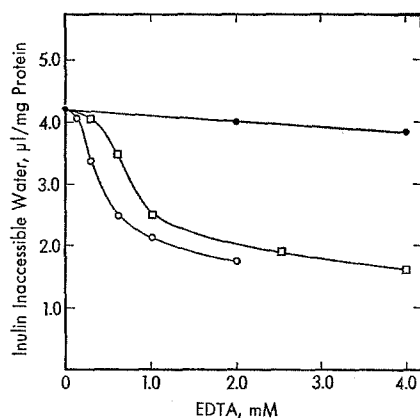


FIGURE 2. The effect of increasing EDTA concentrations on the inulin inaccessible water of microsomes. Inulin inaccessible water was determined as described in the Experimental procedures. The microsomal protein concentration was 18 mg/ml at pH 7.2 (solid circles), 25 mg/ml at pH 8.0 (open squares), and 22.1 mg/ml at pH 9.0 (open circles).

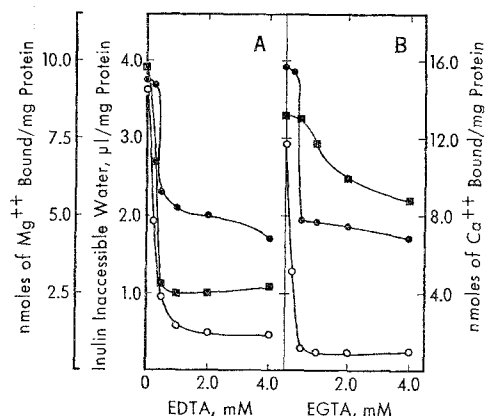
TABLE I  
EFFECT OF EDTA ON THE DIVALENT CATION  
CONTENT AND INULIN INACCESSIBLE WATER SPACE  
OF RABBIT SKELETAL MUSCLE MICROSOMES

Microsomes (18 mg protein/ml) were incubated at 25°C; without or with 2 mM EDTA in 0.7 ml medium containing 10 mM Tris-HCl buffer of the stated pH. After 15 min 0.1 ml of carrier-free inulin-<sup>14</sup>C solution containing 32,000 cpm was added (specific activity 3.6 mCi/g inulin) and 15 min later the suspension was centrifuged for 1 hr at 41,000 *g*. Inaccessible water space was determined by subtracting the intermicrosomal water as measured by the inulin radioactivity trapped in the pellet, from the total pellet water as described under Experimental procedures.

Additions	Ca <sup>++</sup> content, nmoles/mg protein			Mg <sup>++</sup> content, nmoles/mg protein			Inulin inaccessible water, μl/mg protein			Total pellet water, μl/mg protein		
	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9
None	18.0	16.0	18.0	10.0	8.0	9.0	4.0	3.9	3.6	6.5	7.0	8.0
EDTA, 2 mM	2.0	1.8	3.5	2.7	2.5	2.4	4.0	2.5	2.0	6.8	7.5	8.6

inaccessible space measured. With some quantitative variations between preparations, significant, but never complete, restoration of inulin impermeability was observed. Addition of Mg instead of Ca produced a similar effect (Table III A). (b) In the second type of experiment microsomes were exposed to 0.5

mm EDTA for 10 min at pH 8.2 followed by centrifugation. The sedimented microsomes were resuspended in buffer solution and  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  was added to a final concentration of 0.5 mM. No significant restoration of inulin impermeability was found in any of these experiments (Table III B).



FIGURES 3 A and B. The effect of EDTA (3 A) and EGTA (3 B) on the inulin inaccessible water, bound  $\text{Mg}^{++}$ , and bound  $\text{Ca}^{++}$  contents of rabbit skeletal muscle microsomes. Inulin inaccessible water was determined as described in the Experimental procedures. The protein concentration in both experiments was 18.0 mg/ml. Solid circles, inulin inaccessible water; solid squares, bound  $\text{Mg}^{++}$  content; open circles, bound  $\text{Ca}^{++}$  content.

TABLE II  
EFFECT OF EDTA AND Ca-EDTA ON THE INULIN  
INACCESSIBLE SPACE OF MICROSOMES

Microsomes were incubated at 25°C in a solution which contained 10 mM Tris-HCl buffer, pH 9.0, 14 mg microsomal protein, and the additions indicated in the table in a total volume of 0.7 ml. After 15 min 0.1 ml of inulin carboxyl- $^{14}\text{C}$  (30,000 cpm) was added to each tube and 15 min later centrifugation was carried out at 41,000 *g* for 1 hr. Inulin inaccessible water was determined as described under Experimental procedures. For the determination of bound  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  content in the presence of high concentration of total  $\text{Ca}^{++}$ , the  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  content of ultracentrifugally sedimented microsome pellets was corrected by the divalent cation content of the included medium as derived from the inulin content of the pellet and from the separately measured inulin,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$  concentration of the microsome-free supernatant.

Additions	Bound $\text{Ca}^{++}$	Bound $\text{Mg}^{++}$	Inulin inaccessible space	Pellet water
	nmoles/mg protein	nmoles/mg protein	$\mu\text{l}/\text{mg}$ protein	$\mu\text{l}/\text{mg}$ protein
Control	19.0	10.0	3.7	7.6
EDTA, 2 mM	3.0	2.5	1.9	8.3
EDTA, 2 mM + $\text{CaCl}_2$ , 2 mM	23.0	10.0	3.3	7.2



TABLE III A  
PARTIAL REVERSAL OF THE EDTA-INDUCED  
INCREASE IN INULIN PERMEABILITY BY THE  
ADDITION OF  $\text{Ca}^{++}$  OR  $\text{Mg}^{++}$

To 0.6 ml samples of microsomal suspensions (18 mg protein/ml) in 10 mM Tris buffer, pH 8.1, 0.05 ml 8 mM EDTA, pH 8.2 was added followed, 10 min later, by 0.05 ml of 8 mM  $\text{CaCl}_2$  or  $\text{MgCl}_2$ . After 15 min incubation at room temperature 0.1 ml inulin- $^{14}\text{C}$  (20,000 cpm) was added and the inulin inaccessible water determined as described under Experimental procedures. For the determination of bound  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  see Table II legend. Essentially similar effects were obtained when  $\text{CaCl}_2$  was added 30 or 60 min after the addition of EDTA.

