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CATION EXCHANGE AND BINDING IN SMOOTH MUSCLE

by

Ⓒ SATISH BATRA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

of

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The undersigned hereby certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled

"CATION EXCHANGE AND BINDING IN SMOOTH MUSCLE"

submitted by SATISH BATRA, in partial fulfilment of the
requirements for the degree of Doctor of Philosophy.

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.....*Oct. 31/65*..........
Date

A B S T R A C T

Part I

The effects of iodoacetic acid (IAA), IAA and 2:4
dinitrophenol (DNP), and ethacrynic acid (ETCA) (10^{-3} M)
on Na, K, Ca and water movements and Na, K, Ca, and Mg
binding in rat uterus were studied. With each of these
treatments tissues gained Na, Ca and water and lost K.
Net Ca movements showed a definite lag compared to those
of Na and K in every case. The gain in tissue water with
IAA or IAA and DNP was greater than that with ETCA, which
also occurred much later. The loss of K was initially
greater than the Na and Ca gained unaccompanied by bathing
solution. Subsequently the Na and Ca gained unaccompanied
by bathing solution was in excess of K loss. These results
suggested that the metabolic dependence and/or the trans-
port mechanism controlling Ca distribution differed from
those mechanisms controlling Na and K distribution.

Only small differences were found in the binding of
Na, K, Ca and Mg to the washed homogenates of normal
compared to inhibited tissues. Homogenates from normal
and inhibited tissues bound additional amounts of any of
these cations added in excess. Total binding was the
highest with Mg, closely followed by Ca. Total binding of
Na was similar to that of K but binding of either was much
less than Mg or Ca. The small degree of altered binding

in the inhibited tissues was insufficient to explain any of the large differences which were observed in the electrolyte composition of these tissues.

A B S T R A C T

Part II

Ca uptake was studied in mitochondrial and microsomal fractions from rat myometrium. Succinic dehydrogenase assay and electron microscopy were used to characterize the fractions. Both fractions showed Ca uptake from a medium containing ATP and Mg at 25°C and negligible uptake at 4°C, or without ATP. Ca uptake was inhibited by 0.5 mM sodium azide in mitochondria and by 0.5 mM mersalyl in mitochondria and microsomes. High Na and K decreased Ca uptake by mitochondria, but had no effect on microsomes. Aging up to 6 hours at 4°C considerably lowered uptake in mitochondria and had little effect on microsomes. Therefore the Ca uptake by microsomes differed in mechanism from that by mitochondria. Oxalate (5 mM) and inorganic P (5 mM) did not enhance Ca uptake in either fraction.

Ca uptake in the microsomal fraction was increased as the Ca concentration in the medium was increased. Sr, Ba, and La in concentrations over 4 times of that of Ca had no effect on Ca uptake, while higher concentration of Sr and Ba inhibited Ca uptake; Sr more than Ba. High concentrations of Sr inhibited Ca^{45} uptake more than high concentrations of Ca. Oxytocin, ergot alkaloids, caffeine, cyclic AMP had no effect on microsomal Ca

uptake whereas epinephrine increased it by 20%. Ca
uptake increased with increasing protein concentration.
Mg was displaced when Ca was taken up by the micro-
somes. There was no detectable extra splitting of
ATP accompanying Ca uptake in microsomes. The role
of Ca uptake by microsomes in contraction and relaxation
of smooth muscle was discussed.

TO MY PARENTS

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T A B L E   O F   C O N T E N T S

P A R T   I

L I S T   O F   T A B L E S

L I S T   O F   F I G U R E S

PART I

Effect of metabolic inhibitors on the cation
movements and binding in the rat uterus.

I N T R O D U C T I O N

The growing interest in and the importance of dis-
tribution of ions in tissues and cells is clearly reflected
by the increasing frequency of publications in the past
decade devoted entirely to this subject (1-6). Of special
interest to pharmacologists has been the demonstration that
electrolytes shifts may alter the potency of a variety of
drugs and that they may in turn, be influenced by low levels
of these drugs (7-9). Great strides have been made in
recent years by physiologists and biochemists in the invest-
igation of ion transport and related problems and several
distinguished articles have appeared. While not a compre-
hensive list, the following publications cover the field
adequately. Hodgkin (10) has considered in detail the
work of his colleagues and himself in the elucidation of
ionic shift underlying electrical activity of nerve. Shanes
(11) covered the same ground and also gave information on
the nature of drug interaction with natural and artificial
membranes. Much biochemical information is given by
McIlwain (12) and Whittam (13). An excellent review on
electrolytes and smooth muscle contraction has been written
by Bohr (14). Despite all this activity, the molecular
basis for ion transport has scarcely been approached and
it is fair to say that our present knowledge is rudimentary
although there is no shortage of speculation and even of
controversy concerning the few facts available.

It is well known that most mammalian cells contain large amounts of potassium and relatively little sodium, while the surrounding medium in most cases contains large amounts of sodium and little potassium. Similarly among alkali earth metal ions, calcium is the major constituent of the extracellular compartment while magnesium is primarily in the intracellular compartment (5). What is the mechanism by which these differences are maintained? And how are they restored after disturbance from the steady state? Partial answers to these questions with respect to sodium and potassium have in large part been given by physiologists. Sodium ion is pumped out of cells against its electrochemical gradient. Potassium may then flow in, either by a passive movement to replace sodium, the efflux of which leaves the cell interior negative (electrogenic pump) or by an active participation in the transport of sodium (the coupled sodium-potassium pump). Glynn in 1959 (15) has discussed the evidence for the latter, the existence of which in red cells was postulated by Harris and Maizels in 1951 (16) (see also Maizels 17 and Harris 18). Glynn (19) has provided convincing evidence of the existence of the coupled sodium and potassium exchange in red cells by elegant experiments with tracer. Recently, Hoffman (20) has further analysed the components of the pumping mechanism for two separate pumps. From what has been said above, it may be concluded that sodium is actively transported from

mammalian cells. For many tissues studied there is evidence that the involvement of potassium is active whereas in other tissues this has not been established. In fact, in the rat uterus the recent evidence (21) for an electrogenic sodium pump is quite convincing.

There is relatively little information available on the electrochemical gradients for Ca and Mg across the cell membranes and their dependence on tissue energy supply. Evidence for active calcium exclusion has been presented for guinea pig taenia coli (22,23) and rat uterus (24); and for active Ca extrusion in red cells (25). Distribution of Mg in muscle has been discussed by Gilbert (25) and in brain, nerve and muscle by Hilmy and Somjen (27).

Another feature of ion transport in many tissues - if not all - is the process known as exchange-diffusion described by Ussing (28). It is as its name implies, simply a process of exchange of one ion for another. Various models, with and without the use of mobile carriers have been postulated for such a transport system. These have been competently discussed by Stein (29).

ENERGY FOR ION TRANSPORT

Though there is no question that the energy for transport against an electrochemical gradient must come from cellular metabolic processes, the means by which such energy is made available to the transport system is the

subject of considerable argument. Two points of view hold the stage. The first due mainly to Conway and his colleagues (30) suggests that energy is derived directly from vectorial movements of electrons or other charged particles without the mediation of ATP. It is generally known as the 'redox pump' hypothesis. In the second ATP is considered to be the source of free energy for vectorial transport of ions. Most workers and the weight of direct evidence favour the second point of view (see 3 for discussion on the problem).

What is the mechanism by which ATP energy is utilized to cause vectorial ion transport? Numerous ideas have been discussed, schemes for reactions drawn up, and controversy has arisen over points which remain obscure (31, 32). It is difficult to relate any process directly to the utilization of ATP within whole cells since the level of ATP tends to remain steady. The reason for this is that metabolic reactions constantly phosphorylate the nucleotides so that even when there is serious drain, reserves (such as phosphocreatine) are available to rephosphorylate ADP. These reserves have to be depleted before ATP levels fall (33,34). For this purpose Cain, Infante and Davies used muscles poisoned with 1-fluoro-2,4-dinitro benzene (FDB), which inhibits selectively, the phosphate transfer from creatine phosphate. Metabolic inhibitors have been widely used to elucidate the

energetics of living cells (11,35,36,37).

The widely different chemical structures of these agents give promise that they may also provide important leads in identifying the chemical structure of intermediates of enzyme reactions and of active sites of enzyme inhibition. Iodoacetate and fluoride have been used as inhibitors of glycolysis, while cyanide, and azide have employed as inhibitors of oxidation. Dinitrophenol is the classical example of the compounds that uncouple oxidative phosphorylation (38). Several other inhibitors of which the antibiotic oligomycin is the prototype inhibit phosphorylating electron transport (39). However, as pointed out by Webb (35) the interpretation of the results using metabolic inhibitors should be made with caution. Reviewing the voluminous literature in this area Webb (35) comments that even in concentration below 1 mM, iodoacetate which appears to have a selective action on the enzyme 3-phosphoblyceraldehyde dehydrogenase can by no means be considered as a specific inhibitor of this enzyme as it affects certain alcohol dehydrogenases, succinate dehydrogenases, proteolytic enzymes etc. At higher concentrations the drug inhibits a number of other enzymes (lactate dehydrogenase glycerol kinase etc.) but these are not concerned with the supply of energy. Iodoacetate may further affect oxidative phosphorylation but only slightly (35) as it is not a true uncoupler like dinitrophenol. Seidman and Cascarano

(39) have further emphasized that the experiments using IAA and DNP should be interpreted with caution. In spite of these short comings the use of metabolic inhibitors has provided workers with important tools for studying the energetics of living cells and as a result has yielded considerable information.

Metabolic inhibitors particularly iodoacetate (IAA) and dinitrophenol(DNP) have been used to study a number of energy requiring processes in the tissues. Using the squid axon Hodgkin and Keynes (40) showed that the active recovery process following depolarization was inhibited by DNP and cyanide. This suggested that energy derived from oxidative phosphorylation was utilized for the recovery processes. Subsequently Caldwell (41) showed that immersion of axons of the squid Loligo forbesi in sea water containing 2 mM cyanide for 100 minutes or 3 mM azide for 150 minutes considerably lowered the levels of ATP and phosphagen. Immersion in DNP (0.2 mM for 100 minutes) reduced the phosphagen fraction to the low level but had little effect on the ATP level. A correlation between the function of sodium extrusion process and the amount of these phosphate esters was demonstrated. Injection of ATP into the axoplasm restored sodium efflux from poisoned axons (42).

The metabolic requirement for ion movement and contraction in skeletal muscle has also been studied. Carey,

Conway and Kernan (43) using sodium loaded muscle showed that sodium extrusion was completely inhibited by IAA, low temperature and ouabain, but only partially by cyanide or anoxia. Dydynska and Harris (44) found that metabolic inhibition of ion movements in frog sartorius could be correlated with reduction in ATP. Similarly relationship between net cation movement against a gradient and the break down of high energy phosphate esters has been demonstrated in brain by McIlwain (45), in liver by Judah and Ahmed (46) and in red cells by Whittam (47) and Glynn (48).

The effect of metabolic inhibitors has been studied on several smooth muscle preparations from various angles (49-56). Bülbring and her colleagues (49-51) showed that metabolic inhibitors altered both the electrical and the mechanical activity of intestinal muscle. For example DNP, IAA and azide initially stimulated and then depressed the muscle contraction. Daemers and Lambert (57) found that immersion of carotid artery for 30 minutes in IAA containing Tyrodes solution (60-nM KCl) caused a marked decrease in ATP and creatine phosphate content of this muscle. Daniel (54) studied the effect of a number of metabolic inhibitors and ouabain on the contractility of the uterine muscle. He found that ouabain, fluoride, potassium depletion, cold, IAA and DNP, all caused a loss of contractility. Paton (58) showed

that contractile responses of uterine muscles were in-
hibited by IAA in concentrations as low as 0.25 mM and
that these could be incompletely reversed by pyruvate.
Similar results were obtained by Paton (55) later on
rabbit detrusor muscle showing that DNP only depressed
responses which could be reversed to normal after
washing out DNP. The contractile responses were not
abolished by DNP provided that D-glucose was present in
the medium. On the other hand responses were abolished
with IAA and could not be reversed by washing out IAA.
It was concluded that the energy of contraction of these
smooth muscles can be supplied by glycolysis alone and
that the metabolic pathway of the Krebs cycle is present
in these cells. Daniel et al have recently shown that
IAA, 1 mM, produced very marked effects on estrogenized
rat uterine horns; after 1 hour marked downhill ion
movements had occurred, ATP levels had fallen by 90% and
contractility was abolished. Marshall and Miller (52)
found that both DNP and IAA inhibited the electrical
and mechanical activity of the uterus and greatly
diminished its response to oxytocin.

As pointed out above, metabolic energy is needed in
muscle cells not only for contraction but also for the
establishment and maintenance across the cell membrane
of the electrochemical gradients essential for normal

excitability. Until quite recently there was little that could be said about the ionic gradients across the smooth muscle membrane or the permeability of membranes for ions such as Na, K, Cl, Ca, etc. The lack of information on smooth muscle electrolytes compared with the data on skeletal muscle was noted by Manery (59). Kao (60) has further emphasized this observation and has pointed out the reasons responsible for this difference.

In general, recent studies have tended to confirm the findings of those of earlier workers who found that smooth muscle has a relatively high NaCl content and high permeability to ions and water (61,62). Daniel (63) also showed that although the K concentration of smooth muscles was generally less than that of striated muscle, the sodium concentration was much higher and sums of these cations (Na & K) were greater and therefore the possibility of binding of these cations was considered. Binding of monovalent cations has been shown in frog, lobster and crab striated muscles (64,65). McLaughlin and Hinke (66) using cation sensitive microelectrodes found that 84% of the Na was unavailable to the electrode in the myoplasm of giant barnacle muscle fiber. There is now convincing evidence that some electrolytes in smooth muscle are found in an osmotically and electro-chemically inactive form. Goodford (67,68) has presented evidence for the binding of Na, K, Ca and Mg to super-ficial anionic sites in the smooth muscle of guinea pig

taenia coli. It was suggested, that the negative regions
in this smooth muscle were either relatively unselective
in their choice of cations or that there were different
types of anionic sites in different regions. Headings
et al (69) reported that a relatively large proportion of
Na was bound in the dog carotid artery and presented
evidence that this Na was bound to the mucopolysaccharides
in this tissue. They further showed that K was able to
compete with Na for the binding sites. Earlier studies
of Goodford and Hermansen (70) also suggested an extra-
cellular binding of Na in taenia coli. Schatzmann (71)
reported that nearly half of the Ca in intestinal smooth
muscle was present in an electrochemically inactive form.
Kao (72) and Daniel and Daniel (73) have given estimates
of bound Na and K in the uterine muscle. Recently Van
Breemen et al (24) estimated the amount of bound calcium
in the rat uterus. The importance of the role of the
bound ion in smooth muscle has been discussed by Bohr
(14).

Daniel and Robinson (74) studied the effects of
metabolic inhibitors on the extrusion of Na from cat and
rabbit uterine muscle which were depleted of K and en-
riched with Na. They showed that inhibitors of glycolysis
(iodoacetate and fluoride) were both effective inhibitors
of active transport. Inhibitors of the electron transfer
chain (cyanide, azide and antimycin A) were ineffective. DNP

produced an inhibition of active ion transport and since cocaine significantly restored the depressed potassium reaccumulation after DNP it was suggested that DNP may have altered resting permeability to potassium. Siegman and Kao (75) using cold stored myometrium of estrogen dominated rabbit uterus showed that Na and K movements could be dissociated. According to these authors, Na extrusion appeared to be dependent on oxidative metabolism whereas K accumulation derived its energy from glycolysis. Recent studies of Daniel and Robinson (76) using estrogen dominated rat uteri have shown that with IAA, DNP, IAA and DNP, the onset of the downhill ion movement corresponded to the reduction in the tissue ATP level. These inhibitors prevented the extrusion of Na and accumulation of K in sodium rich tissues as well as caused net Na gain and K loss in fresh tissues. With prolonged glucose depletion which also eliminated most of the ATP and ADP, there was equivalent gain of Na and loss of K, but Na efflux was not slowed to the same extent as with IAA.

A study of calcium distribution and exchange in the rat uterus by Van Breemen et al (24) proposed an active transport for calcium. They identified it as an exclusion mechanism that derived its energy from cell metabolism, since metabolic inhibition by IAA and DNP caused a downhill Ca movement into the cells and had no effect on Ca efflux. Metabolism dependent Ca exclusion has also been

demonstrated for guinea pig taenia coli (22,23). However, as pointed out in the above study (24); active Ca transport appears to have several unusual features. Firstly it was not inhibited by ouabain and therefore differed from liver and cardiac muscle in this respect (77). In the rat uterus, 10^{-3} M ouabain has been shown to inhibit Na and K transport (54,78) and Mg and Na+K activated ATPase (79). Secondly the concentration of IAA required to cause down-hill Ca movement was tenfold of the concentration for Na and K downhill movements. Finally, cooling to 4°C for 24 hours did not cause Ca gain while cold (below 7°C) causes downhill movements of Na and K in this tissue (74) and in the guinea pig taenia coli a net Ca gain was induced by cold (22).

The dissimilar features of Ca transport on one hand, and Na and K transport on the other hand in the same tissue led to the possibility of separate mechanisms for Na-K transport, and for Ca transport across the cell membrane of the rat uterus. To investigate t is possibility it was decided to study the time course of the effect of metabolic inhibitors, namely IAA and DNP on the downhill Ca and Na, K movements in the rat uterus. Ethacrynic acid (ETCA) a newer diuretic which increases excretion of Na, Cl, and water, and to a lesser extent K and H (80,81) was also included to compare its action on the electrolyte movements to that of metabolic inhibitors. Ethacrynic

acid has also been reported to inhibit Na efflux from red cell, and this inhibition is ouabain insensitive, but sensitive to the external Na (20).

It has previously been shown (24,53,76) that these procedures lead to extensive changes in the electrolyte composition of the tissue. For example IAA (10^{-3} M) treated uterine tissues (after 3 hours) contain 758 mmoles Na, 155 mmoles K, 20 mmoles Ca and 30 mmoles Mg, whereas the content of these cations in the normal tissues are 480, 410, 12 and 27 mmoles/Kg dry weight of Na, K, Ca, and Mg respectively (24,53,76,87).

There is the possibility that an altered binding of these cations in the inhibited tissues might be responsible to some degree for the observed electrolyte shifts. A method was therefore developed to estimate the relative binding and exchange of these cations in the normal and inhibited tissues.

M E T H O D S

Total Uterine Electrolyte Concentration

Experimental Animals and Preparation of Tissues

Female Wistar rats weighing between 100 and 150 grams were pretreated with 50 micrograms of diethylstilbesterol administered subcutaneously in oil to stimulate the growth of the myometrium. Each rat was killed by a blow on the head, its peritoneal cavity opened, the uterine horns removed and freed of fat. Each horn was slit open and placed in Krebs Ringer (K.R.) medium. This procedure took less than 2 minutes.

For most of the experiments each horn was cut into two halves, one was treated and the other half was run as the control.

Solutions

Krebs Ringer Bicarbonate solution (K.R.) had the following composition: NaCl 115 mM, KCl 4.63 mM, $CaCl_2$ 2.47 mM, $MgSO_4$ 1.16 mM, NaH_2PO_4 1.16 mM, $NaHCO_3$ 21.9 mM, glucose 49 mM, pH 7.4 when bubbled with 95% O_2 and 5% CO_2.

Drugs

Iodoacetic acid (IAA), 2,4-Dinitrophenol (DNP) and Ethacrynic Acid (ETCA).

Iodoacetic acid was purchased from Eastman Organic

Chemicals, Dinitrophenol from Fisher Scientific, while Ethacrynic acid was a gift from Merck Sharp & Dohme of Canada.

Media

IAA, DNP and ETCA were added in K.R. solution to give a final concentration of 10^{-3} M. To facilitate dissolution ETCA was first dissolved in the appropriate amount of $NaHCO_3$ solution before it was added to the K.R.

Tissue Incubation

Uterine horns were allowed to recover for 15 minutes in K.R. medium before transferring them into the incubation medium at 0 time, because, the electrolytes content of the tissues varied considerably depending on the speed and handling during the dissection. Tissues were then incubated in appropriate K.R. medium; i.e. control or IAA or IAA and DNP or ETCA for appropriate time intervals (5 minutes to 5 hours) at 25°C and bubbled with 95% O_2 and 5% CO_2 during incubation. After incubation tissues were taken out blotted and placed in weighed test tubes, and the test tubes weighed again for tissues wet weight. These test tubes containing tissues were dried in an oven at 100°C for 25 hours.

Determination of Total Tissue Na, K and Ca Concentration

After weighing the dried tissues, they were wet ashed

by heating to 200°C in concentrated nitric acid with a drop of hydrogen peroxide added to hasten the digestion. The digestion took approximately 48 hours. The ash was dissolved in 10 cc. of 0.1N HCl. For Na and K determination, this solution was further diluted by 1 to 5. Ca, Na and K contents of the dissolved ash were measured by flame emission using Unicam S.P. 900 spectrophotometer and matching standards.

Preparation of Standards

Standards matching Na, K, Ca and Mg content of the samples were prepared. The values obtained by calibration using these standards were not significantly different from the values obtained by the Internal Standard Method used earlier in this laboratory (24,82). Since the external standard method is simpler, it was used throughout for the analysis of the cations in this study.

Magnesium Carbonate, Calcium Carbonate, Sodium Chloride and Potassium Chloride were used. These salts were dried at 100°C for 48 hours, and then cooled in a desiccator before weighing the exact amount to make a stock solution. This stock solution was a 100 fold concentrate of the lowest standard used for the calibration curve. The final standards were made by appropriate dilution of the stock solution, and also contained 0.1N HCl. The concentration of electrolytes in the experimental
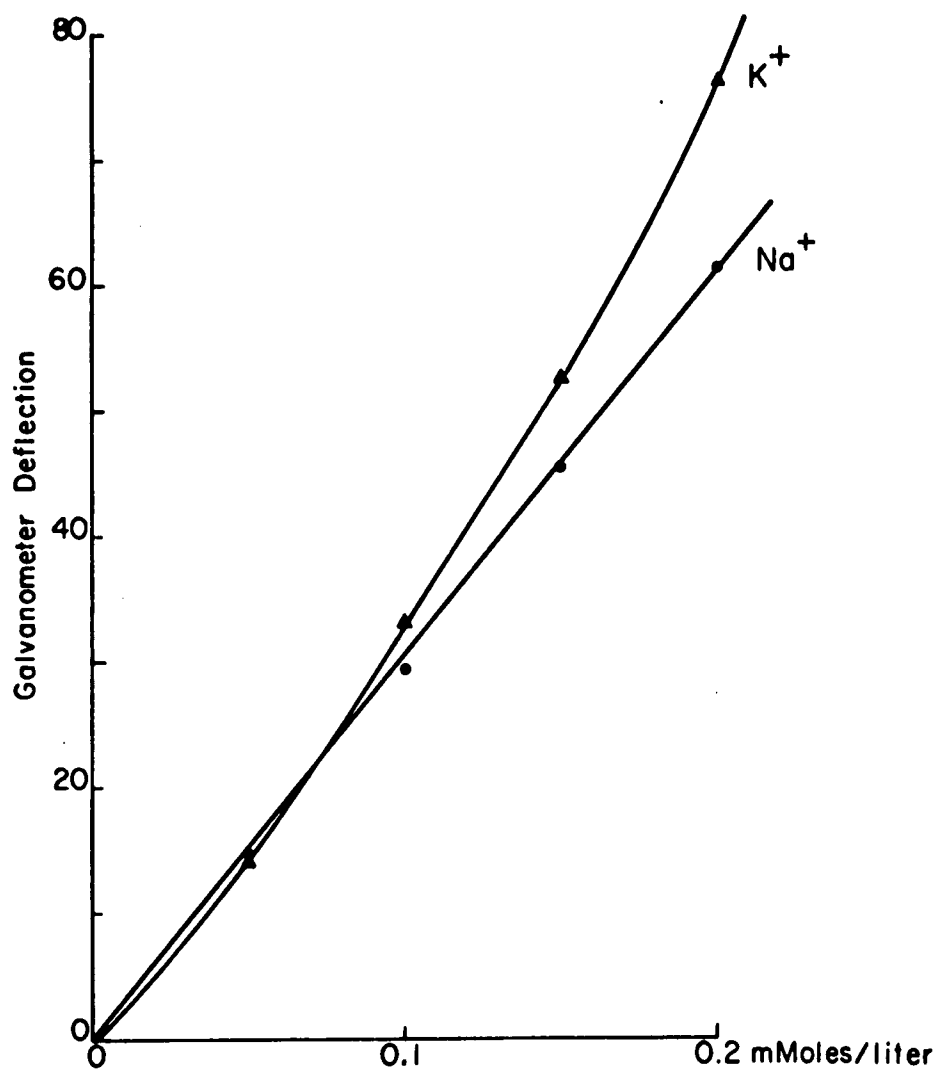
FIGURE 1

Calibration curve for the determination of Na
and K using Unicam SP 900 Flame Spectrophotometer.
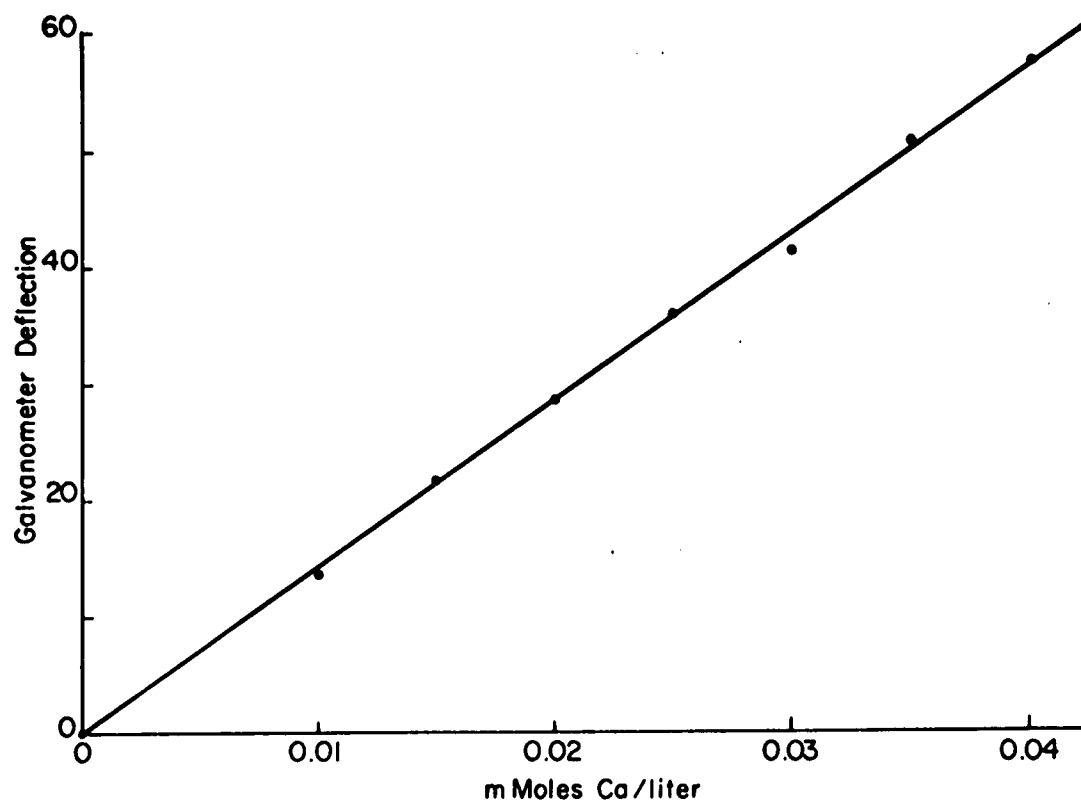
FIGURE 2

Calibration curve for the determination of Ca using
Unicam SP 900 Flame Spectrophotometer.

samples was determined from the calibration curves for Na, K, Ca shown in Figs. 1 and 2.

Cation Binding and Cation Exchange

In most of these experiments six rats were used. Each of 4 horns taken at random from a pool of 12, were incubated in control, IAA, or ETCA Krebs Ringer medium for 5 hours as detailed in the previous section. Tissues were then taken out and a part from each treatment saved for electrolyte analysis.

The remaining tissues of each treatment were taken out, rinsed for 2 to 3 seconds in cold (4°C) 0.25 M sucrose and then homogenized with the polytron 20 ST. to give approximately a 2% W/V suspension.

Separation of Particulate Bound Cations

Four ml of the homogenates from control, IAA or ETCA tissues were taken in centrifuge tubes (Oak Ridge type, Fisher) and centrifuged for 1 hour at 110,000x g in a Model L2 Spinco Ultracentrifuge. Usually the supernatant was discarded and the pellet resuspended in 0.25 M sucrose after rinsing once with deionized water and recentrifuged for 1 hour at the same speed. These are called washed pellets. In some earlier experiments however, the pellet was not resuspended and was used after the first centrifugation. These are called unwashed pellets. The supernatant after the second centrifugation was dis-
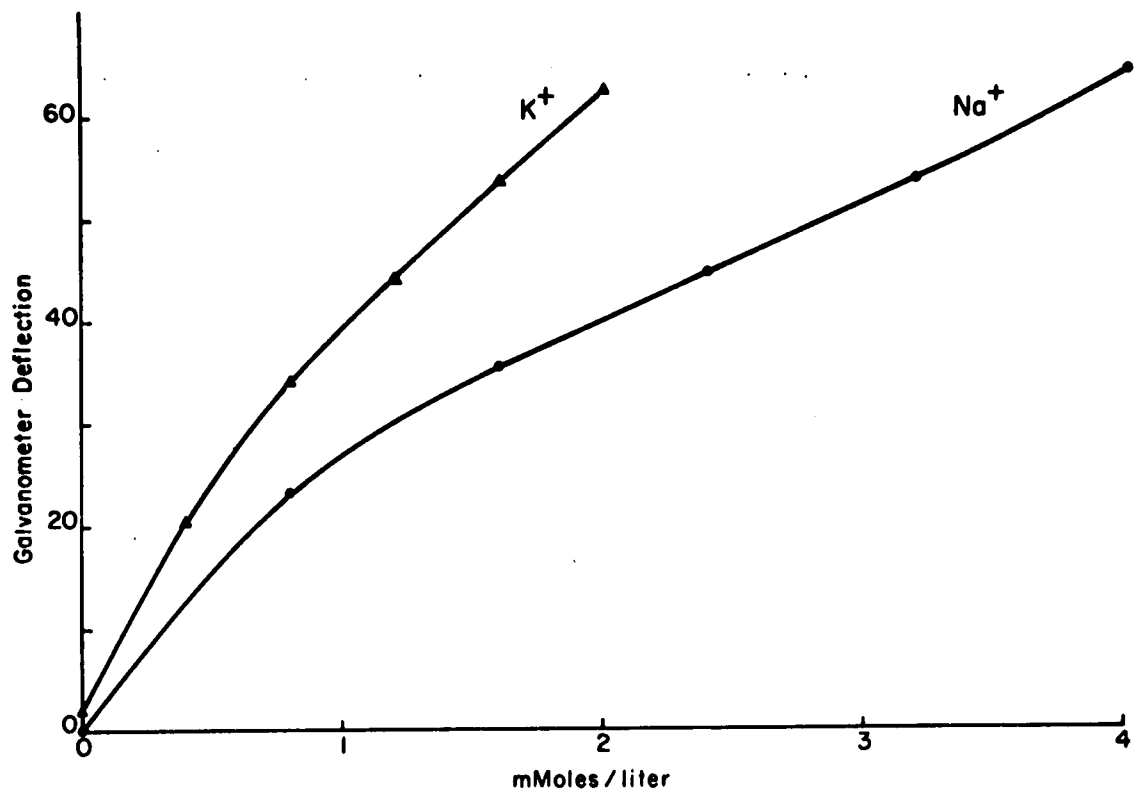
FIGURE 3

Calibration curve for the determination of Na and K using EEL Flame Photometer.
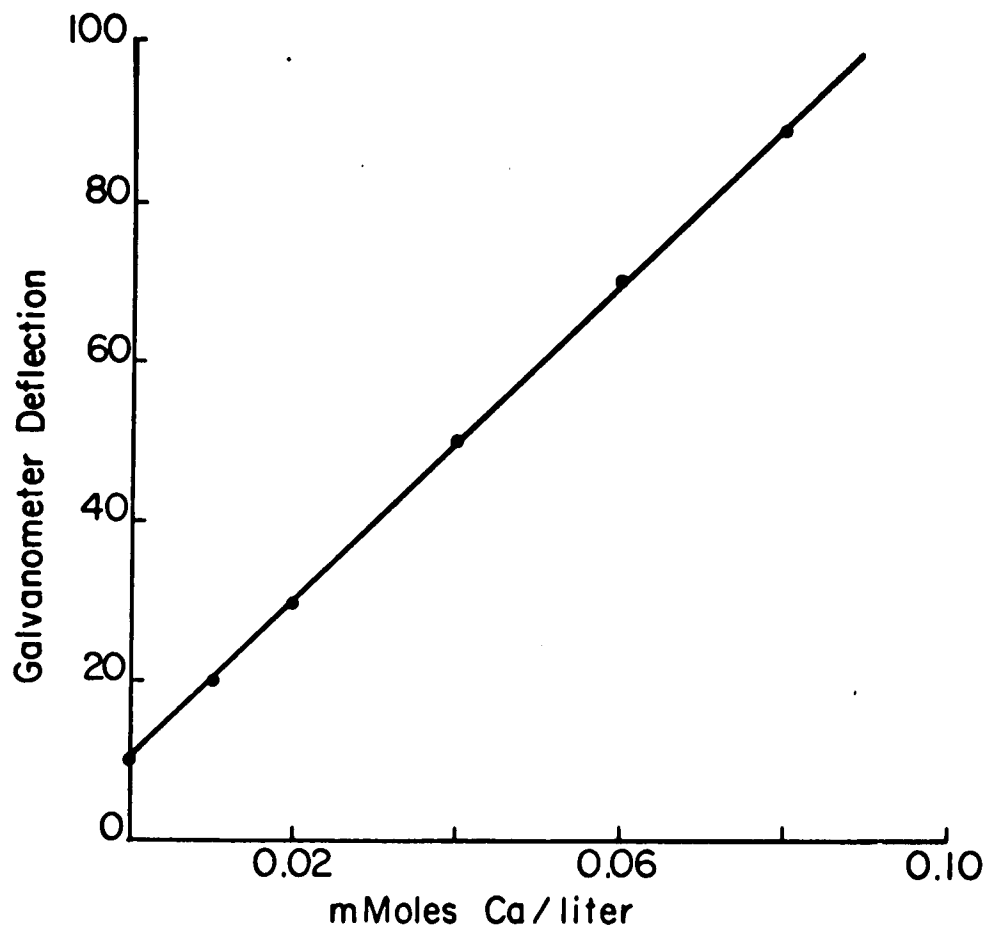
FIGURE 4

Calibration curve for the determination of Ca using
Unicam SP 90 Atomic Absorption Spectrophotometer.
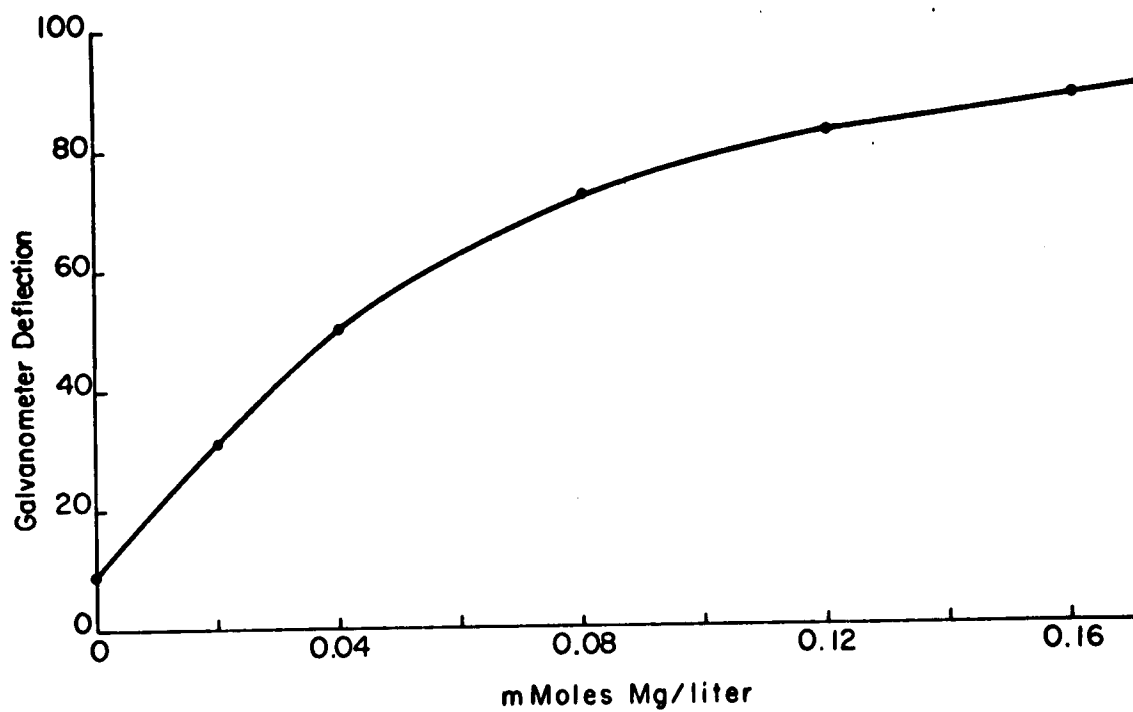
FIGURE 5

Calibration curve for the determination of Mg using
Unicam SP 90 Atomic Absorption Spectrophotometer.

carded again. The pellet and the sides of the tube were rinsed three times with deionized water. The pellet was then suspended in about 1 ml of water and dried at 100°C to a constant weight. The drying of the pellet suspension took between 48 to 60 hours. After taking their dry weight they were wet ashed as described in the previous section.