Additions	Inulin inaccessible water	Pellet water	Bound $\text{Ca}^{++}$	Bound $\text{Mg}^{++}$
	$\mu\text{l}/\text{mg protein}$	$\mu\text{l}/\text{mg protein}$	<i>nmoles/mg protein</i>	
Control	4.20	8.5	14.0	8.7
0.5 mM EDTA	2.87	8.9	5.7	5.4
0.5 mM EDTA + 10 min later 0.5 mM $\text{CaCl}_2$	3.70	8.5	14.0	8.5
0.5 mM EDTA + 10 min later 0.5 mM $\text{MgCl}_2$	3.82	8.5	9.4	21.0

TABLE III B  
PRETREATMENT OF MICROSOMES WITH EDTA  
FOLLOWED BY WASHING AND SUBSEQUENT ADDITION  
OF CALCIUM OR MAGNESIUM

Microsomal suspensions (18 mg protein/ml) were treated with 0.5 mM EDTA in 10 mM Tris-HCl (pH 8.1) for 15 min followed by centrifugation for 1 hr at 41,000 g. The sediments were resuspended in 10 mM Tris-HCl (pH 8.1) to the original volume and EDTA,  $\text{CaCl}_2$ , or  $\text{MgCl}_2$  were added to final concentrations of 0.5 mM when indicated. Inulin inaccessible water was determined as described under Experimental procedures. For the determination of microsome-bound  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  see Table II legend.

Additions	Inulin inaccessible water	Pellet water	Bound $\text{Ca}^{++}$	Bound $\text{Mg}^{++}$
	$\mu\text{l}/\text{mg protein}$	$\mu\text{l}/\text{mg protein}$	<i>nmoles per mg protein</i>	
Control	4.2	8.5	14.0	8.7
Pretreatment with 0.5 mM EDTA followed by centrifugation and resuspension in 10 mM Tris pH 8.0	2.5	9.2	5.7	8.8
Pretreatment with 0.5 mM EDTA followed by centrifugation and resuspension in 0.5 mM $\text{CaCl}_2$	2.5	8.9	20.5	8.3
Pretreatment with 0.5 mM EDTA followed by centrifugation and resuspension in 0.5 mM $\text{MgCl}_2$	2.35	8.9	6.5	29.0

The volume of microsomal pellet after ultracentrifugation progressively decreased with  $\text{Ca}^{++}$  concentrations exceeding 1 mM and visible aggregation of microsomes occurred at pH 8.0 above 4 mM  $\text{CaCl}_2$ . These effects may contribute to the fact that no reversal of the increased inulin permeability of microsomes caused by 2 mM EDTA could be demonstrated by the addition of Ca or Mg in millimolar concentrations.

Treatment of microsomes with Dowex chelating resin substantially decreased the inulin inaccessible water space, provided that the calcium content of the microsomes was lowered to values below 4.0 nmoles/mg protein. Addition of calcium (0.2–5 mM), Mg (0.1–5 mM), or  $\text{La}^{+++}$  (0.01–1 mM) to Chelex-treated microsomes did not restore the original inulin permeability characteristics.

Preliminary experiments on the relationship between the inulin penetration and the free calcium concentration of the medium at pH 8 indicate that the affinity of calcium binding sites involved in the regulation of the inulin permeability of microsomes is unexpectedly large with complete saturation at a free  $\text{Ca}^{++}$  concentration of  $10^{-5}$  M.

#### *The Effect of EDTA on the $\text{Ca}^{++}$ Transport and ATPase Activity*

In view of the probable physiological significance of the permeability of the microsomal membrane in regulating the rate of  $\text{Ca}^{++}$  uptake and release during the contraction-relaxation cycle, the effect of conditions which influence the inulin permeability of microsomes was investigated on the rate of  $\text{Ca}^{++}$  transport and associated ATPase activity (Fig. 4). Pretreatment of microsomes with 0.25–0.5 mM EDTA at pH 8.0 sharply decreased the rate of  $\text{Ca}^{++}$  uptake accompanied by an activation of ATPase activity while the inulin permeability changed only moderately. Further increase in EDTA concentration caused a progressive decrease in inulin inaccessible space and in the rate of  $\text{Ca}^{++}$  uptake without further change in ATPase activity.

**THE EFFECT OF TEMPERATURE ON THE INULIN PERMEABILITY OF MICROSOMAL MEMBRANES** Temperature exerted a marked effect on the inulin permeability of microsomes as at 40°C inulin entered the microsomes at pH 9.0 even in the absence of EDTA and at pH 7.3 with 2 mM EDTA, in contrast to the observation made at 25°C (Table IV). Paralleling these changes there was a progressive increase in the rate of release of accumulated  $\text{Ca}^{++}$  from microsomes with increasing temperature in the range of 4° to 40°C (Fig. 5).