In experiments for the release and exchange of cations using Na_4 EDTA and chloride salts of Ca, Mg, Na and K^+, 0.2 ml of the appropriate salt solution were added to the 4 ml of the homogenates in the centrifuge tubes. Deionized water was used for the controls. The tubes were centrifuged and the rest of the procedure was the same as above for the separation of particulate bound cations.

Determination of Electrolytes in the Pellet Ash

Ca and Mg in these samples were measured by atomic absorption using S.P. 90 spectrophotometer while Na and K were measured with EEL flame photometer. The ash after digestion was dissolved in 10 ml of 0.1N HCl containing 0.5% $LaCl_3$, and the concentration of Na, K, Ca and Mg determined from the calibration curves such as those shown in Figs. 3 to 5. The standards used for the calibration curves also contained 0.5% $LaCl_3$. $LaCl_3$ was added to overcome any interference in calcium absorption by phosphate.

R E S U L T S

Whole Tissue Experiments

Total Tissue Electrolytes

Electrolytes contents have been presented in terms of tissue dry weight. Dry weights were shown in separate studies (76) not to change more than 1 to 2% even after long exposure to metabolic inhibitors. The mean electrolyte content of the tissue has been expressed as mmoles per kilogram solid, and water as grams of water per gram of solid. The figure after the mean is its standard error.

Fig. 6 shows the Na, K and Ca and water content of the control tissues with time of incubation. Although there were no significant changes in their amounts with time, separate control was run with each treated tissue to minimize the individual variations.

The means and standard errors of changes in the treated tissues compared to paired controls were first calculated as percentage of control values. After judging the significance of the standard error values on the basis of null hypothesis, the mean percent differences and standard errors were multiplied by the pooled control values at 0 time divided by 100 and added or subtracted from the control values, as the case may be. These values were then plotted as shown in Figs. 6 to 9. This method of representation makes it easier to see the comparison between the values obtained by various treatments by eliminating the differences between the control groups.
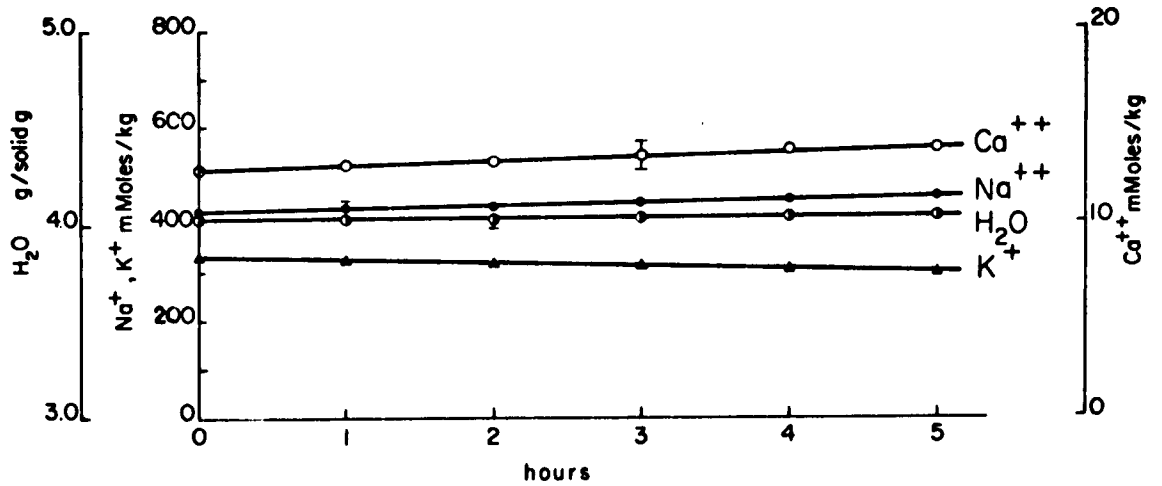
FIGURE 6

Effect of time of incubation in Krebs Ringer on the
electrolytes content of rat uterus. Na (●),
K (▲), Ca (O), H₂O (◑).
Each point is mean of 5 determinations. Vertical bars
on either side of points indicate S.E. In the absence
of a bar S.E. is within the point.

Effect of Drugs on Tissue Electrolytes

Iodoacetic acid: In the presence of 10^{-3} M IAA (Figure
7), tissues lost K (from 338 ± 4.5 to 263 ± 15.3 mmoles)
and gained Na (from 427 ± 2.8 to 458 ± 9.2 mmoles(in
the first hour, but they did not gain either Ca or water
in this time. After 2 hours their Ca content also in-
creased (from 12.8 ± 0.25 to 16.8 ± 0.51 mmoles) as did
their water contents (from 4.03 ± 0.08 to 4.33 ± 0.10 g).
After 2 hours in IAA, tissue Ca and Na continued to in-
crease and K to decrease steadily until the last measure-
ment at 5 hours. The rates of uptake of water and Na
slowed between the 2nd and 3rd hour after which they in-
creased for the remainder of the experiment.

Iodoacetic acid and dinitrophenol: When IAA and DNP
(10^{-3} M each) were used together, Na and K downhill move-
ments began immediately. Tissue K decreased from 338 ±
4.5 to 300 ± 5.7 mmoles and tissue Na increased from 427 ±
2.8 to 466 ± 5.1 mmoles in the first 5 minutes and contin-
ued to increase over the time intervals shown [Fig. 8(a)].
Ca gain did not occur until after 15 minutes [Fig. 8(b)].
Then Ca increased slightly (from 12.8 ± 0.25 to 13.7 ± 0.36
mmoles). Over the same time period Na content increased
from 427 ± 2.8 to 535 ± 14.1 mmoles, and K decreased from
338 ± 4.5 to 229 ± 11.4 mmoles.   After one hour Ca in-
creased to 15.3 ± 0.54 mmoles while Na increased to
664 ± 14 mmoles and K decreased to 108 ± 11.1 mmoles.
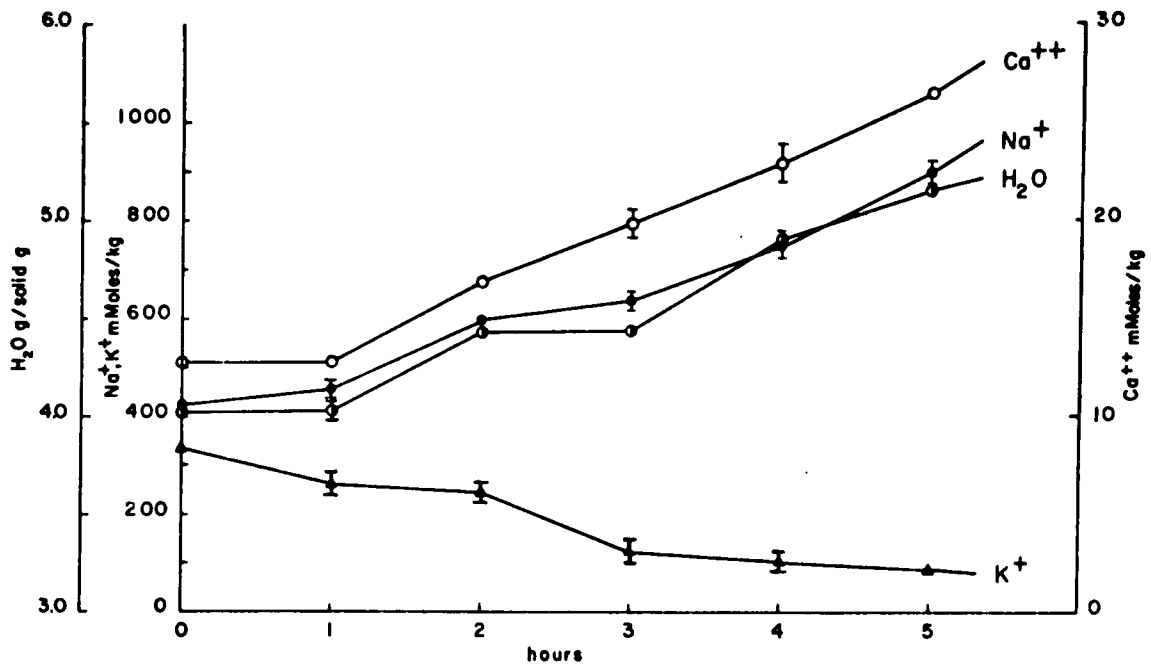
FIGURE 7

Effect of IAA $(10^{-3}$ M) on the electrolytes content of
rat uterus. Na (●), K (▲), Ca (O), H_2O (◑).
Each point is mean of 5 determinations. Vertical bars
on either side of points indicate S.E. In the absence
of a bar S.E. is within the point.
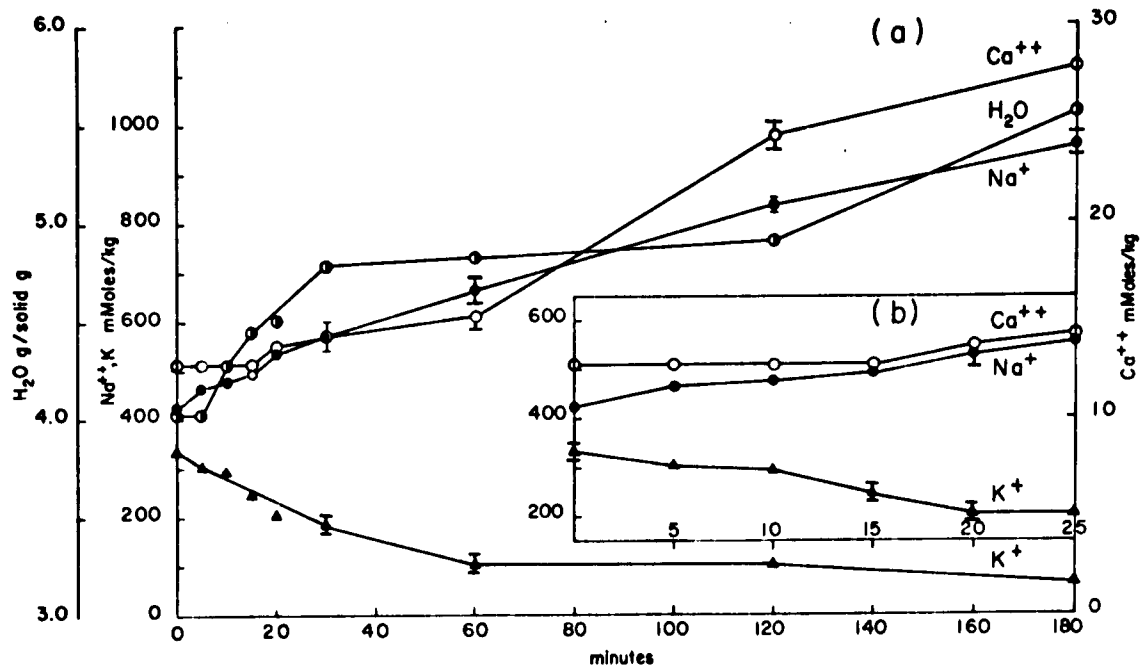
FIGURE 8(a)

Effect of IAA and DNP (10^{-3}M each) on electrolytes content of rat uterus. Na (●), K (▲), Ca (O), H_2O (◑). Each point is mean of 5 determinations. Vertical bars on either side of points indicate S.E. In the absence of a bar S.E. is within the point.

FIGURE 8(b)

Same as Figure 8(a) but on expanded scale for time on abscissa.

The effect of combining DNP with IAA affected Na
and K movements more than Ca movements. Comparison of
electrolytes contents with IAA (Fig. 7) and with IAA
and DNP (Fig. 8) shows this. During the period between
60 and 180 minutes, tissues in IAA and DNP gained
additional Ca and also gained Na in excess of the K lost.
Ca increased from 15.3 $\pm$ 0.54 to 27.7 $\pm$ 0.25 mmoles, Na
from 664 $\pm$ 14 to 952 $\pm$ 14 mmoles and K decreased from
108 $\pm$ 11.1 to 69 $\pm$ 6.4 mmoles. Gain of Na and Ca during
this period was not closely associated with the uptake
of water. Water content in fact showed a partly indepen-
dent pattern, steady increase in the first hour and no
significant change in the second, followed by another
large increase in the third.

Ethacrynic Acid: Tissues in 10^{-3} M ETCA (Figure 9)
for one hour lost K (from 338 $\pm$ 4.5 to 299 $\pm$ 10.3 mmoles),
gained Na (from 427 $\pm$ 2.8 to 449 $\pm$ 6.3 mmoles), but did
not change their Ca or water contents. Ca increased
after 2 hours from 12.8 $\pm$ 0.25 to 15.6 $\pm$ 0.47 mmoles per
kilogram solid. Between 3 hours and 5 hours Ca and Na
continued to increase while K decreased. These changes
are similar to those with IAA, but slower in onset. For
example, after 3 hours in ETCA (Fig. 9), Ca, Na, and K
contents were similar to those after 2 hours in IAA (Fig.7).
However, the K loss in ETCA lagged behind Na gain com-
pared to net ion movements in other inhibitors (see
Discussion). Water content was not affected even after
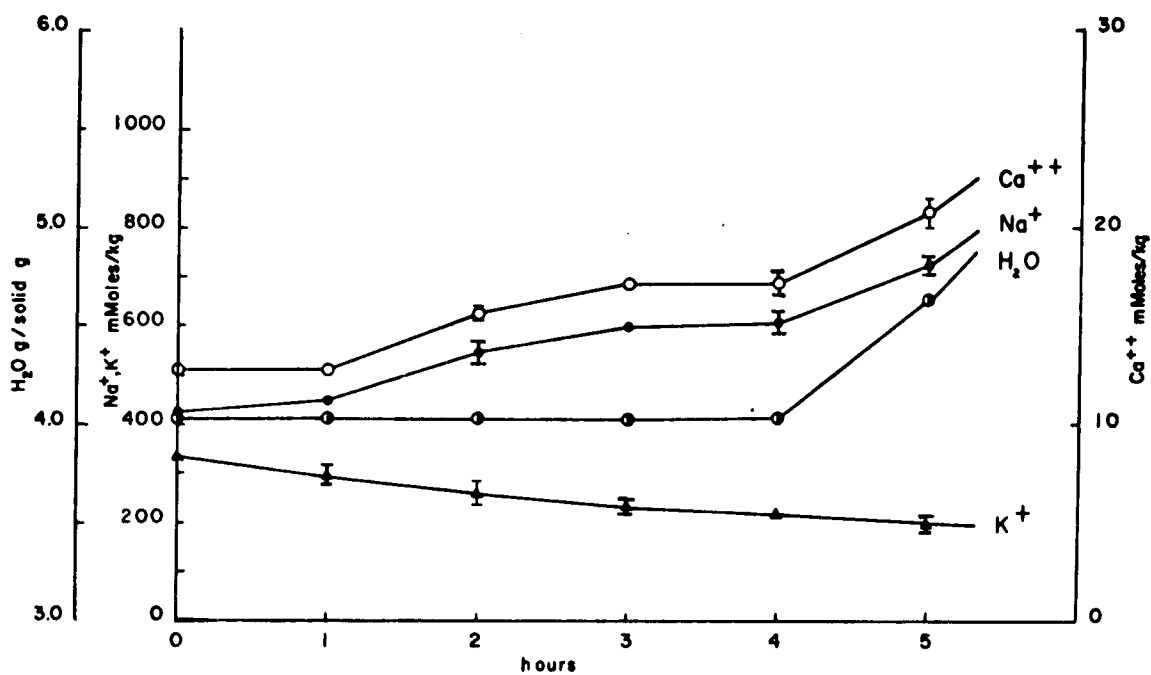
FIGURE 9

Effect of ETCA (10^{-3} M) on the electrolytes content of rat uterus. Na (●), K (▲), Ca (O), H_2O (◐).

Each point is mean of 5 determinations. Vertical bars on either side of points indicate S.E. In the absence of a bar S.E. is within the point.

4 hours in ETCA, but increased markedly between 4
and 5 hours.

Cation Binding and Exchange

Tissue cations and cations in the pellets of
homogenized tissues were measured. Mg along with
Na, K and Ca was determined in these experiments. Total
Mg was found to be lower in IAA inhibited tissues
(Table 1) as compared to the normal tissues, but ETCA
treated tissue showed no change in Mg content.

Of total Na and K only the small amounts (29.97
mmoles and 21.70 mmoles respectively) were found in
the washed pellet (see Methods). A large portion of
Ca and Mg remained bound as shown in Table 2. Large
differences exist in the electrolyte composition of the
normal and IAA treated tissues as seen in Table 1. How-
ever, small but significant differences in the
tightly bound Ca (increase) and K (decrease) and
an insignificant difference in the tightly bound Na
(increase) and Mg (decrease) were observed in the pellets
from these tissues (Table 2). ETCA produced no signifi-
cant changes in the tightly bound cations as compared to
the control.

In Tables 3, 4 and 5 the effects of adding EDTA and
Na, K, Ca and Mg (chloride salts) on the release and
exchange of pellet cations are shown. Using 0.25 mM EDTA
(tetra sodium, adjusted to PH 7 with HCl) one could

TABLE 1

Tissue Electrolytes as mmoles/Kg Dry Wt.

Treatment	Na^+	K^+	Ca^{++}	Mg^{++}
Nil	443.95 ± 17.92	371.16 ± 11.04	18.37 ± 0.74	26.73 ± 0.72
ETCA	639.54 ± 51.47	156.76 ± 17.55	29.11 ± 2.62	27.83 ± 1.86
IAA	723.28 ± 23.88	67.41 ± 6.70	33.51 ± 3.10	20.45 ± 0.87

Values are means of 5 experiments ± S. E. M.

TABLE 2

Pellet Electrolytes as mmoles/kg Dry Wt.

Treatment	Na	K	Ca	Mg	Total meq/kg
Nil	29.97 ± 1.79	21.70 ± 1.13	11.96 ± 0.94	19.30 ± 1.76	114.19 ± 8.32
ETCA	29.65 ± 2.00	18.90 ± 1.64	13.67 ± 1.43	18.11 ± 2.38	112.11 ± 11.26
IAA	31.83 ± 1.55	15.22 ± 1.47	16.70 ± 1.13	16.20 ± 2.63	112.85 ± 10.54

Values are means of 5 experiments ± S.E.M.

remove (Table 3) up to 64% of pellet Ca and 61% of pellet Mg, although the added amount of EDTA was calculated as to be sufficient to remove all the pellet Ca and Mg. There was no change in K content and the small increase seen in Na is due to the addition of Na with Na_4 EDTA.

Addition of 5 mM Ca (Table 3), replaced a small, but significant amount of pellet Na and an insinificant amount of K. Ca replaced more than half of the pellet Mg. Ca itself was bound (59.20 - 11.96 = 47.24 mmoles) considerably more than required if it replaced the cations [Na + K + Mg equalled $\frac{11.62}{2} + \frac{6.25}{2} + 10.38 =$ 19.31 mmoles] released. Addition of 5 mM Mg removed insignificant amounts of Na and K. It released about one third (11.96 - 8.09 = 3.87 mmoles) of Ca, but this is much lower than the amount (10.38 mmole) of Mg released by Ca. A large amount of Mg itself (70.61 mmoles) was bound which is higher than the amount of Ca (59.20 mmoles) bound when an equal concentration of Ca (5 mM) was added. Thus the total cation binding capacity is higher in the presence of Mg than in the presence of Ca. Addition of 150 mM of Na or K removed as much Ca as was removed by 5mM Mg and removed as much Mg as was removed by 5 mM Ca. The total binding capacity however in the presence of Na or K is much lower than in the presence of divalent cations.

In Table 4 and 5 the effects of the addition of these salts on the pellet bound cations of the ETCA treated

TABLE 3

Electrolytes in the Pellet as mmoles/Kg Dry Wt.

Control Tissues

Treatment	Na	K	Ca	Mg	Total meq/kg
Nil	29.97 ± 1.79	21.70 ± 1.13	11.96 ± 0.94	19.30 ± 1.76	114.90 ± 8.32
EDTA 0.25 mM	37.85 ± 2.77	21.90 ± 2.20	4.27 ± 0.80	7.52 ± 1.32	83.35 ± 9.21
Ca 5 mM	18.30 ± 2.47	18.45 ± 1.42	59.20 ± 1.64	8.92 ± 0.28	169.99 ± 7.33
Mg 5 mM	22.78 ± 3.51	18.27 ± 1.15	8.09 ± 0.88	70.61 ± 3.0	198.45 ± 12.42
Na 150 mM	72.58 ± 7.88	16.40 ± 1.80	7.52 ± 1.20	7.89 ± 1.76	119.80 ± 14.60
K 150 mM	23.5 ± 2.10	69.23 ± 6.30	7.64 ± 0.92	8.12 ± 1.26	124.25 ± 12.76

Values are means of 5 experiments ± S.E.M.

TABLE 4

Electrolytes in the Pellet as mmoles/Kg Dry Wt.

ETCA Tissues

Treatment	Na	K	Ca	Mg	Total meq/kg
Nil	29.65 ± 2.00	18.90 ± 1.64	13.67 ± 1.43	18.11 ± 2.38	112.11 ± 11.26
EDTA 0.25 mM	36.73 ± 3.56	17.37 ± 0.95	3.72 ± 1.02	6.31 ± 0.89	74.16 ± 8.33
Ca 5 mM	15.69 ± 1.67	15.76 ± 0.14	61.59 ± 3.09	9.17 ± 0.39	172.97 ± 8.77
Mg 5 mM	24.67 ± 2.11	17.33 ± 1.08	7.93 ± 1.49	73.24 ± 5.99	204.34 ± 18.15
Na 150 mM	76.95 ± 7.59	15.53 ± 2.81	8.52 ± 2.11	7.48 ± 1.38	124.48 ± 17.38
K 150 mM	22.91 ± 1.85	68.36 ± 5.86	7.82 ± 1.13	8.52 ± 1.16	123.95 ± 12.29

Values are means of 5 experiments $\pm$ S.E.M.

TABLE 5

Electrolytes in the Pellet as mmoles/Kg Dry Wt.

IAA Tissues

Treatment	Na	K	Ca	Mg	Total meq/kg
Nil	31.83 ± 1.55	15.22 ± 1.47	16.70 ± 1.13	16.20 ± 2.63	112.85 ± 10.54
EDTA 0.25 mM	37.56 ± 3.26	13.35 ± 1.17	3.78 ± 1.04	5.27 ± 1.00	69.01 ± 8.51
Ca 5 mM	16.89 ± 2.67	15.68 ± 1.54	56.25 ± 4.20	8.54 ± 0.58	162.15 ± 13.77
Mg 5 mM	23.77 ± 2.04	17.62 ± 0.98	7.61 ± 1.04	77.18 ± 6.15	210.97 ± 17.40
Na 150 mM	81.08 ± 1.75	9.86 ± 1.14	8.18 ± 0.54	9.27 ± 2.83	125.84 ± 9.63
K 150 mM	20.85 ± 2.06	68.85 ± 7.16	8.55 ± .92	8.39 ± 1.32	124.46 ± 13.70

Values are means of 5 experiments ± S.E.M.

TABLE 6

Electrolytes in the Pellets (Not Washed) as mmoles/Kg Dry Wt.

Tissues	Treatment	Na^+	K^+	Ca^{++}	Mg^{++}
Control	Nil	40.65	33.37	11.21	30.36
	EDTA	50.32	38.16	5.35	9.21
	Mg^{++}	25.98	23.59	8.72	79.85
ETCA	Nil	46.45	27.24	13.45	20.27
	EDTA	72.13	29.37	5.59	9.37
	Mg^{++}	33.45	25.26	8.13	80.98
IAA	Nil	50.39	18.45	17.59	15.23
	EDTA	75.68	20.36	5.68	9.31
	Mg^{++}	35.45	19.21	7.85	85.52

Values are means of 2 experiments.

and IAA treated tissues are shown.

In ETCA treated tissues (Table 4) effects of the above procedure were almost identical to their effects on the control tissues (Table 3) discussed above.

In IAA treated tissues (Table 5) the effects of the addition of EDTA, Na, K, Ca and Mg were similar to the control tissues except for one difference. On the addition of excess Na (150 mM) a significant amount of K was displaced. This was the only experiment in which any significant amount of pellet K was replaced by any other cation.

Table 6 shows the results of two earlier experiments in which tissues were homogenized in sucrose solution, but the pellets were not resuspended after the first centrifugation. Effects of added EDTA and Mg are also shown. As compared to the results in Tables 3, 4 and 5 in which the pellets were resuspended and rerinsed, these pellets (Table 6) have similar amounts of Ca and Mg but higher amounts of Na and K bound. Also, when neutralized Na_4 EDTA was added Ca and Mg were removed to nearly the same extent (Tables 3, 4 and 5). However the Na added with Na_4EDTA (Table 6) was bound considerably more in IAA and ETCA treated tissues as compared to the control tissues, and K was released in its place. Thus the difference in the Na and K binding is more marked in these pellets. Addition of Mg in this case removed most of the excess Na and K which otherwise remained bound because the pellets were not resuspended and not washed.

D I S C U S S I O N

Whole Tissues

The results clearly showed a dissociation in time
of the downhill Ca movement from downhill Na and K move-
ments produced by metabolic inhibition in whole tissues.
The difference in sensitivity of the mechanism controll-
ing the gradient of these electrolytes to metabolic
inhibition is quantitative rather than qualitative.
Calcium downhill movements always lagged behind those of
Na and K. The lag period observed in Ca accumulation
might be related to the degree of depletion of metabolic
energy. Recent experiments by Daniel and Robinson (76)
show a good correlation between the time course of ATP
depletion to values not significantly different from zero
by these metabolic inhibitors and the onset of downhill
Na and K movements (Table 7). This together with the
evidence that cooling to 4°C did not affect the Ca gradient
(24) raises the question whether calcium exclusion in this
tissue is ATP dependent. Ouabain failed to cause downhill
Ca movements in amounts which inhibited membrane Na-K
ATPase (79), and caused downhill Na and K movements (54,79).
Hence the membrane ATPase does not appear to be involved in
controlling calcium distribution despite evidence that
inhibition of membrane ATPase in rat uterus caused Ca
dependent contractures (54).

TABLE 7

Effect of Metabolic Inhibitors on Adenine Nucleotides and Electrolytes

Content of the Tissue*

Inhibitor	Time	ATP loss %	ADP loss %	Na gain %	K loss %	Ca gain %
IAA	1 hr.	90.0	44.7	7.4	22.0	0.00
IAA	2 hrs.	90.8	71.9	39.6	27.3	0.00
IAA + DNP	5 mins.	61.4	1.9	9.1	11.0	0.00
	15 mins.	83.5	39.8	15.6	28.3	0.00
	1 hr.	94.2	83.8	55.6	68.0	19.6

* Data on Adenine Nucelotide is taken from Daniel and Robinson (76) who used similar experimental conditions to those used in this study.

The onset of downhill Ca movement with metabolic inhibitors observed in these experiments can be correlated with the decline in tissue ADP level found by Daniel and Robinson (76) and shown in Table 7. Whether this correlation is causal or is coincidental cannot be answered with certainty. In any case, the presence of adenylate kinase in rat uterus (Allen, J.C. and Daniel, E.E., unpublished data) suggests that ATP may be supplied for Ca pumping from ADP in the absence of appreciable levels of ATP. However, cooling to 4°C did not decrease ATP levels markedly, so utilization of nucleotides must have diminished at this temperature. Nevertheless, Ca content did not increase (24), so the mechanisms for control of uterine Ca content do not depend upon continuous utilization of nucleotides.

The effect of cooling on Ca permeability should also be considered. The possibility of any significant change in Ca permeability by cooling can be excluded by the results of the experiments I have done (not yet reported) showing that there was only a small decrease in Ca efflux at 4°C as compared to 25°C. This is in agreement with the low Q10 of 1.34 for Ca efflux reported by van Breeman et al (24).

The most likely explanation for the delay in net Ca gain after onset of downhill Na and K movements and ATP depletion (76) is a slow change in a structural element of the membrane which controls Ca permeability and requires

ATP for maintenance.

The delay in the onset of the downhill Ca movement may be related to internal compartmentalisation of ATP. For example a small compartment may contain a level of ATP sufficient to keep permeability to Ca unchanged after the inhibition of the energy supply for sodium transport. When the ATP level in this compartment eventually falls below a critical value, this compartment might become permeable to Ca resulting in the delayed uptake of Ca observed in the inhibited tissues. I have no evidence to exclude this possibility.

The possibility of a sodium-calcium exchange diffusion mechanism which could explain an increased inward movement of Ca ion consequent to increased intra-cellular sodium concentration may be mentioned. This can however be ruled out by the fact that the tissue gained sodium either by cooling or by ouabain (54,74,78) whereas Ca movement remained unaffected (24). Furthermore van Breeman et al (24) showed that a changing external Na concentration from 10 to 200 mM had no effect on tissue Ca content or ^{45}Ca afflux.

In a report by Krejci and Daniel (82), evidence was presented that IAA or IAA and DNP in the concentrations used here inhibited ^{45}Ca uptake from Ca-poor solutions into a saturable uptake mechanism. There also was an uptake mechanism which took up increasing amounts of ^{45}Ca at increasing external Ca concentrations. The increased

uptake of ^{45}Ca and total Ca seen in this and previous

studies from either 1.5 or 2.5 mM Ca solutions (24)

after inhibition with IAA or IAA and DNP would therefore

appear to be a consequence of increased influx via the

non-saturable mechanism. IAA or IAA and DNP depolarized

uterine cells (24). IAA also decreased the chemical

gradient inward. van Breeman et al (24) showed that the

binding of Ca in the IAA inhibited tissues was not suf-

ficient to lower Ca concentration (< 10^{-6}M) believed to be

present in normal tissues. Therefore, IAA decreased the

electrochemical gradient for Ca. IAA-increased Ca uptake

has to be attributed to increased permeability and not to

an increased driving force. Efflux of ^{45}Ca did not

diminish while uterine horns were gaining Ca following

inhibition of IAA (24). Thus total Ca efflux probably

increased, though efflux of tracer did not, because it was

diluted by the extra uptake of non-tracer calcium.

Depolarization of cells by IAA might have caused an

increased ^{45}Ca efflux by increasing the electrochemical

gradient outward (24) if calcium crosses the membrane

as an ion (but see Krejci & Daniel, 83). Thus metabolic

inhibition probably increased Ca efflux as well as influx,

and probably increased Ca permeability. Damage to the

nonsaturable uptake mechanism may reflect specific or

general damage to the membrane which caused the increased

permeability to Ca.

The possibility that the delayed increase in Ca uptake

(increased Ca permeability) might be secondary to the decline in membrane potential which occurred in the presence of metabolic inhibitors in this tissue (24) may be considered. The observations of van Breeman et al (24) and Krejci and Daniel (83) that K depolarization had no significant effect on the net Ca movement or Ca fluxes tends to rule out the above possibility.

If a recent proposal of Somlyo and Somlyo (56) is correct, there are two permeability barriers to Ca penetration between smooth muscle cells and interstitial fluid. One is external to the plasma membrane and saturated at low external Ca concentration. According to the model of these authors, this barrier is the one which determines the rate of influx and efflux (see Krejci and Daniel, 82, for further discussion). On that model, metabolic inhibition increased Ca permeability at this external barrier. Any decrease in Ca extrusion by the plasma membrane may have escaped detection in our studies because of increased permeability at this external barrier in inhibited tissues.

van Breeman et al (24) have presented evidence that Ca binding in uterine muscle occurs after metabolic inhibition, but is inadequate to lower the intracellular Ca concentration to 10^{-6} or 10^{-7} M, the concentration range believed to be consistent with relaxation in normal uterine horns. Uterine horns made Ca-rich and ATP depleted following metabolic inhibition were, however, relaxed and flaccid; their length was increased 20 to 60 per cent.

Therefore the Ca taken up in these ATP-depleted horns was unable to activate the contractile mechanism because the mechanism was unresponsive. This raises a question about whether rigor occurs in smooth muscle.

ETCA may cause calcium accumulation by the same mechanism as that suggested for IAA, increased permeability to Ca via drug induced impairment of the non-saturable uptake mechanism in the cell membrane. That ETCA had less effect on the K gradient than on the Na gradient is in agreement with data reported by Daniel (84) on rabbit uterus and aorta. Na extrusion was inhibited by ETCA. However, there was no significant effect of ETCA on K uptake in Na-rich rabbit tissues, but the concentration used (100 µg/ml, maximum) and incubation time (90 minutes) were much less than in this study. Daniel (84) also did not observe any change in water content. In our present experiments, the water content showed an increase only after 4 hours in ETCA. The ETCA-sensitive pump (20) in red cells had been shown to have an ETCA-sensitive K influx component equal in magnitude to ETCA-sensitive Na efflux. Our results showed loss of K less than equivalent to the gain of Na. Therefore, 1:1 coupling of Na and K movements does not exist in the rat uterus treated with ETCA.

If the principle of microscopic electroneutrality applies to rat uterus, then the net ion movements produced by metabolic inhibitors must not lead to accumula-

TABLE 8

Calculated data showing electrolytes that could be gained by water movement.

Inhibitor	Time Hrs.	Na gain m moles/kg			Total K loss m moles/kg	Ca gain m moles/kg		
		Total Na gain	Na gained with H_2O	Net Na gain $(C_1 - C_2)$		Total Ca gain	Ca gained with H_2O	Net Ca gain $(C_5 - C_6)$
		C_1	C_2	C_3	C_4	C_5	C_6	C_7
	3	209.2	55.5	153.7	209.5	7.12	0.98	6.14
IAA	4	323.0	122.2	200.8	225.3	10.19	2.17	8.02
	5	466.8	155.5	311.3	251.2	13.61	2.76	10.85
	0.5	148.1	105.5	39.6	149.9	1.40	1.87	---
IAA	1	237.6	111.1	126.5	229.8	2.51	1.97	0.54
&	2	409.9	122.2	287.7	233.2	11.52	2.17	9.35
DNP	3	525.2	211.1	314.1	268.1	14.93	3.75	11.18
ETCA	4	180.7	-0-	180.7	122.8	4.26	-0-	4.26
	5	296.0	83.3	212.7	143.0	8.06	1.48	6.58

Net Na and Ca gains are compared with K loss.