**THE EFFECT OF PH ON THE RATE OF RELEASE OF ACCUMULATED  $\text{Ca}^{++}$  FROM MICROSOMES** Following  $\text{Ca}^{++}$  accumulation at pH 7.0 for 1 min, the pH was rapidly adjusted to values ranging from 7.0 to 9.0 by the addition of solutions

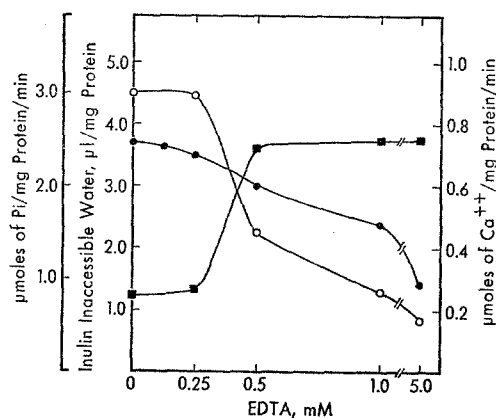


FIGURE 4. The effect of EDTA treatment on the inulin inaccessible water, calcium uptake, and ATPase activity of muscle microsomes. Graph represents the mean of three experiments; all data fell within  $\pm 12\%$  of the mean. Microsomes (17.0–17.2 mg protein/ml) were preincubated with EDTA at pH 8.0 at concentrations indicated on the abscissa for 30 min in 10 mM histidine-NaOH, pH 8.0. The inulin inaccessible water was then measured as described under Experimental procedures (solid circles) and aliquots were diluted for measurement of the calcium uptake (open circles) and ATPase activity. In the calcium uptake assay 0.1 mg of microsomal protein was added to 3 ml of medium containing 5 mM ATP, 5 mM  $MgCl_2$ ,  $10^{-4}$  M  $^{45}CaCl_2$ , 5 mM oxalate, 100 mM KCl, 10 mM histidine, pH 7.4 at 23°C. Uptake was measured over a 2-min period. In the assay for ATPase activity (solid squares) 20  $\mu M$  Ca was included in the medium due to the transfer of EDTA (maximum concentration 14.0  $\mu M$ ) from the preincubation medium. 0.1 mg of microsomal protein was added to 2.0 ml solution, containing 5 mM ATP, 5 mM  $MgCl_2$ , 100 mM KCl, 20  $\mu M$   $CaCl_2$ , 10 mM histidine, pH 7.4, at 23°C and the released inorganic phosphate was measured after 5 and 10 min.

TABLE IV  
THE EFFECTS OF TEMPERATURE AND  
EDTA ON THE PERMEABILITY OF MUSCLE  
MICROSOMES TO INULIN

Microsomes (18–20 mg/ml) were incubated in 20 mM Tris-HCl solution of the stated pH and temperature with 28,000 cpm/ml inulin- $^{14}C$  (specific activity 1.56 mCi/g inulin). Total volume was 0.9 ml. EDTA (2 mM) was included when indicated. After 30 min the suspensions were centrifuged at 41,000 *g* for 60 min and the inulin inaccessible water determined as described under Experimental procedures. The numbers in parentheses indicate total pellet water expressed in microliters per milligram of protein.

Temperature	Inulin inaccessible water, $\mu l/mg$ protein					
	No additions			2 mM EDTA		
	pH 7.3	pH 8.2	pH 9.0	pH 7.3	pH 8.2	pH 9.0
°C						
25	3.0 (6.0)	3.15 (6.5)	3.0 (7.5)	2.8 (6.2)	1.85 (7.0)	1.3 (8.2)
40	3.3 (6.8)	3.1 (7.5)	2.2 (7.7)	1.7 (6.9)	1.1 (7.0)	1.3 (—)

of varying pH's containing no EDTA (Fig. 6 A) or EDTA in concentrations slightly in excess of the concentration of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (Fig. 6 B). The release of  $\text{Ca}^{++}$  was followed for a period of 2 min.

The rate of  $\text{Ca}^{++}$  release markedly increased with increasing pH up to and probably beyond pH 9.0, implying an increased  $\text{Ca}^{++}$  permeability of microsomal membranes at alkaline pH's. The pH-dependent increase in the rate of  $\text{Ca}^{++}$  release in the presence of EDTA cannot be explained entirely by the increased affinity of EDTA for Ca as an increase in the rate of Ca release at alkaline pH also occurs in the absence of EDTA.

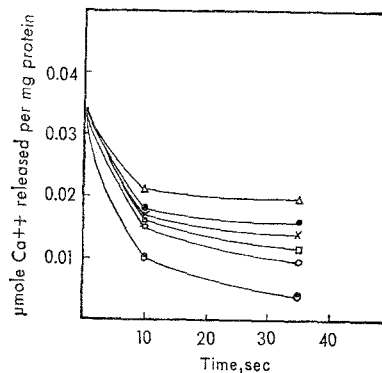


FIGURE 5. Effect of temperature on calcium release. 0.35 ml of medium containing 20 mM ATP, 20 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{Ca}^{45}\text{Cl}_2$ , 100 mM KCl, and 10 mM histidine, pH 7.4, was added to 0.35 ml of microsomal suspension containing 3.5 mg protein, at 25°C. After 1 min 0.5 ml was withdrawn and added to 5 ml of "release" medium containing 1 mM EDTA, 100 mM KCl, and 10 mM histidine at pH 7.4 held at 4°C (open triangles); 10°C (solid circles); 20°C (X); 30°C (open squares); 35°C (open circles); and 40°C (half-filled circles). Aliquots were filtered after 5–15 sec and 30–40 sec using syringes fitted with Swinny adapters containing Millipore filters of 0.45  $\mu$  pore size. Calcium uptake was determined in the initial suspension after 1 min, by cooling in ice water, centrifuging for 20 min at 41,000 *g*, and assaying the supernatant.