47.

tion or loss of positive charge. Na and Ca gain must be accompanied either by equivalent gain of anions or loss of K. We assumed that the water gained in our experiments was accompanied by Na and by anions in concentrations similar to those in the bathing medium and calculated the proportion of Na gain which was accounted for in this way (C_2 in Table 8). The Na gained without water (C_3 in Table 8) was initially less than the K lost in the presence of IAA or IAA and DNP. Ca gain was insufficient to make up the difference and other studies have shown that in these circumstances some Mg is lost from the tissue (Moawad, A.H. and Daniel, E.E., unpublished experiments). Therefore there must have been an unmeasured gain of cellular cations or loss of cellular anions, or alternatively some cellular cation such as K must have been released from anionic binding sites destroyed by metabolic inhibition. Later the Na gained without water exceeded the K lost. Then either Na was bound at newly formed anionic sites or unmeasured loss of cations or gain of anions had occurred. Net movements of sodium without water in the presence of ETCA always exceeded K loss.

In studies of Daniel and Robinson (76) it was found that IAA or IAA and DNP accelerated efflux of a large cellular K fraction seven times, up to the efflux rate of a smaller cellular fraction. These inhibitors also increased sodium influx two to three times and slowed Na

efflux from cells. The above results and the ones described here could be explained on the hypothesis that metabolic inhibition with IAA or IAA and DNP initially leads to release of bound K from binding sites and consequent swelling by uptake of a hypotonic solution containing Na as the principal cation (the Na pump being inhibited). Net K loss initially exceeded net Na gain. Apparently sodium in the inhibited cell is, like potassium, not effectively bound. Eventually as the exposure of the tissue to IAA and DNP continues, internal potassium is depleted, and net gain of sodium becomes the driving force for swelling. However, sodium gain without water then exceeds potassium loss. In tissues treated with IAA and DNP (Fig. 8) two phases of water uptake are discernible. The first, associated with potassium loss in excess of sodium gain and complete in 30 minutes, and the second, associated with continued sodium gain and little or no potassium loss and beginning at 120 minutes. Two similar phases of water gain can be seen in IAA-treated tissues (Fig. 7). With ETCA no unbinding of K or swelling seems to occur after 4 hours, but the sodium pump is inhibited. Leakage of internal K is less than Na gain. Excess Na (gained without water and not exchanged for K) throughout exposure to ETCA and late after exposure to IAA or IAA and DNP appears to be osmotically inactive (bound) or exchanged for an unmeasured cation. (See discussion on cation binding).

It has been demonstrated (85) that ETCA in 1/10
concentration (10^{-4} M) used here inhibits oxidative
phosphorylation in mitochondria. If this was the
mechanism of its action in the rat uterus, one would
expect an increased production of lactic acid and an
increase in H ions, which might be exchanging for those
Na ions which are not balanced by K ions. However, it
is difficult to explain how this occurs without swelling.

Cation Binding and Exchange in Particulate Fractions

One of the possible explanations suggested in the
above discussion for the excess Na gained over K lost
with IAA or ETCA was that Na might be bound at newly formed
anionic sites. In other words the binding capacity of Na
and K might have been altered by these compounds either
due to a change in affinity for these cations or due to
exposure of additional anionic sites.

The term binding has been used as synonymous with
osmotically or electrochemically inactive, inextractable,
inexchangeable and slowly exchangeable by various authors
(64,65,67,72,73). In the present study the term bound
is meant to include that portion of cations which is
attached to the particulate material and can be divided
into three parts; one that could be removed by washing,
second that could be exchanged with cations, or removed
by EDTA, and the third which could not be removed by any
of these procedures. This third fraction may be in a
compartment which is unaffected by or inaccessible to

EDTA or any of the cations used. However this seems unlikely.

The experiments with the homogenates of the control and treated tissues showed no significant difference in bound Na and K in the pellets which were resuspended and washed but differences in pellet content were found when pellets were unwashed (see p.19). This may mean that there is an alteration produced by the inhibitors on the relative binding of Na and K and this binding is not so tight but that resuspension and washing or the presence of 5 mM Mg could remove the bound Na and K. In addition to the fraction removed by washing and to the fraction which could not be removed by any of the procedures used, there was a fraction of bound Na which was replaced by Ca in the resuspended and washed pellets. The two exchangeable fractions of bound Na, one removed by resuspension and washing, and the other by Ca, could be similar to the two bound fractions of Na shown by Palaty et al (86) in the arterial wall. These authors showed that some weakly bound Na could be removed by using an ion free surrounding medium which is essentially equal to washing in our experiments. According to the above report this process corresponded to the hydrolysis of a sodium salt of a weak acid which could be observed with carboxylic cation exchange resin. The second type of binding was ascribed to the strongly acidic sulfo groups ($R-SO_3^-$) and the bound Na of

this fraction could be exchanged readily for a polyvalent cation. In the present results an additional amount of Na was exchanged with Ca or K, but the amount of Na exchanged with Mg was not significant except in IAA treated pellets. This may mean that in normal tissues, Mg is not an effective counter cation for a part of the anionic sites where Ca, Na, and K are able to interact.

The difference in replacement of K by Na in the IAA treated tissues as compared to the control tissues is an important observation which implies that with IAA, even some of the tightly bound K, which was not exchanged by any other cation now becomes available for exchange but only for Na in these tissues. In the control tissues this was not found to be the case as neither Na nor the other cations tested could replace any significant amount of K. Thus an alteration in the membrane and intracellular structure by IAA may increase the lability of K binding.

These results also show that a small fraction of Na and K is tightly bound in the tissue and unaffected by other cations, the amount of Na being slightly higher than that of K. Kao's (72) estimate for bound Na in the uterus is higher than for the bound K, while Daniel & Daniel (73) estimated a slightly higher value for bound K than for bound Na. These figures cannot however be compared with the present data or in fact with each other

due to the differences in the conditions used in the experiments and the interpretation of the term "binding" or "bound". Although Kao (72) and Daniel and Daniel (73) both used isotonic sucrose for extraction, the time allowed for extraction was 6 to 8 days in the first case and 6 hours in the second. Furthermore, whereas Kao (72) used rabbit myometrium in his extraction experiment, Daniel and Daniel (73) used the whole uterus.

In the present study the differences from the methods used in the above mentioned studies are obvious. Firstly the tissue was homogenized which exposed more cellular material to leaching of the bound cations and secondly there may have been some bound cations in the supernatant, which in the whole tissue may have not been extracted. Thirdly, it is possible that the nature of binding sites may have been altered by homogenization.

Bound Ca and Mg

A considerable amount of Ca and Mg was found to be bound to the particulate material. The amount of Mg bound was higher (60%) than the amount of bound Ca (Table 2). Moawad and Daniel (87) have recently shown in the rat uterus that a much larger portion of the Mg remained in the tissue after incubation in Mg free medium than the amount of Ca which remains after incubation in Ca free medium as shown by van Breeman et al (24). It can be seen in these results that a part of the bound fraction

of either of these cations could be exchanged for the other or for Na or K. This portion again probably represents less tightly bound fraction of these cations. A further quantity of Ca could be removed by EDTA in all pellets, and Mg only in IAA treated tissue pellets. Thus in the case of Mg, it could be argued that IAA enhanced the lability of tightly bound Mg. This would be in agreement with the observations: (a) that IAA treated tissues had lower total Mg than the control of ETCA tissues (Table 1), (b) an additional amount of Na could be exchanged with Mg in IAA treated pellets only (Table 5) whereas this fraction of Na was exchanged with Ca or K in all pellets. There still remained a fraction of these cations which was not removed even by EDTA and may have been very tightly bound and inaccessible to EDTA. van Breeman et al (24) showed that about 20% of the uterine tissue Ca was inexchangeable. This might represent the fraction not removable with EDTA in this study.

Total Cation Binding Capacity

The cation binding capacity of the control and the inhibited tissues were identical (Tables 3, 4 and 5). The fact that Na, K, Ca and Mg were able to exchange for each other in the washed pellets, at some of the binding sites implies the existence of a portion of anionic sites unselective in their binding of these cations. Goodford (68) studying ion binding in taenia coli with a relatively

indirect method, but under more physiological conditions, found a fraction of superficial anionic sites which were also unselective. There are however apparently other anionic sites where Na or K are bound less effectively than Ca and Mg, since the total binding capacities in the presence of 150 mM Na or K were less than in the presence of 5 mM Ca or Mg.

The total cation binding capacity in the presence of Mg (5 mM) is higher than in the presence of Ca (5mM). If one assumes that there has not been a gross change in the nature or number of the binding sites with homogenization, it would appear that tissue has a higher capacity for binding Mg than Ca. However some of the same sites can be occupied by either of these cations, since Ca could compete for Mg sites and vice versa.

The binding capacities for Na and K were nearly equal but the binding capacity of each of them was much less than for Ca or Mg. Goodford (67) suggested a preference for K rather than for Na by superficial anionic sites in taenia coli mentioned above. Whether such a preference exists at sites in the rat uterus cannot be answered with certainty from the experimental results in this study, since the relative binding of these two ions was studied only at one high concentration. Under these conditions however, there was no difference in their exchangability with other cations and in

the total binding capacity in the presence of either. In
the control as well as treated tissue, pellets, the bind-
ing capacity for Na and K, is far from being exhausted,
assuming homogenization does not affect binding sites.
A higher concentration of these cations leads to a
greater binding, presumably according to the law of mass
action. A similar binding of Ca by the rat uterus was
demonstrated by van Breeman et.al. (24) using IAA in-
hibited tissues, and by increasing Ca concentration in
the external medium. The small but net significant
increase in binding of Na and a significant increase in
binding of Ca in the inhibited tissue is probably due
to a higher concentration of these cations made available
as a result of the depletion of ATP, as shown in the
case of Na by Daniel et.al. (76) and presumably by
alteration in membrane structure leading to an increase
permeability to Ca.

C O N C L U S I O N S

Part I

From the results of Part I of this study, the following conclusions are drawn:

(1)   While sodium, potassium and calcium movements of rat uterus can be affected by metabolic inhibitors, the former can be dissociated from the latter.

(2)   Metabolism appears to be essential for maintaining the Na, K, and Ca gradients across the plasma membrane in the tissue. However, there is a qualitative difference in sensitivity to metabolic inhibition of Na, K movement and Ca movement; the Ca gradient being relatively more resistant to metabolic inhibition.

(3)   Accumulation of Na and Ca and the loss of K by their downhill movements caused by metabolic inhibition, cannot completely be explained on the basis of an altered passive binding of the tissue for these cations as measured in the homogenates of the tissue.

(4)   A portion of all cations measured (Na, K, Ca, and Mg) was found to be bound in the tissue. Ca and Mg were bound in much larger proportion than Na or K.

(5)   Among the cations studied, tissue seemed to possess the highest total passive binding capacity for Mg.

P A R T   I I

Calcium uptake by the microsomal fraction of rat myometrium

I N T R O D U C T I O N

Calcium in Excitation Contraction Coupling

A.   Skeletal Muscle

The importance of Ca ions in muscle physiology was recognized as early as 1883 when Ringer (1) showed that frog ventricles in Ca free saline will fail to contract and that addition of calcium restores spontaneous contraction.   Later it was shown that in the absence of Ca the rhythmic spontaneous action potentials of frog heart were still present in only slightly modified form (2,3). At that time it was also known from studies on skeletal muscle that depolarization of the fiber membrane was the electrical event for initiation of mechanical response (4). One obvious explanation of these findings at that time was that the action potential or depolarization of the muscle fiber membrane somehow allows or facilitates the entrance of Ca ions from the surface to the interior of the fiber and that these Ca ions then initiate a mechanical response.

In recent years a vast amount of literature has accumulated on the subject, and at the present time there is considerable evidence that Ca is intimately and importantly involved in all these stages of muscular contraction, namely; excitation or electrical events, mechanical events

or contraction and the link between electrical and mechanical events. Heilbrunn and Weircinski in 1947 (5) with their ingenious experiments of injecting various ions into single muscle fibers were the first to demonstrate that an elevated myoplasm calcium concentration activated the contractile mechanism. Calcium was the only physiologically occurring cation which would cause shortening of the skeletal muscle fibers. This effect was later confirmed by Niedergerke (6) under more physiological conditions. He controlled the amount of Ca ions injected by iontophoresis, and demonstrated local reversible contractions under these conditions. More recently, Podolsky and Costantin (7) using a preparation of isolated frog fibers in oil were able to activate the contractile mechanism with local application of calcium. In this preparation in which sarcolemma was dissected away, application of Ca to the surface in concentrations exceeding 10 μM always activated the contractile response. However the unknown extent of dilution made it impossible to know the exact threshold concentration. This difficulty was overcome by using Ca-EGTA buffers. Portzehl et al (8) injected Ca-EGTA buffers of relatively high concentration, adjusted to a certain concentration of free Ca ions, into isolated living giant muscle fibers of Maia Squinado by means of micropipettes. They showed that when a Ca-EGTA buffer solution containing $5-10 \times 10^{-7}$M free Ca was injected,

fibers underwent a fairly marked contraction. However, when a buffer containing Ca ions below 3×10^{-7}M was injected only a small percentage of the fibers used, showed any tendancy to contract. Because of the high buffering capacity of the Ca-EGTA solutions injected, the influence of the dilution of the solution in the muscle volume was considered to be negligible.

The above evidence shows that Ca ions are involved in the contraction-relaxation process. When the contraction of Ca ions exceeds threshold value, evidently between $10^{-6} - 10^{-7}$M, muscle fibers shorten and when Ca ion concentration falls below this threshold value, relaxation occurs.

The molecular mechanism whereby Ca initiates contraction is not yet completely understood despite much investigation (9,10). Weber et al (11-14) related effects of Ca to the reversible binding to the myofibrils. The mechanism by which Ca activates contraction and the site at which Ca binds remains obscure.

In 1964 Ebashi and Ebashi (15) showed that the Ca sensitivity of actomyosin was dependant on the presence of another protein closely associated with actin. They elaborated on earlier observation (11,16) indicating a difference in Ca sensitivity between natural and reconstituted actomyosin. Ebashi and Ebashi (15) partially

purified the new protein and designated it native tro-
pomyosin in view of its physico-chemical similarities
to the classical Bailey tropomyosin (17). However,
tropomyosin prepared by the Bailey procedure was ineffect-
ive in conferring Ca sensitivity on Ca insensitive
actomyosin. The ability of tropomyosin-like proteins to
sensitize actomyosin to Ca and the inability of Bailey
tropomyosin to do so were confirmed by several workers
(18-22) and led to the suggestions that the Ca sensitizing
factor was either a different molecular form of tropomyo-
sin (15) or some other protein associated with tropomyosin
(18,19,21). Apparent support for the first possibility
came from Müeller's observation (22,23) that tropomyosin
prepared in the presence of sulfhydryl protecting agent
(SH-tropomyosin) had Ca sensitizing activity whereas un-
protected tropomyosin (S-S tropomyosin) did not. That
the state of the SH groups might be related to the diff-
erence in activity of Bailey tropomyosin and native tro-
pomyosin appeared reasonable in the light of earlier
observations (24-27) that treatment of myosin B with SH
reagents rendered it insensitive to Ca. This view was
modified, however, by the discovery of Ebashi and Kodama
(28) of a new protein troponin found in close association
with tropomyosin. They further reported (29) that the Ca
sensitizing factor is a tropomyosin-troponin complex. This
was recently confirmed by Hartshorne and Müeller (30).

The previous observations of Müeller (22,23) on the role
of the SH groups can now be explained by the finding of
Yasui et al (31) that the SH groups of troponin, not
tropomyosin, are essential for Ca sensitizing activity.

These discoveries on the nature of Ca-sensitizing
factor raised the question of whether the Ca which
activated the myofibrils was bound to actin, myosin, or
to a component of the Ca-sensitizing complex. Fuchs
and Briggs (32) have recently measured the extent of Ca
binding to isolated myofibrils and various proteins
derived from myofibrils. They found that tropomyosin
bound very little Ca, whereas troponin bound Ca with an
apparent affinity constant which was identical to the
affinity constant of the myofibrils. Since on a weight
basis troponin has a much greater number of Ca binding
sites than the myofibrils (32) it was suggested that Ca
activates myofibrillar contraction by binding to the
troponin molecule.

Most of the available information on the mechanism of
contraction-relaxation process has come from the experi-
ments on isolated systems. Contraction in vitro can be
measured by the development of tension in or shortening
of the glycerol extracted fibre bundles. While using
myofibrils, syneresis is measured by different methods.
On centrifugation at low speeds, myofibrils form a sedi-

ment of much higher protein concentration after syn-
eresis than before syneresis.

Suspensions of actomyosin have been used more
commonly to study the contraction-relaxation mechanism.
These suspensions exhibit the phenomenon of superpre-
cipitation attended by ATP hydrolysis which is taken
to represent contraction in vivo, and the system is
considered to be in a relaxed state if there is no inter-
action between actin and myosin (33,34) because the two
proteins are dissociated. Enzymatically dissociation
or relaxation is indicated by a reduction of ATP hydroly-
sis to values approaching that of myosin alone. Weber
(35) pointed out that since ATP hydrolysis can be
inhibited by means other than separation of co-factor
actin from myosin, the inhibition of ATPase activity
alone is no more indicative of relaxation than the pre-
vention of contraction or syneresis. She further
emphasizes that the inhibition of ATPase activity or
contraction may be used for quantitative studies only
if it can be demonstrated that the factors responsible
for inhibition also produce phenomena specific for
dissociation. Preparations of myofibrils and actomyosin
have the advantage that diffusion problems are minimal
and the preparations are of greater purity than fibres
or fibre bundles (35).

The effect of Ca ions has been studied on all these

preparations. Hasselbach and Makinose (36), and Weber and Herz (12) studied the range of Ca ion concentration at which myofibrils relax or the syneresis and ATP splitting inhibited. They found it to be lower than $2 \times 10^{-7}M$ (36), and $1.3 \times 10^{-6}M$ (12), but the conditions of the experiment such as ionic strength and pH were different in the two studies. Weber and Winicur (11,14) also studied the threshold Ca concentration for super-precipitation and of activation of ATPase of actomyosin and reported it to be in the range of 5×10^{-7} to $10^{-6}M$.

Glycerated preparations of skeletal and smooth muscle in which the cell membrane is damaged and thus the myofibrils are in direct contact with the external medium have also been used (37). The threshold for Ca ion concentration for contraction is reported to be between $10^{-6} - 10^{-7}M$ in either of these muscles. Bozler (38) has recently reported a significant difference in the Ca ion sensitivity of glycerated preparations of smooth and cardiac muscle. Whereas the cardiac muscle preparation contracted at the lowest concentration of Ca ions attainable by chelating agents ($< 10^{-8}M$), a concentration of 4×10^{-7} M had no effect in the smooth (stomach) muscle preparation. The type of curve obtained by plotting increase in tension against increase in pCa were, however, parallel for the two preparations. He also confirmed previous work by Schalder (39) on mammalian smooth and cardiac muscle, who showed that the Ca sensi-

tivity increased markedly with pH and suggested that the effect implies that activation of contractile mechanism was due to binding of Ca by protein.