The pH-dependent changes in  $\text{Ca}^{++}$  permeability occur in the same pH range in which marked changes in inulin permeability were also observed in the presence of EDTA or at elevated temperatures. Interestingly the same equilibrium level of microsome-bound  $\text{Ca}^{++}$  is obtained at a certain pH whether it is reached by uptake or release (Fig. 6 A), but the amount of  $\text{Ca}^{++}$  bound by microsomes progressively decreases with increasing pH in the range of 7 to 9.

**THE EFFECT OF SH REAGENTS ON THE INULIN PERMEABILITY OF MICROSOMES**  
Salyrgan in low concentrations (0.1  $\mu\text{mole/mg}$  protein) at pH 7.5 caused a rapid release of actively accumulated calcium from microsomes (Martonosi and Feretos, 1964 *b*) without major change in the inulin inaccessible water space or microsome-bound  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  content (Table V). At a concen-

tration of 1.0  $\mu$ mole of salyrgan per mg microsomal protein a marked increase in inulin permeability was observed and the pellets became strongly hydrated although the calcium and magnesium contents decreased by only 25%. *N*-Ethylmaleimide even at a concentration of 3  $\mu$ moles/mg protein did not affect the permeability of microsomes to inulin.

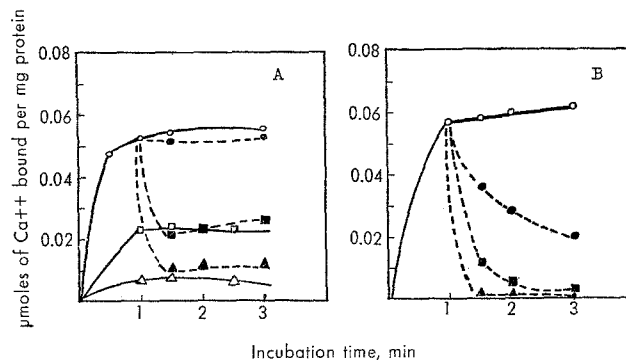


FIGURE 6. The effect of pH and EDTA on the uptake and release of calcium. A, to 4 ml aliquots of a solution containing 5 mM ATP, 5 mM  $MgCl_2$ , 50  $\mu M$   $^{45}CaCl_2$ , 100 mM KCl, and 10 mM histidine at pH 7.0, 0.5 ml of 0.1 M Tris-HCl buffer of pH 7.0, 8.1, and 9.4 was added to give final pH values of 7.0, 8.0, and 9.0, respectively. The  $Ca^{++}$  uptake was initiated with the addition of 0.1 ml of microsomal suspension containing 2.2 mg microsomal protein, and aliquots were taken for filtration after incubation at room temperature for times indicated on the abscissa (open circles, pH 7.0; open squares, pH 8.0; open triangles, pH 9.0). In a separate set of three samples microsomes were added to a medium containing 5 mM ATP, 5 mM  $MgCl_2$ , 50  $\mu M$   $^{45}CaCl_2$ , 100 mM KCl, and 10 mM histidine, pH 7.0. After 1 min incubation the  $Ca^{++}$  release was initiated by the addition of Tris-HCl buffer of the same composition as described above to give final pH's of 7.0, 8.0, and 9.0, respectively. Samples were withdrawn for filtration at times indicated on the abscissa (solid circles, pH 7.0; solid squares, pH 8.0; solid triangles, pH 9.0). B, to 4 ml samples of incubation mixtures containing 5 mM ATP, 5 mM  $MgCl_2$ , 50  $\mu M$   $^{45}CaCl_2$ , 100 mM KCl, and 10 mM histidine, 0.1 ml of a microsome suspension containing 1.87 mg microsomal protein was added to initiate  $Ca^{++}$  uptake. After 1 min incubation at 25°C the pH of the solutions was rapidly changed by the addition of 0.5 ml of 50 mM EDTA of the required pH with rapid stirring to obtain final pH values of 7.0 (solid circles), 7.7 (solid squares), and 9.0 (solid triangles), respectively. To the control sample (open circles), 0.5 ml 0.1 M KCl, 10 mM histidine, pH 7.0, was added at the same time.

**OTHER EFFECTS** Treatment of microsomes by trypsin or phospholipase under conditions known to inhibit the Ca transport of skeletal muscle microsomes (Martonosi et al., 1968; Martonosi, 1968) decreased the inulin inaccessible water space from 4.4 to 1.2 and 1.5  $\mu$ l/mg protein, respectively, measured at pH 7.2 in 10 mM Tris-HCl buffer solution.

*Selective Solubilization of Microsomal Proteins With EDTA at Alkaline pH*

The partial restoration of inulin impermeability on addition of divalent cations to a microsomal suspension still containing EDTA (Table III A) is in apparent contradiction with the finding that  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  added to EDTA-treated microsomes after removal of the EDTA washing fluid by centrifugation (Table III B) proved ineffective. The following experiments were designed to test the possibility that EDTA removes reversibly some microsomal membrane constituent, other than Ca or Mg, which is involved in the regulation of permeability.

Washing with buffer solutions at pH 7–9 solubilized a trace amount of pro-

TABLE V  
TREATMENT OF MICROSOMES WITH  
SALYRGAN AND *N*-ETHYLMALEIMIDE

Microsomes (20 mg protein/ml) were incubated with salyrgan or *N*-ethylmaleimide in 10 mM histidine solution (pH 7.4–7.6) for 15 min at 25°C. 0.1 ml of an inulin- $^{14}\text{C}$  solution containing 30,000 cpm (specific activity 1.56 mCi/g) was added and the inulin inaccessible space and bound divalent cation content were measured as described under Experimental procedures.