The interactions between the contractile proteins ATP and other co-factors (Mg and Ca) leading to contraction-relaxation cycle are under the control of the fibre membrane. The electrical changes occurring during excitation initiate chemical changes inside the fiber which activate the contractile system. After a short period, the system is inactivated and returns to the relaxed state.

As early as 1952, Sandow (40) postulated that the membrane depolarization during the action potential liberates some substance, probably Ca from the membrane structure itself into the myoplasm. This Ca then would move into the interior of the fibre by a mechanism other than diffusion, possibly an exchange reaction in order to activate the entire fiber. Diffusion of a substance from the surface inward as a mechanism for activation of a twitch in skeletal muscle fibers was ruled out by Hill (41) who showed that this process was far too slow to explain the rapid development of full activity in a twitch by assuming that it is initiated by arrival at any point of some substance from the surface. Hill (41) suggested that a physical or physico-chemical process is propagated into the fiber interior.

Frank (42) tested the effect of the removal of the

external Ca on the contractile response of the frog's extensor longus digitus IV incubated in choline ringer to high potassium depolarization. He obtained rapid and complete elimination of mechanical responses under these conditions. He further showed that the rate of loss of mechanical response was determined by the rate at which Ca ions left the extracellular spaces of the muscle. On the basis of these results he suggested that the external Ca or a Ca fraction in equilibrium with it played an essential role in excitation contraction coupling during potassium depolarization in skeletal muscle. In answer to Hill's (41) argument mentioned above, he suggested that it may be necessary for Ca ions only to reach the inner surface of the muscle membrane to initiate some other process which eventually led to a mechanical response.

Later experiments by Frank (43,44), in which he used divalent cations or caffeine in Ca free medium to observe their effect on contractile responses elucidated some of his and other investigators earlier observations (42,45,48). He showed a large number of multivalent cations could restore potassium-induced contractures when they were added to the Ca free medium. Caffeine-induced contractures, which occurred independently of membrane potential changes, were not modified when the muscles were kept in Ca free solution for a sufficient

length of time to eliminate completely the potassium

induced contractures. From these observations Frank

(44) suggested that the divalent cations, and caffeine

were able to release Ca from cellular binding sites, which

might be located in the sarcoplasmic reticulum. There was

however a difference between the effects of these chemi-

cals. Whereas caffeine had the ability to accelerate the

loss of calcium from the binding sites, the multivalent

cations could only make it possible for this bound Ca to

support potassium induced contractures. In accordance with

this recently Weber et al (47) have demonstrated that Ca

efflux from the microsomal vesicles derived from the sar-

coplasmic reticulum of skeletal muscle was increased

by stontium and by caffeine in a more recent report (48).

Just over a decade ago, Bennett, (49) and Porter and

Palade (50) reported their ingenious observation made

with electron microscopy that the elements of triad grouping

of the endoplasmic reticulum of skeletal muscle which

surrounds each fibril appeared to be connected transversely

across the fibre. They made this observation on many

muscles. Triad structures by Porter and Palade (50) were

defined as tubular-like sarcoplasmic elements flanked

by larger vesicles. They also hypothesized that some

polarized part of the triads conducted excitation from

the sarcolemma into the fibre. A. F. Huxley and co-workers

(51-53) provided striking evidence that sarcoplasmic

reticulum was involved in the conduction of the excita-
tory impulse from the surface membrane to the contractile
system. They demonstrated that a local depolarization
of a very small area of the fibre membrane produces a
local contraction only at those levels of the sarcolemma
where the triad structures are located, which in differ-
ent striated muscles are placed at different levels of
the A and I band (52). Huxley and his colleagues further
showed that increasing depolarization caused contraction
in increasing numbers of sarcomeres and the direction
of spread of excitation was always in the transverse
direction across the fibre. Thus the electrical spread
inward appeared not to be a propagated action potential which
is all or none in nature. Later it was also demonstrated
that the central tubules of the triads which ran trans-
versely across the muscle fiber are continuous with the
extracellular space (54,55).

B.   Smooth Muscle

Thus presently it is generally accepted that in fast
skeletal, and cardiac muscle which possess an extensive
sarcoplasmic reticulum, excitation is conducted into the
fibre interior along the central tubules of the triads.
However, in some smooth muscles studied (56,57) and slow
striated muscle (50,58) the reticulum is only poorly
developed. Depolarization in smooth muscle probably
only takes place at the surface of the cell to activate

the contractile mechanism.

There is also considerable evidence that drugs can affect excitation-contraction in smooth muscle by a common mechanism that is not entirely dependent on depolarization of the membrane. A variety of smooth muscles contract when stimulated by drugs after depolarization in high K media (59-61). This could however be interpreted as a greater increase in permeability of cell membrane to Ca than that caused by K depolarization. But, several smooth muscles bathed in Ca free media contract in response to drugs for some time after the loss of the contractile response to high K or electrical depolarization. Examples of these are the effects of norepinephrine on rat tail artery (62) and of acetylcholine on rat uterus (63), the taenia coli (64) and the toad stomach (65). A related property of a number of smooth muscles is their greater maximal contractile response to drugs than to complete depolarization with K. Examples are the maximal responses of vascular smooth muscle to norepinephrine (66-69) and the responses of uterine (63) intestinal (70), and tracheal (71) smooth muscle to acetylcholine. Somlyo and Somlyo (72) further observed that the maximal responses of vascular smooth muscle to different drugs also varied within the same vascular segment and the difference persists in the depolarized state (67). These characteristics of smooth muscle could be explained by the ability to these drugs to trans-

locate Ca into the cytoplasm from a compartment not access-
ible to depolarization.

Several authors have suggested the existance of two
separate Ca sites to account for the different responses
of smooth muscles to drugs and to K (73-76). Somlyo and
Somlyo (77) however seem to prefer the alternative pos-
sibility that drugs produce a longer and more persistant
increase in the permeability of plasma membrane to Ca
than does K, and thus postulate two permeability barriers
to Ca in series in order to explain some of the experimental
findings.

The Relaxing Factor System

A.   Skeletal Muscle

As pointed out above, the membranes of the reticular
system appear to conduct excitation inward at least in the
muscles in which this system is markedly developed. The
second function of the reticulum as pointed out by
Hasselbach (78) is that the system must further be able
to interfere with the interaction between ATP and the
contractile system in order to produce activation and in-
activation.

Striking evidence for the existence of an extractable
factor in the muscle was provided by Marsh (79). He
discovered that an aqueous extract of muscle prevented the
syneresis of bundles of myofibrils in the presence of ATP.

To explain these findings, a "relaxing factor" present in the muscle homogenates inhibiting contraction and ATP splitting was postulated (79).

The inhibitory action of the isolated supernatant was proved by testing its effect on tension development of glycerol-extracted muscle fibers by Bendall (80) and its inhibition of contraction and ATP splitting of well washed myofibrils by Hasselbach and Weber (81). On the addition of small amount of Ca ions (79) the inhibiting effect of the factor disappeared instantaneously.

In the following years it was shown by Kumagai et al (82) that the activity was associated with the Kielley Meyerhof ATPase (83,84) and like it, was destroyed by phopholipase (85). These workers were able to precipitate the factor activity with 20% ammonium sulfate. Subsequently it was shown by Portzehl (86), Lorand et al (87) and Ebashi (85) that 100% of the activity could be collected by high speed centrifugation.

Kumagai (82) and Ebashi (85), who tested the relaxing effect on extracted fiber bundles, demonstrated an auxiliary requirement for transphosphorylating system which tempted a number of workers (88-90) to speculate that the inhibiting effect of the factor was a consequence of the resynthesis of ATP in the muscle extract. These speculations were later abandoned since it was shown (78) that relaxing precipitate of Portzehl (86) did not resynthesize ATP.

The possible identification of the relaxing factor with the sarcoplasmic reticulum or the sarcotubular system was first suggested by Porter (91) and Muscatello et al (92,93). Consistent with this view, the first electron micrographs of Hasselbach's relaxing factor preparation obtained by H. E. Huxley (94) showed that it consisted of vesicles measuring 500 - 3000 $\overset{o}{A}$ in diameter. This concept received further support from Ebashi and Lipman (95) who found complete triads in their preparation. They pointed out that often fragmentation of the tubular elements apparently seal off into closed vesicles, a fact which is of considerable importance for the uptake of Ca by the vesicles, discussed below.

Ebashi (96-98), Ebashi and Lipmann (95), as well as Hasselbach and Makinose (36), discovered that the relaxing factor in the fragments of the reticulum removed calcium from the medium in the presence of ATP and Mg. Ebashi considered this binding by the reticulum as the basis of its relaxing effect (97,98) thus concurring with an earlier suggestion by Weber (99) that the relaxing factor may exert its effect by Ca binding. In support Ebashi demonstrated that aging and ADP were as inhibitory to Ca uptake as they were for the relaxing effect (95). Furthermore, he showed that the reticulum reduced the amount of Ca bound to actomyosin (97). More detailed quantitative

studies by Weber et al (13) showed that reticulum caused
maximal inhibition of syneresis and ATPase activity of
myofibrils after reducing their Ca content from 2 μmoles
to about 1 μmole/gm protein. In addition it was shown
that the time course of Ca removal was strictly parallel to
the time course of increasing inhibition of ATPase activity
and reversal of syneresis. The reticulum did not seem to
change the binding constant of the myofibrils for Ca, but
Ca dissociated from the myofibrils only because the
reticulum raised the pCa of the medium. Furthermore, the
extent of inhibition of myofibrillar ATPase activity
depended on the level to which the reticulum raised the
pCa (13,100).

When Ca accumulation by reticulum was inhibited by
salyrgan (36,78) olinate (78,101), cetyltriethylammonium
chloride (78), phospholipase C (85,102), amytal (92,103)
ADP (95,101), relaxation by reticulum was inhibited.
Caffeine despite some earlier disagreement (104,105) can
now be included in the above list of inhibitors (48).

Reagents which augment the Ca uptake by reticulum
such as oxalate (36,106), pyrophosphate (78,106,107),
citrate (78), and inorganic phosphate (78) also increase
the relaxing effect of the reticulum. In view of the find-
ing that potentiation of Ca uptake could be caused by
inorganic pyrophosphate, Seidal and Gergely (109) suggested

that the dialysable cofactor for relaxing activity reported by some investigators including Gergely et al (110-112) could be accounted for by the inorganic phosphate content of the supernatant used in these experiments.

According to several published reports reticulum does not inhibit contraction in the presence of nucleotide tri-phosphates other than ATP (106,113) although these nucleo-tides support Ca accumulation (103,107,114). At variance with these observations is the recent report of A. Weber (115) who found complete inhibition of myofibrillar syn-eresis by reticulum with Mg ITP.

Reticulum from cardiac muscle has also been shown to remove Ca from skeletal myofibrils (13) and prevent syneresis of skeletal (13,116) and cardiac myofibrils (117) and cardiac actomyosin (118) which depended on the level to which it raised the pCa of the medium by accumu-lating Ca (100,116,117).

Coupling Between Ca Uptake and ATP Hydrolysis

As mentioned above, Ca uptake by the reticulum requires the presence of ATP. Hasselbach and Makinose were the first to show that Ca activated the rate of ATP hydrolysis and that the rate of ATP hydrolysis and Ca transport were linked (36,78,101,118). Reticulum preparations also hydrolyse ATP in the absence of Ca but at rates 10 (78) to 30 times lower (47) than during maximal transport depending on the ATP concentration

(47).

The ATPase active during Ca uptake has been called "extra ATPase", while the ATPase active in the absence of Ca is generally designated as the "basic ATPase". Recently Weber et al (47) have shown a difference in the dependence on ATP concentration between the basic and the extra ATPase. It has also been shown that in the absence of Ca, ATP hydrolysis is not accompanied by ADP-ATP exchange which takes place during Ca-activated ATPase activity (94,95). This implies that there is a phosphorylated intermediate associated with the Ca transport in the reticulum. Recently evidence has been presented for such an intermediate (119,120). The rate of exchange is reported to be 10 times the rate of hydrolysis (78,101,104).

It was shown by Hasselbach and Makinose (36) that on the addition of Ca to reticulum, the rate of ATP hydrolysis increased to a value proportional to the rate of Ca uptake and returned to the original low value when the added Ca had been removed from the medium restoring a high PCa.

A figure of 2 Ca accumulated for each ATP hydrolyzed in the presence of oxalate was given by Hasselbach and Makinose (118) and confirmed by Weber et al (47) who also found the same ratio in the absence of oxalate.

Apparently the rate of ATP hydrolysis is coupled to a net Ca flux across the membrane. Changes in flux induced by changing the ATP concentration are attended by proportional

changes in the rate of ATP hydrolysis. These and other parameters of Ca uptake and ATP hydrolysis are discussed in detail by Weber et al (47) and Weber (35).

A number of parameters of Ca uptake by reticulum of skeletal and cardiac muscle have been measured and discussed (35,121,122,123).

Although there remain several unsolved problems, particularly the molecular mechanism of Ca uptake (119,120,124), there is general concurrence on the following observations.

1. Vesicular Ca uptake is against an electrochemical gradient, and there is a stoichometric relation between the ATP hydrolysis and Ca uptake (47,119).

2. Ca uptake is greatly augmented by oxalate and inorganic phosphate (35,78,107).

3. Ca uptake and ATP hydrolysis can be inhibited by a number of inhibitors, and specifically by salyrgan (36,48,78,101).

4. Ca uptake in the reticular fragments can be clearly distinguished from the Ca uptake in the Mitochondria (107,35).

*5. The velocity of calcium uptake and the release of accumulated Ca, and the capacity of the vesicles to lower Ca ion concentration are consistent with

the _in vivo_ contraction-relaxation speed and the
threshold values for Ca ion for contraction
(47,78,119).

Thus from the above it is clear that there is at
least in skeletal muscle, a well documented instance
of a Ca-reversible relaxation by the sarcoplasmic
reticulum as a result of its ability to remove Ca.

B.   Smooth Muscle

In smooth muscle the existence of intracellular
Ca translocation comparable to the sarcoplasmic reticular
system cf skeletal and cardiac muscle has been postulated
from studies on Ca distribution and Ca movements in this
muscle (127). On the other hand alternative suggestions
such as diffusion of extracellular Ca into the cells and
intracellular Ca into the extracellular space resulting
in contraction and relaxation respectively have been put
forward (67,77,129,128). These suggestions were made
primarily because of the small amount of endoplasmic
reticulum (56,57) and the short diffusion distance in
smooth muscle and slow rates of contraction and relaxation
in these tissues (78). Peachy and Porter (128) suggested
that influx of extracellular Ca activates contraction.

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* In cardiac muscle this observation is not yet completely
  established (35,125,126).

Attempts to demonstrate active extrusion of Ca in taenia coli (129,130) or uterus (127) have not been successful. The scarcity of smooth endoplasmic reticulum in aortic smooth muscle has been noted (131,132) but no comparative studies of the proportional volume of smooth endoplasmic reticulum in different types of smooth muscle have been made. Somlyo and Somlyo (77) have made the suggestion that the relative contribution of, respectively, the Ca stored in the sarcoplasmic reticulum and the extracellular Ca may vary in different types (e.g., phasic or tonic) of smooth muscles.

Hasselbach and Ledermair (133) failed to obtain an active preparation of a relaxing factor from cow uterus and rectum.

Bozler's (38) recent study on the glycerinated preparation of cardiac and smooth muscle strongly supports the postulate for the existence of a Ca accumulating mechanism, perhaps analogous to the reticular system of skeletal muscle.

In the past 2 years some preliminary reports on the demonstration of Ca uptake in the microsomal fraction from arterial (134) and intestinal (135) smooth muscle have appeared. More recently, a more complete report on the demonstration of Ca uptake by the sarcoplasmic reticulum of cow uterus was published (136). The possibility of the intracellular release of accumulated Ca to account

for contraction was suggested, although the amount of Ca stored by the reticular preparation was found to be grossly inadequate for the calculated amount of Ca required to be available to cause contraction. An extra ATPase activity with the addition of Ca was shown to be present in the above study (136). However, its role for Ca transport was not discussed.

Obviously there is lack of information and an abundance of speculation on the possible existence of a Ca accumulating system in smooth muscle. It was, therefore, decided to study whether an ATP-dependent Ca accumulating system can be found in the subcellular particulates of rat myometrium, and if so, what are its basic properties?

M E T H O D S

Experimental Animals and Preparation of Tissues

Rats of 175 - 200 grams were pretreated as described in Part I of the thesis. The horns were removed and placed in cold Krebs Ringer solution. A layer of endometrium and circular muscle was stripped away leaving the longitudinal layer. This was accomplished by splitting the horn lengthwise, laying it flat on a piece of filter paper soaked with cold sucrose histidine solution (0.25 M sucrose and 5 mM histidine) and peeling off the endometrium and circular muscle layers. The resulting longitudinal muscle was placed in a weighed beaker containing cold sucrose histidine solution. Longitudinal muscle from each horn weighed about 75 - 100 mg. Six to eight rats were usually used for each experiment. When skeletal muscle was used, muscle from the rat thigh was excised, weighed, and the rest of the procedure for the isolation of the microsomes was the same as that for myometrium.

Homogenization

The beaker containing the longitudinal muscle was weighed. The tissue was chopped into fine pieces with a pair of scissors and transferred into a chilled plastic tube of 1 1/8" diameter and 4" in length. This tube size was found to be the best for use with

the Polytron homogenizer. Tissues were homogenized in cold sucrose histidine medium with a Polytron 20 ST homogenizer, in all experiments, except a few in which homogenization was done with a glass tube and Teflon pestle.

The Polytron 20 ST was used for homogenization, at a speed of 3/4 of the top speed of the instrument was used for 4 seconds. This speed and duration of homogenization gave a good yield of microsomes and little, if any, contamination with mitochondria. When a Teflon homogenizer was used 30 strokes with a pestle driven by a 1/150 H.P. motor at about 1550 r.p.m. were applied. The tube containing the tissue was kept in ice throughout this procedure. Final homogenate suspension was usually 5% (W/V). Thereafter all operations were carried out in the cold (0-4°C).