Additions	Inulin inaccessible water	Pellet water	Ca content	Mg content
	$\mu\text{l}/\text{mg protein}$	$\mu\text{l}/\text{mg protein}$	$\text{nmoles}/\text{mg protein}$	
Control (salyrgan)	3.6	7.7	23.5	13.5
0.25 $\mu\text{mole}$ of salyrgan/mg protein	3.1	9.1	24.5	11.5
0.5 $\mu\text{mole}$ of salyrgan/mg protein	3.1	8.6	25.0	11.5
1.0 $\mu\text{mole}$ of salyrgan/mg protein	1.4	12.3	20.0	10.5
Control (NEM)	3.5	7.0	17.5	9.5
3.0 $\mu\text{moles}$ of NEM/mg protein	3.5	7.0	16.5	8.5

teins from microsomes which migrated in two distinct bands on polyacrylamide electrophoresis (Fig. 7). The extraction of two additional bands was greatly promoted by 1 mM EDTA at pH 8–9 (Fig. 7) accompanied by a number of fainter bands. Addition of 1 mM  $\text{CaCl}_2$  10 min later resulted in the rebinding of a major part of the proteins released by EDTA, to the microsomal membrane (not shown). Repeated extraction of microsomes with 1 mM EDTA at pH 8.0 (Fig. 8) led to the nearly complete removal of the two protein fractions from the membrane (marked with arrow) while others were quantitatively retained. These latter included band M which was previously identified as a component of the enzyme system involved in ATPase activity and Ca transport of skeletal muscle microsomes (Martonosi, 1969). The possible role of the proteins solubilized by EDTA in the regulation of the permeability of microsomal membranes is under investigation.

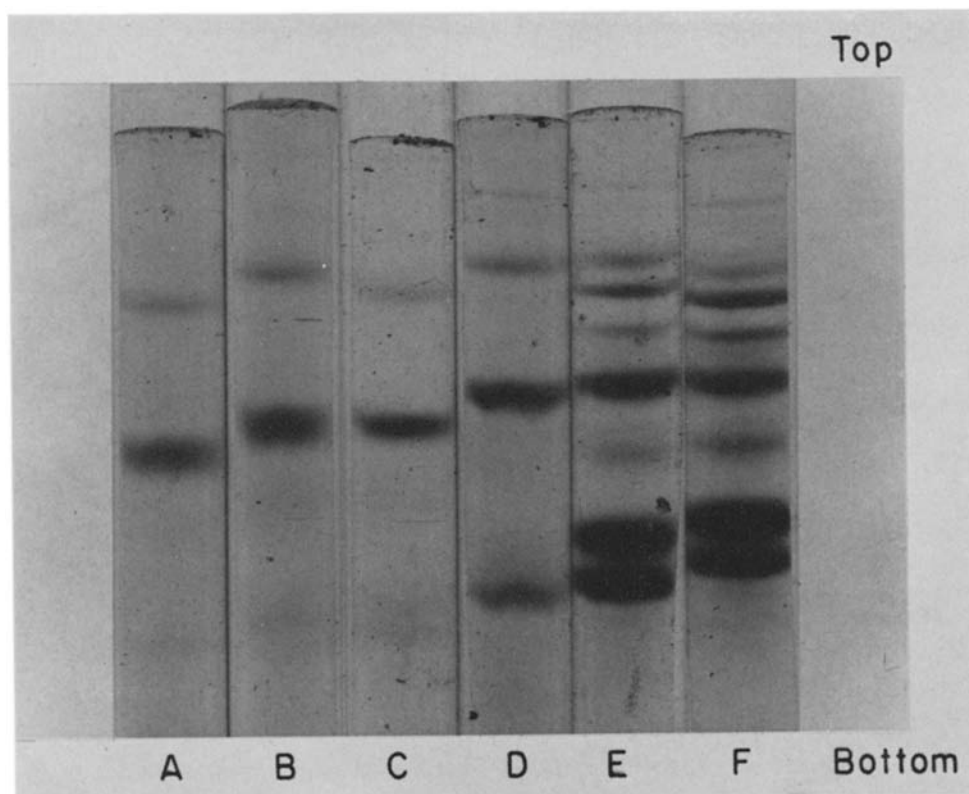


FIGURE 7. Extraction of proteins from muscle microsomes at alkaline pH. Microsomes (20 mg protein/ml) were incubated at room temperature in a medium of 20 mM Tris buffer at the pH stated below, with or without 1 mM EDTA for 15 min, followed by centrifugation at 41,000 *g* for 1 hr. The supernatants were decanted and after addition of small volumes of sucrose and sodium dodecylsulfate to final concentrations of 7.5 and 0.7%, respectively, 0.1 ml aliquots were placed on 5% polyacrylamide gels containing 0.1 M Na-phosphate buffer, pH 6.0 and 0.1% sodium dodecylsulfate. Sodium dodecylsulfate is used for solubilization of membrane proteins; sucrose for increasing the density of the solution which permits the formation of a sharp sample layer on the surface of the gel (Martonosi, 1969). Electrophoresis was carried out with a buffer containing 0.1 M Na-phosphate, pH 6.0, and 0.1% sodium dodecylsulfate at 5 ma per tube for 14 hr. A, pH 7.2; B, pH 8.2; C, pH 9.2; D, pH 7.2 with 1 mM EDTA; E, pH 8.2 with 1 mM EDTA; F, pH 9.2 with 1 mM EDTA.