Differential Centrifugation

The centrifugal force and duration used were mainly based on the method of Wichmann (137). The homogenate was centrifuged in a clinical centrifuge (International Model CL) at 1000 x g for 15 minutes. The sediment obtained was called the nuclear fraction as it was assumed to contain nuclei and all debris.

The supernatant from this fraction was centrifuged at 10,000 x g for 15 minutes in a Spinco Model L2-40 centrifuge to obtain the mitochondrial fraction.

diamond knife. The sections were mounted on 200 mesh copper grids and after staining with lead citrate and uranyl acetate, were examined with a JEM-7A electron microscope.

Protein Determination

The method of Lowry et al (138) as modified by Miller (139) was used.

Reagents

1. 10% Sodium carbonate (Na_2CO_3) in 0.5N Sodium hydroxide (NaOH).

2. 2% Potassium tartrate ($K_2C_4H_4O_6 \cdot \frac{1}{2}H_2O$)

3. 1% Copper sulfate ($CuSO_4 \cdot 5H_2O$)

4. Folin phenol reagent (Fisher) 1 to 11 dilution.

5. Copper reagent was freshly prepared by mixing 10 parts of (1) with a 1:1 mixture of (2) and (3).

6. A standard stock solution 1 mg/ml of crystallized bovine serum alubumin was prepared in sucrose-histidine medium. Standards were prepared by a known dilution of the stock solution with sucrose-histidine solution since all the fractions were suspended in this medium and histidine was found to interfere in these determinations. The blank also contained an equal amount of this solution (see Standard curves, Fig. 10).
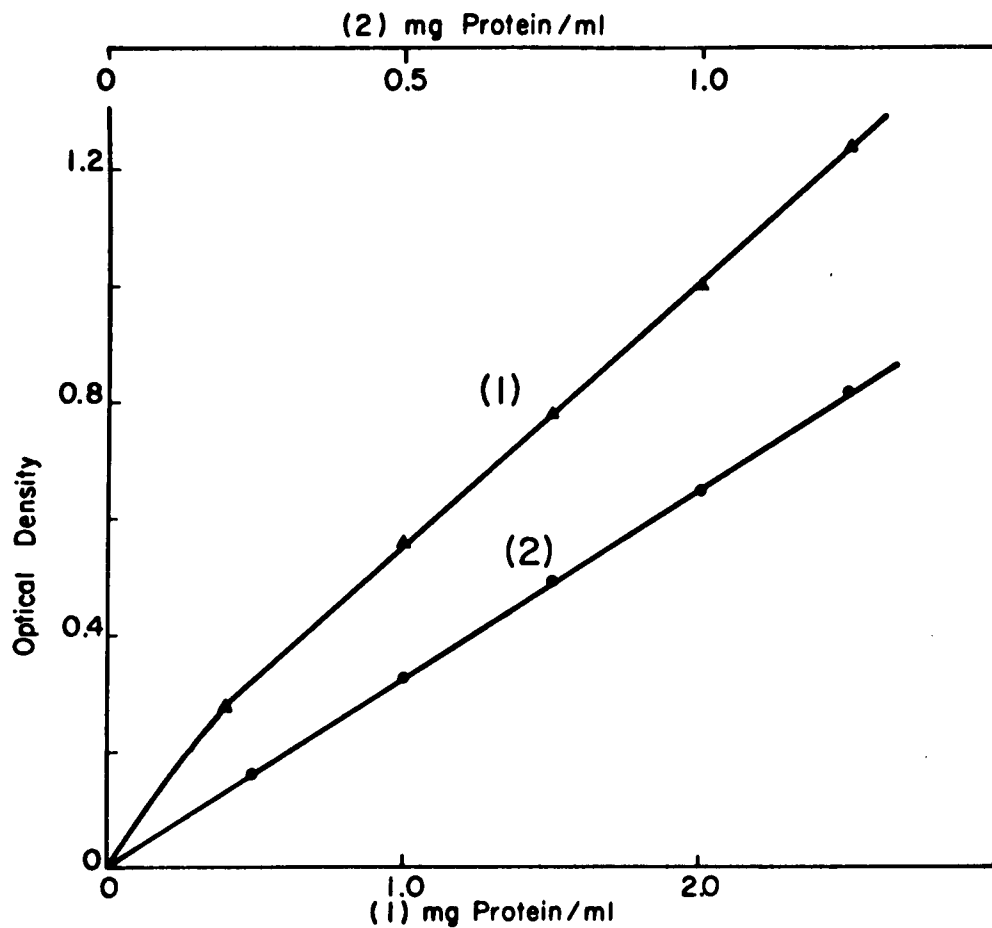
FIGURE 10

Average calibration curve for the determination of protein. Standards in sucrose histidine solution (▲). Standards in deionized water (●).

Procedure

1. 0.2 ml of 1N NaOH was added to 0.2 ml of the suspended fraction. Similarly 0.2 ml of NaOH was added to 0.2 ml of a series of standard solutions, and also in 0.2 ml of sucrose-histidine solution for the blank.

2. All tubes were then transferred to a beaker containing near boiling water (90°C) for 5 minutes to obtain a clear solution. This was found to be necessary especially with nuclear suspensions such as the tissue homogenate or the nuclear fraction.

3. After cooling the tubes, 0.6 ml of water followed by 1 ml of copper reagent was added.

4. After 10 minutes 3 ml of the Folin phenol reagent were added as forcibly as practicable.

5. The mixtures were heated for 10 minutes at 50°C in a constant temperature water bath.

6. After cooling the tubes to room temperature, absorbance was read at a wave length of 540 mμ using Bausch and Lomb spectronic 20 colorimeter.

7. A standard curve was constructed and the protein in the samples read from the curve.

Succinic Dehydrogenase Assay

The spectrophotometric method described by Slater

and Bonner (140) was used. This method depends on measuring the reduction rate of $K_3Fe(CN)_6$ in the presence of sufficient KCN to inhibit cytochrome oxidase. All available methods for the estimation of succinic dehydrogenase activity, as emphasized by Bonner (143), give comparative activities only.

Reagents

0.1 M Potassium cyanide, neutralized.

0.01 M $K_3Fe(CN)_6$

0.2 Sodium succinate

0.1 M Tris buffer (PH 7.2)

Procedure

1. To a 1 cm cell were added 0.3 ml each of the KCN and $K_3Fe(CN)_6$ and 0.2 ml of Na succinate.

2. 2.2 ml of the Tris buffer were added to make the volume to 3 ml.

3. 3 ml water were used in the reference cell.

4. At zero time 0.2 ml of appropriate suspension (mitochondrial or microsomal) was added to both cells and the optical density at 400 mμ was followed as a function of time using the Beckman DB spectrophotometer.

5. Activity was expressed as the decrease in optical density per minute at the room temperature (24°C) per mg protein.

Ca Uptake

Reaction Mixture

The standard reaction mixture contained 0.25 M sucrose, 5 mM histidine, 5 mM ATP, 5 mM $MgCl_2$, 10 μM to 15 μM Ca Cl_2 (containing 2.5 μc Ca^{45}) and 100 mM of Tris (pH 7.2), microsomal protein 0.15 to 0.25 mg and the total volume was 1 ml. In some experiments 50 mM histidine replaced 100 mM Tris used above, and there was no difference in the results obtained.

A solution containing twice the concentration of the above chemicals except the sucrose and histidine was prepared. This was called medium. It was then diluted to one half concentration by adding equal volume of the microsomal or mitochondrial suspension to start the reaction. In the blanks, the medium was diluted to one half by adding equal volume of sucrose histidine solution. Although a known concentration of Ca was added in the reaction mixture, the total Ca concentration was found to vary depending on Ca contamination in the chemicals. ATP was the major source of Ca contamination. Total Ca concentration of the medium was therefore determined with atomic absorption method, each time a new batch of ATP or other stock solutions were prepared.

Incubation

The microsomal suspension and the medium were brought

to room temperature before mixing the two. 0.5 ml of
the microsomal suspension was pipetted into glass tubes
and at zero time 0.5 ml of the medium was added to start
the reaction while stirring continuously with teflon
coated magnetic stirrers at room temperature.

Whenever any additions (drugs, inhibitors, metal
ions) were desired in the reaction medium, 0.1 ml of the
appropriate compound was added to give the desired con-
centration in 1 ml of the reaction medium. Microsomal
suspension in this case was reduced to 0.4 ml so that
the total volume still remained 1 ml.

Separation of the particulate material

Two methods were used.

1.   Filtration Method: A method similar to that of
      Martonosi and Feretos (107) was used.
      At specified time intervals 0.4 ml of the reaction
      mixture was filtered through Millipore filters
      (0.30 μ diameter) into glass tubes placed inside
      Millipore flasks connected to the vacuum line.
      Filtration was generally complete within five
      seconds. Blanks were filtered simultaneously.

2.   Centrifugation Method: This method was used
      only when it was desired to determine the total
      Ca in the particulate in order to compare it with
      the radiocalcium uptake.

3 ml of the medium was added to 3 ml of microsomal suspension in 10 ml beakers to start the reaction. At specified time intervals the contents of the beakers were transferred into the chilled centrifuge tubes (Oak Ridge type) and spun at 150,000 x g for 20 minutes in Spinco L2-65 ultracentrifuge. After centrifugation the pellets and walls of the tubes were rinsed twice with cold deionized water, without disturbing the pellets.

Measurement of Ca Uptake

Calcium accumulated by the microsomes was calculated by measuring the radioactivity of the filtrate or the supernatant following centrifugation and subtracting the value obtained from that for the radioactivity found in the blank.

Radioactivity Measurements

0.1 ml of the sample to be measured was taken into the scintillation vials containing 10 ml of Bray's (141) counting solution prepared as follows:-

Reagents

1. Napthalene 60 Gm.

2. PPO (Picker Nuclear) 4 Gm.

3. POPOP (Picker Nuclear) 200 mg.

4. Methanol 100 ml.

5. Ethylene Glycol 20 ml
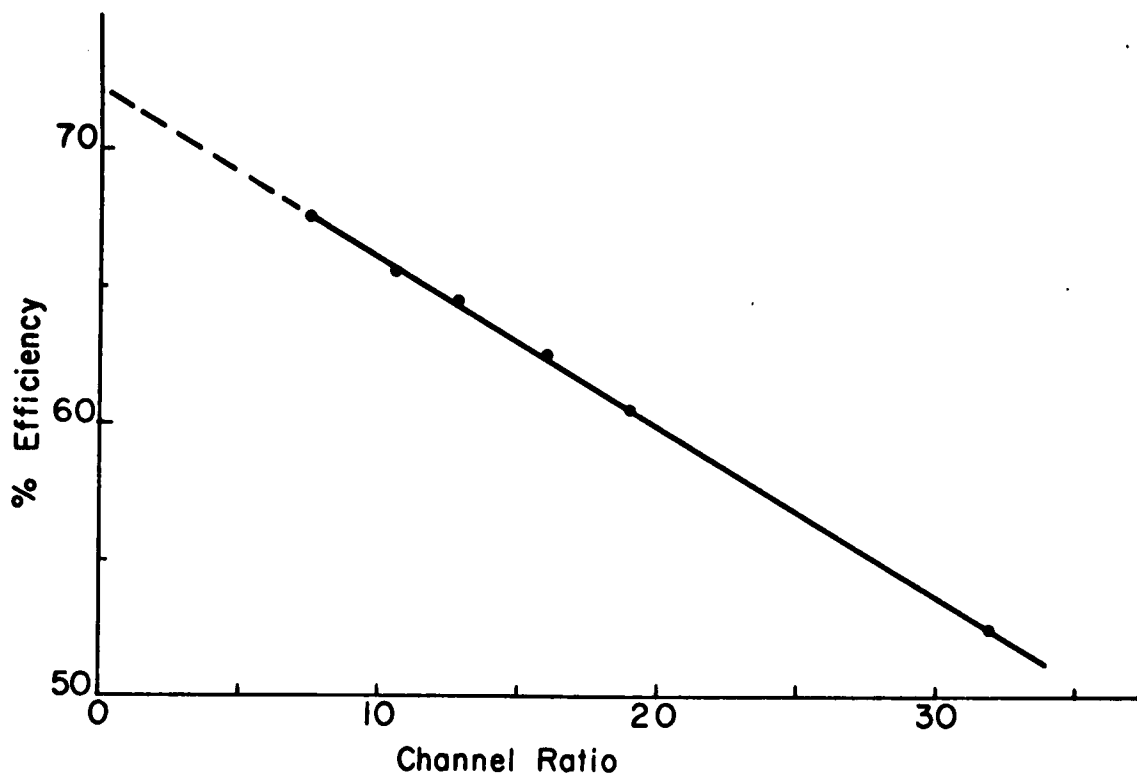
FIGURE 11

Calibration curve for the determination of counting efficiency.

6.   The above chemicals were mixed and dioxane added to

make 1 liter.

Samples were counted by liquid scintillation counting

in a Picker Nuclear Liquimat Model 650, using the channel

ratio method for quench correction.

Correction for quenching was made by plotting channel

ratio against percentage efficiency. The standards for

quenching were prepared with Ca^{45} of known activity quenched

by a stepwise addition of acetone. A linear relation is obtained

as seen in Fig. 11. The intercept and the slope were known and

determined an equation for the straight line. This data was

used to calculate the counting efficiency for the samples using

an Olivetti-Underwood programma 101.

Calculation of Ca Uptake

Calcium taken up by the microsomes was calculated by

dividing the difference in counts between the filtrates of

the blank and of the sample, by the specific activity of

the blank. Specific activity of the blank was calculated

from the radioactivity of the blank and the total

Ca in the blank as described earlier. The value in μ moles

of Ca thus obtained was divided by milligrams of the pro-

tein in the sample in order to get μ moles Ca per mg

protein. This is shown in the simple equation given below.

$$(\text{CPm in blank filtrate} - \text{CPm in sample filtrate}) \times \frac{\mu \text{ moles Ca in blank}}{\text{CPm in blank} \times \text{mg protein in sample}}$$

µ moles of Ca in blank is the total Ca in the reaction mixture.

Determination of the total Ca in the blank filtrate showed no difference from the total Ca in the blank before filtration.

Determination of Inorganic Phosphate (Pi)

The method of Lecocq and Inesi (142) was used.

Reagents

1. Ammonium molybdate $(NH_4)_6MO_7O_{24} \cdot 4H_2O)$, 100 g and NH_4OH (sp. gravity 0.90), 10 ml were dissolved in deionized water to make 1 litre. (R-1).

2. 2.35 g Ammonium vanadate (NH_4VO_3) were dissolved in 400 ml hot deionized water, the solution was cooled and then 6.16 ml HNO_3 (sp. gravity 1.42) diluted with 14 ml deionized water added and the volume made up to 1 litre with deionized water. (R-11).

3. 150.5 g TCA were added to 100 ml R-1 and 100 ml R-11 and made up to 500 ml with deionized water. (R-111).

Procedure:

1. At the desired time 0.2 ml of the enzyme reaction medium was added to 0.2 ml of cold 15% TCA in conical tubes and centrifuged at top speed in a clinical centrifuge for 5 minutes. In some experiments 0.5 ml of enzyme reaction medium and 0.5 ml of TCA were used. The supernatant from each was decanted into
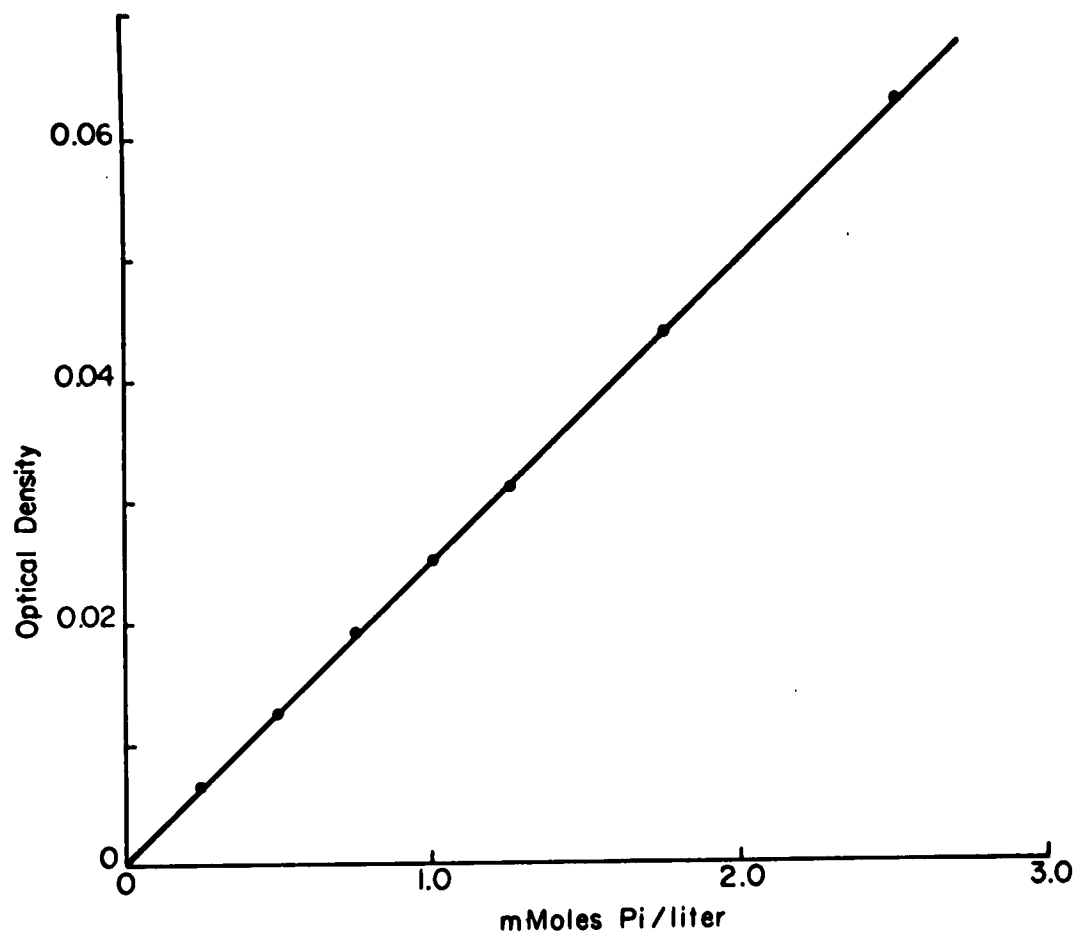
FIGURE 12

Calibration curve for the determination of Pi.

another set of tubes and 0.2 ml of the supernatant was used for Pi determination.