*The Anion Permeability of Fragmented Sarcoplasmic Reticulum Membranes* The determination of the inaccessible water space for acetate, chloride, and EDTA requires the consideration of charge effects. Presumably as a result of the Donnan effect acetate- $^{14}\text{C}$ ,  $^{36}\text{Cl}$  (Table VI), and EDTA- $^{14}\text{C}$  (Table VII) appear to be partially excluded from microsomes especially at elevated pH's and at low ionic strength. An increase in the concentration of potassium- $^{14}\text{C}$ -

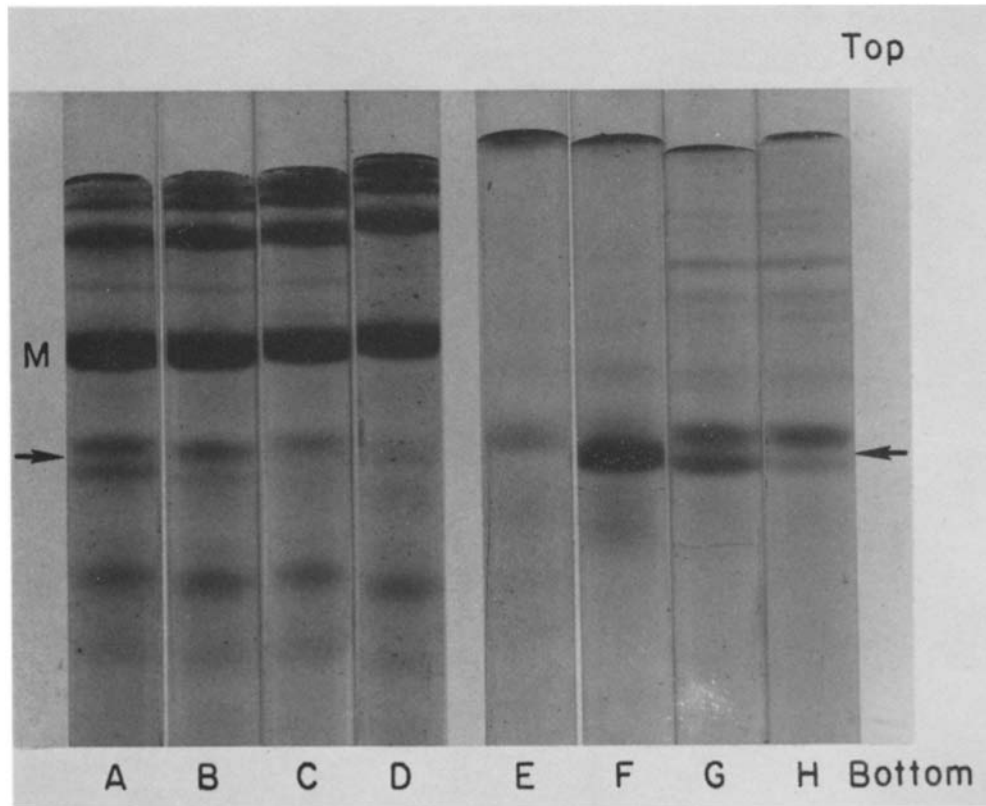


FIGURE 8. Extraction of proteins from muscle microsomes at pH 8.0 with 1 mM EDTA. A microsomal suspension containing 1 mg protein/ml was extracted three times with 1 mM EDTA in 10 mM Tris-HCl, pH 8.0. Microsomes were separated from the suspending medium by centrifugation at 50,000 *g* for 45 min. The supernatants were dialyzed against 1 mM Tris, pH 7.0, overnight, lyophilized, and resuspended in one-tenth of the original volume with water. The sediments were resuspended in 10 mM Tris, pH 8.0, to approximately 1 mg protein/ml and electrophoresis was carried out on polyacrylamide gels as described in the legend for Fig. 7. Sediments: Samples A–D, A, microsomes after one washing with 10 mM Tris, pH 8.0; B, one washing with 1 mM EDTA, pH 8; C, two washings with EDTA; D, three washings with EDTA. Supernatants: E–H, E, supernatant after one washing with 10 mM Tris, pH 8; F, supernatant after one washing with 1 mM EDTA, pH 8; G, supernatant after the second washing with EDTA; H, supernatant after the third washing with EDTA. No protein was extracted during four to six washings.

acetate or potassium- $^{36}\text{Cl}$  from 0.5 to 25 mM (Table VI) or the addition of 0.1 M KCl to a dilute solution of acetate (Table VI) or EDTA (Table VII) permits nearly complete equilibration of microsomal water space with the various anions, presumably by neutralizing charge repulsions. EDTA had little effect on the distribution of acetate in the microsomal suspension even at pH 9.2



(Table VI) where it strongly promoted the penetration of inulin into microsomes (Table VII). The decrease in inulin or EDTA inaccessible water space on addition of 0.1 M KCl (Table VII) is usually accompanied by a decrease in the total pellet water raising the possibility that the volume of intramicro-

TABLE VI  
EFFECT OF IONIC STRENGTH AND  
EDTA ON THE PENETRATION OF ACETATE  
AND Cl INTO MICROSOMES

To microsome suspensions containing 10 mM Tris-HCl buffer of stated pH and 17-20 mg protein in a total volume of 0.8 ml, inulin-<sup>14</sup>C, acetate-<sup>14</sup>C, <sup>36</sup>Cl, KCl, and EDTA were added in concentrations indicated in the table. Measurement of inaccessible water space was carried out as described under Experimental procedures. The difference in inulin space between pH 7.2 and 9.2 is unusual (see Fig. 1).

	Inaccessible water space, $\mu\text{l}/\text{mg protein}$		
	pH 7.2	pH 8.2	pH 9.2
Inulin- <sup>14</sup> C	5.05	5.4	6.2
0.5 mM acetate- <sup>14</sup> C	1.45	2.3	3.1
25 mM acetate- <sup>14</sup> C	1.4	1.65	1.65
0.5 mM acetate- <sup>14</sup> C + 0.1 M KCl	0.0	1.3	1.05
0.5 mM acetate- <sup>14</sup> C + 0.75 mM EDTA	2.55	3.0	2.5
0.5 mM <sup>36</sup> Cl	2.3	2.65	4.1
25 mM <sup>36</sup> Cl	0.4	1.35	0.9

TABLE VII  
PENETRATION OF EDTA AND  
INULIN INTO MICROSOMES

Microsomes (18 mg protein/ml) were incubated at 25°C for 10 min in 10 mM Tris-HCl buffer of pH 9.2 with the additions indicated in the table, followed by centrifugation at 41,000 *g* for 1 hr. Inaccessible space and total pellet water were measured as described under Experimental procedures.

	Inaccessible water	Total pellet water
	$\mu\text{l}/\text{mg protein}$	$\mu\text{l}/\text{mg protein}$
$2 \times 10^{-5}$ M EDTA- <sup>14</sup> C	6.15	9.40
$2 \times 10^{-5}$ M EDTA- <sup>14</sup> C + 0.1 M KCl	3.20	7.15
1 mM EDTA- <sup>14</sup> C	4.15	10.30
1 mM EDTA- <sup>14</sup> C + 0.1 M KCl	1.50	7.20
Inulin- <sup>14</sup> C	4.4	9.50
Inulin- <sup>14</sup> C + 0.1 M KCl	3.15	7.00
Inulin- <sup>14</sup> C + 1 mM EDTA	2.05	10.20
Inulin- <sup>14</sup> C + 0.1 M KCl + 1 mM EDTA	0.70	7.15

somal water space is altered due to shrinkage of microsomes. The investigation of the relative contribution of microsomal volume changes to the observed changes in inaccessible space caused by 0.1 M KCl is in progress. The permeability characteristics of citrate were similar to those of acetate.

## DISCUSSION

The permeability of microsomal membranes is influenced by pH and the membrane-bound divalent cation-presumably calcium-content. Inulin (mol wt 5000) and carboxydextran (mol wt 15,000–90,000) while completely excluded from microsomes at pH 7.0, rapidly penetrate the membrane at pH 8–9, provided sufficient EDTA is present to reduce the membrane-bound calcium content to levels below approximately 4 nmoles/mg protein. Removal of membrane-bound  $\text{Ca}^{++}$  at pH 7.0 or exposure to elevated pH without a chelating agent had no effect on the permeability of the membrane to inulin at 25°C. Since the inulin used in these experiments had a mol wt of 5000–5500, it is plausible that permeability changes to  $\text{Ca}^{++}$  and other substances of small molecular weight could be induced under even milder conditions.

Makinose and Hasselbach (1965) reported that the bound  $\text{Ca}^{++}$  content of microsomes was of the order of 0.02  $\mu\text{mole}$  of  $\text{Ca}/\text{mg}$  protein. The bound  $\text{Ca}$  was freely exchangeable with added  $^{45}\text{Ca}$ , but surprisingly, treatment of microsomes with EDTA followed by repeated washing did not markedly diminish the membrane-bound  $\text{Ca}^{++}$  content. Since EDTA consistently removed 90% of the membrane-bound  $\text{Ca}^{++}$  in our experiments, it appears that in the earlier work of Makinose and Hasselbach sufficient  $\text{Ca}^{++}$  contamination was introduced during washing of microsomes with KCl to restore the  $\text{Ca}^{++}$  content to starting levels. The 2–3 nmoles of  $\text{Ca}^{++}$  and 1–2 nmoles of  $\text{Mg}^{++}/\text{mg}$  protein which remain bound after treatment of microsomes with 1 mM EDTA, EGTA, or with chelating resin may represent a firmly bound divalent cation pool but further experiments would be necessary to establish this possibility.

In the regulation of the permeability of microsomes to inulin and carboxydextran,  $\text{Ca}^{++}$  appears to play a more significant role than  $\text{Mg}^{++}$ . This is evident from the fact that EDTA and EGTA are about equally effective in increasing the permeability of microsomal membranes to inulin, and the permeability change correlates well with the membrane-bound  $\text{Ca}^{++}$  rather than with the  $\text{Mg}^{++}$  content.

The mode of involvement of  $\text{Ca}^{++}$  in the definition of membrane permeability to inulin is unknown but some indications for an indirect mode of action were obtained. The permeability increase caused by treatment of microsomes with 0.5 mM EDTA at pH 8.0 can be partially reversed if  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  is added to the microsomal suspension with the EDTA still present. No reversal of permeability increase was found if EDTA-treated microsomes were washed free of EDTA followed by readdition of  $\text{Ca}^{++}$ .  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{La}^{+++}$  were also ineffective in reversing the permeability increase caused by Dowex chelating resin. It may be relevant in explaining these findings that treatment of microsomes with EDTA solutions at pH 8–9 (but not at pH 7) causes the selective release of well-defined proteins from the microsomal membranes as

shown by polyacrylamide electrophoresis, which are largely rebound following addition of  $\text{Ca}^{++}$ . Similar observations concerning the effect of EDTA and divalent metal ions on the binding of an ATPase enzyme to the surface membrane of *Streptococcus faecalis* were made earlier by Abrams and his collaborators (1965). Experiments are in progress to establish whether the protein fractions in question are related to the observed permeability changes. As an alternative explanation it is possible that in addition to or perhaps instead of  $\text{Ca}^{++}$ , other unidentified metal(s) are also involved. The effect of Mg in partially restoring the permeability characteristics of microsomes treated with 0.5 mM EDTA may be explained by competition of Mg with Ca for the chelating agent. Release of  $\text{Ca}^{++}$  from EDTA after the addition of Mg is suggested by the increase in the amount of membrane-bound Ca from 5.7 nmoles/mg protein in the presence of 0.5 mM EDTA, to 9.4 nmoles/mg protein when 0.5 mM EDTA and 0.5 mM  $\text{MgCl}_2$  are present (Table III A).

Molnar and Lorand (1962) reported that treatment of fragmented sarcoplasmic reticulum with Chelex resin at pH 7.0 inhibits  $\text{Ca}^{++}$  transport and activates ATPase activity. These observations are similar to ours, with the important difference that the permeability effects connected with cation depletion occurred in our experiments only at pH values above 8 and no effects on the inulin penetration, ATPase activity, or  $\text{Ca}^{++}$  transport were found on pretreatment with EDTA, EGTA, or chelating resin at pH 7.0. Alkaline pH shift during passage of microsomes through columns of Dowex chelating resin was observed in our experiments and might serve as an explanation for these differences.

At EDTA concentrations at which inulin penetration of microsomes just begins, there is an abrupt decrease in the rate of  $\text{Ca}^{++}$  uptake with increased ATPase activity. This suggests that the uncoupling of  $\text{Ca}^{++}$  transport from ATP hydrolysis is due to the increased Ca permeability of the membrane which develops prior to the appearance of major change in the inulin permeability of microsomes.

At room temperature and neutral pH sarcoplasmic reticulum membranes are relatively impermeable to  $\text{Ca}^{++}$  as judged from the slow rate of  $\text{Ca}^{++}$  release from  $\text{Ca}^{++}$ -loaded microsomes. At elevated temperatures and at alkaline pH (pH 9) marked increase in the rate of  $\text{Ca}^{++}$  release occurs implying some change in the  $\text{Ca}^{++}$  permeability of microsomes; this is also supported by observed changes in the inulin permeability. Increased loss of  $\text{Ca}^{++}$  may in part explain the drastic inhibition of net  $\text{Ca}^{++}$  uptake which occurs between pH 8 and 9.0, although decline of transport ATPase activity in the same pH range suggests a direct pH effect on the  $\text{Ca}^{++}$  pump as well. Direct measurement of the permeability of microsomal membrane to  $\text{Ca}^{++}$  or  $\text{K}^+$  is complicated by the binding of various cations to the membrane material, and by the existence

of charge effects. As a consequence, attempts made so far at the direct measurements of  $\text{Ca}^{++}$  or  $\text{K}^+$  permeability did not yield unambiguous results.

Settlemyre, Hunter, and Brierley (1968) observed that the permeability of beef heart mitochondria was altered by EDTA under conditions which are somewhat different from those found with microsomes. The swelling of mitochondria is induced by EDTA only during respiration and in the presence of high concentration of  $\text{Na}^{++}$  and a permeant anion (acetate). The increased permeability of EDTA-treated mitochondria was shown to be related to the removal of Mg, as EGTA, which did not reduce the level of membrane-bound  $\text{Mg}^{++}$ , was ineffective in promoting swelling. The penetration of EDTA into mitochondria increased at alkaline pH but only in the presence of a permeant anion and respiration enhanced EDTA entrance. These differences suggest that the mechanism of EDTA effect on the permeabilities of mitochondria and microsomes is different.

Evidence for penetration of oxalate, phosphate, and pyrophosphate through microsomal membranes was provided by their potentiating effect on  $\text{Ca}^{++}$  transport (Hasselbach, 1964), and by the  $\text{Ca}^{++}$ -linked accumulation of oxalate anions in microsomal particles (Hasselbach and Makinose, 1963; Martonosi and Feretos, 1964 *a*). The penetration of anions through microsomal membranes appears to be influenced by charge effects, as complete penetration of microsomal water space with acetate,  $\text{Cl}^-$ , and EDTA occurs only at salt concentrations exceeding 25 mM or in the presence of 0.1 M KCl; i.e., under conditions in which charge repulsions are expected to be negligible even at pH 9.2.

The ready penetration of microsomal water space by sucrose and by various anions establishes the sarcoplasmic reticulum membrane as a much less restrictive permeability barrier than the inner membrane of mitochondria, and explains the absence of major osmotic response when microsomes are placed in hypoosmotic or hyperosmotic sucrose solutions. It is not known whether this high permeability is the consequence of membrane damage suffered during homogenization or is the natural property of the membranes. If the latter possibility is correct the content of sarcoplasmic reticulum tubules *in vivo* would be expected to contain most of the important metabolic intermediates in equilibrium with the rest of the sarcoplasm. This might be of importance if sarcoplasmic reticulum membranes possess some of the enzymatic activities that are known to be associated with the analogous endoplasmic reticulum of other tissues (Dallner and Ernster, 1968).

The work was supported by research grants GB 7136 from the National Science Foundation and NB 07749 from the National Institutes of Health U.S. Public Health Service.

A preliminary report of this work was presented at the Third International Biophysics Congress of the International Union of Pure and Applied Biophysics held in Cambridge, Massachusetts, on 29 August-3 September 1969.

*Received for publication 17 September 1969.*

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