2. Into 0.2 ml of the supernatant 0.2 ml of R-111 was added and the volume made up to 10 ml with deionized water. The tube contents were well mixed and the optical density of the yellow colour formed was measured at 350 mμ with a Hitachi spectrophotometer, against the reagent blank containing 0.2 ml R-111 in 10 ml deionized water.

3. The standard curve (Fig. 12) was plotted from the optical density measurement using 0.2 ml of a known phosphate standard solution and following the same steps as with the enzyme sample.

Measurement of Total Ca

Total Ca in the wet-ashed particulate or in the media or in the supernatant was measured by atomic absorption as described in Part I. In the presence of ATP, the precipitate formed with lanthanum was centrifuged in a clinical centrifuge and Ca was measured in the supernatant. Weber et.al. (47) have used this method to measure Ca in the presence of ATP.

ATPase Assay

In some of the experiments ATPase assay was done simultaneously with the Ca uptake measurements.

At specified time intervals 0.2 ml of the reaction

mixture was added into 0.2 ml of cold 15% TCA. Inorganic

phosphate was determined as described in the methods.

When ATPase assay was desired in the absence of Ca,

the reaction mixture was the same as for Ca uptake, but

Ca was excluded and EGTA added to give final concentration

of 1 mM.

Solutions, Drugs and Chemicals

Generally, stock solutions of the items to be added in

the Ca uptake reaction mixture contained 10 times the

final concentration, since 0.1 ml was added in 0.9 ml of

the reaction mixture.

L. Epinephrine (Nutritional Biochemical Corp.) was dis-

solved in deionized water with a drop of HCl added to

facilitate dissolution. The pH was then adjusted to

7 with Tris. It was freshly prepared every time.

Butoxamine Hydrochloride (B.W. & Co. Montreal) was dis-

solved in deionized water and pH adjusted to 7 with Tris.

Caffeine (Nutrional Biochemical Corp.) was dissolved in

water and pH adjusted to 7 with Tris.

Ethyleneglycol-bis (β aminoethyl ether) N-N'-Tetra acetic

acid (EGTA, Sigma) was dissolved in deionized water by

adding minimum amount of Tris. At complete dissolution

the pH was 7.45.

Mersalyl, sodium salt (Sigma) was dissolved in deionized

water.

Sodium Azide, K oxalate, K phosphate (KH_2PO_4, adjusted to pH 7).

Chlorides of Strontium, Barium, Lanthanum, Sodium and Potassium (Fisher) were all dissolved in deionized water.

Tris (Hydroxymethyl) Aminomethane (THAM, Fisher) was dissolved in deionized water. For buffer preparation, HCl was added to reach the desired pH, and made up to the final concentration with deionized water.

Adenosine Triphosphate, Tris salt (Tris ATP, Sigma) was dissolved in deionized water and pH adjusted to 7 with Tris. Generally a 50 mM stock solution was prepared, 2 ml of the stock solution was taken into separate tubes, which were left in the freezer. A tube of frozen ATP was taken just before it was required to make the reaction mixture.

Adenosine 3'-5'-cyclic Monophosphoric acid (Sigma) was dissolved in deionized water and pH adjusted to 7.

Ergonovine Maleate (Sigma) was dissolved in deionized water.

Ergotamine Tartarate (Sigma) was dissolved in deionized water and prepared fresh, since it has the tendency to precipitate on standing.

Oxytocin synthetic (Grade IV, Sigma) or synthetic Oxytocin (Parke Davis & Co) received in the powder form,

were made in 0.1 N acetic acid to give a stock solution
containing 5 units/ml.

Ca-EGTA Buffers

In order to calculate the concentration of free Ca
ions, Ca and EGTA were mixed in a certain ratio following the
guidelines of the calculated data by Portzehl et al
(8). The actual concentration of free Ca ions was then
calculated from the equation

$$K = \frac{[CaE]}{[Ca_f][E_f]} = \frac{Ca - Ca_f}{(Ca_f)(E - [Ca - Ca_f])}$$

$$K = \frac{(Ca - Ca_f)}{Ca_f(E - Ca + Ca_f)} \tag{1}$$

where K = apparent association constant for Ca EGTA
complex. (This value was the sum of K_1 and K_2 given by
Portzehl et al (8) since the pH and ionic strength
were adjusted to the same values as those used in their
experiments).

Ca or E = Total Ca or EGTA respectively (moles/liter)

Ca_f or E_f = Concentration of free Ca^{++} or EGTA (moles/liter)

CaE = Concentration of Ca-EGTA complex (moles/liter)

Thus all the values except Ca_f in the equation are known.

The ionic strength in our experiments was adjusted
to 0.1 by adding buffered Tris. The amount of total Ca

was kept constant to 20 µM and the amount of EGTA was varied to obtain the desired $\frac{Ca}{EGTA}$ ratio.

A sample calculation is shown below.

Sample Calculation for Ca_f
_____

Apparent association constant $K = 7.62 \times 10^6 \ M^{-1}$

Ca = 20.0 µM

E = 63.5 µM

Substituting the above values in equation (1),

$$7.62 \times 10^6 = \frac{20 \times 10^{-6} - Ca_f}{(Ca_f)[63.5 \times 10^{-6} - 20.0 \times 10^{-6} + Ca_f]} \quad (1a)$$

or

$$7.62 \times 10^6 = \frac{20.0 \times 10^{-6} - Ca_f}{43.5 \times 10^{-6} Ca_f + Ca_f^2} \quad (1b)$$

The value for Ca_f can be obtained from equation (1b) as follows:

$$Ca_f^2 + 43.5 \times 10^{-6} Ca_f = \frac{20.0 \times 10^{-6}}{7.62 \times 10^6} - \frac{Ca_f}{7.62 \times 10^{-6}}$$

i.e.

$$Ca_f^2 + 43.631 \times 10^{-6} Ca_f - 2.625 \times 10^{-12} = 0$$

$$Ca_f = \frac{-43.631 \times 10^{-6} \pm \sqrt{(43.631 \times 10^{-6})^2 + 10.5 \times 10^{-12}}}{2}$$

i.e. $Ca_f = 0.6 \times 10^{-7}$

R E S U L T S

Protein Distribution.

Protein distribution of the subcellular fractions using two different homogenization techniques is shown in Table 9. With Polytron homogenizer, the protein content was higher in the nuclear fraction, and considerably lower in the mitochondrial fraction. The protein content of the microsomal fraction was slightly higher after Teflon homogenization as compared to Polytron homogenization, but the difference was not statistically significant. After preliminary experiments to compare the homogenization techniques for the yield and purity of the microsomal fractions, only Polytron homogenization was used.

Since in all earlier experiments on Ca uptake, skeletal muscle microsomal fraction was also isolated simultaneously, and used for comparison, the protein content of this fraction using the Polytron homogenizer is also shown. The protein content of this fraction was considerably lower (65%) than the protein content of its identical fraction from myometrium.

Characterization of Subcellular Fractions of Rat Myometrium

(a) Succinic Dehydrogenase Activity. The activity of succinic dehydrogenase in the mitochondrial and microsomal fractions by two different homogenization techniques is

TABLE 9

Protein Content of Subcellular Fractions of Rat Myometrium

by Two Different Homogenization Techniques

Fraction	Polytron Homogenizer		Teflon Homogenizer	
	mg protein/g. wet wt.	% of Homogenate	mg protein/g wet wt.	% of Homogenate
Nuclear	42.50 ± 2.12	52.6 ± 2.51	38.51 ± 2.01	47.5 ± 2.2
Mitochondrial	5.34 ± 0.36	6.6 ± 0.29	8.57 ± 0.52	10.5 ± 0.53
Microsomal	8.08 ± 0.69	10.0 ± 0.72	8.86 ± 0.72	10.9 ± 0.74
Supernatant	24.90 ± 2.91	30.8 ± 3.12	25.00 ± 2.32	31.1 ± 2.91
Total	80.82 ± 4.2	100	80.94 ± 4.5	100
Skeletal Microsomes	2.86 ± 0.16	---	---	---

Values are means of 6 determinations ± S.E.M.

TABLE 10

Succinic Dehydrogenase Activity in
Mitochondrial and Microsomal Fractions
of Rat Myometrium Prepared by Two
Different Homogenization Techniques

Fraction	Activity expressed as optical density change/min/mg protein	
	Teflon Homogenization	Polytron Homogenization
1. Mitochondrial	0.0065	0.0091
2. Microsomal	0.00	0.00
3. Liver Mitochondrial	0.0130	---

Activity in Liver Mitochondrial Fraction by Teflon
Homogenization is also shown to test the validity of
the assay method.

shown in Table 10. On mg protein basis the activity
in the mitochondrial fraction from the homogenate by
Polytron homogenizer is higher (40%) than mitochondria
from the Teflon homogenized tissues. Detectable
succinic dehydrogenase activity was absent in the
microsomal fractions from either homogenate as seen in
Table 10. Activity in liver mitochondria was also
measured to test the validity of the method used, (see
Methods) and as expected was found to be higher than
the myometrium mitochondria obtained either by Teflon
or Polytron homogenization techniques. As pointed out
in the 'Methods section', all available methods for
the estimation of succinic dehydrogenase give comparative
activities only (141). Bonner (141) further emphasized
that succinic dehydrogenase works at far below the
activity in any test than has been devised yet. Thus
the absence of activity in the microsomal fraction
(Table 10) is not a proof of the absence of mitochondrial
material in these microsomal fractions.

(b)   Electron Microscopy.

Electron micrographs of nuclear, mitochondrial, and
microsomal fractions are shown in Figs. 13 to 18.

Nuclei, broken nuclei and mitochondria were found
in the sections prepared for electron microscopy from
the nuclear fraction (Figs. 13 and 14). These sections

FIGURE 13

Eelctron micrograph of the nuclear fraction from
rat myometrium. x 1,800

FIGURE 13

Eelctron micrograph of the nuclear fraction from
rat myometrium. x 1,800

FIGURE 14

Electron micrograph of the nuclear fraction from
rat myometrium. x 14,000.

FIGURE 14

Electron micrograph of the nuclear fraction from rat myometrium. x 14,000.
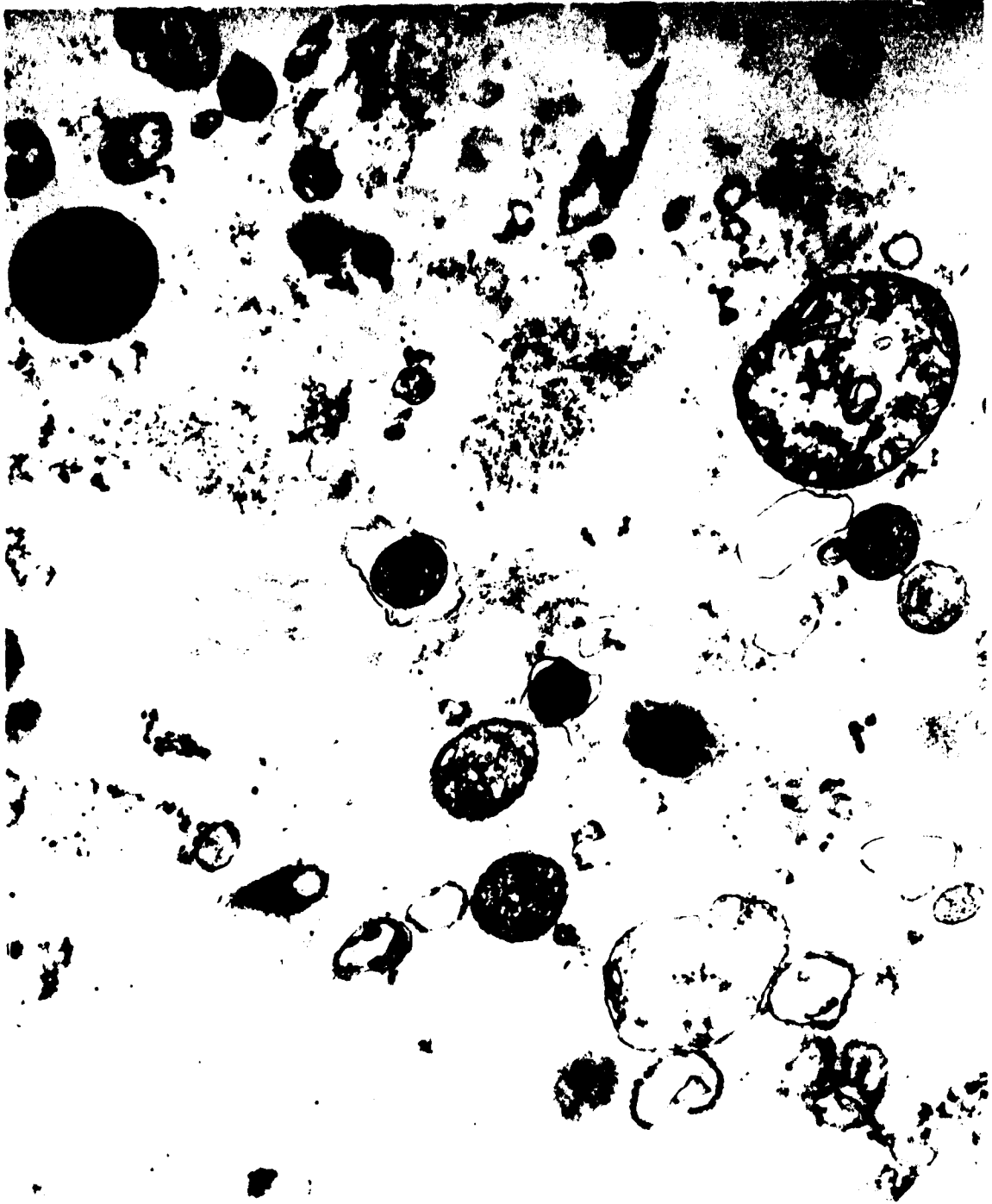
FIGURE 15

Electron micrograph of the mitochondrial fraction
from rat myometrium. x 14,000.
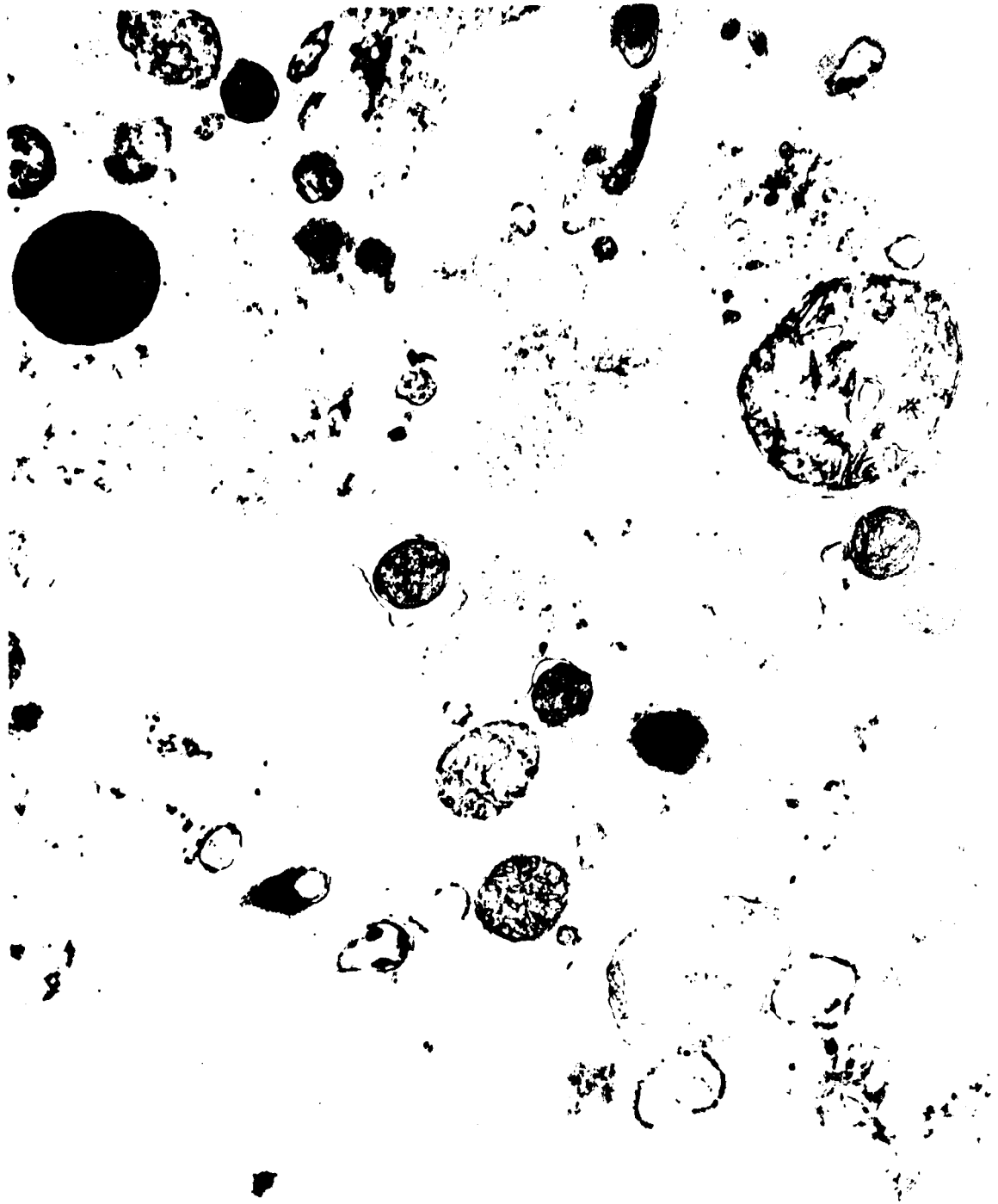
FIGURE 15

Electron micrograph of the mitochondrial fraction
from rat myometrium. x 14,000.
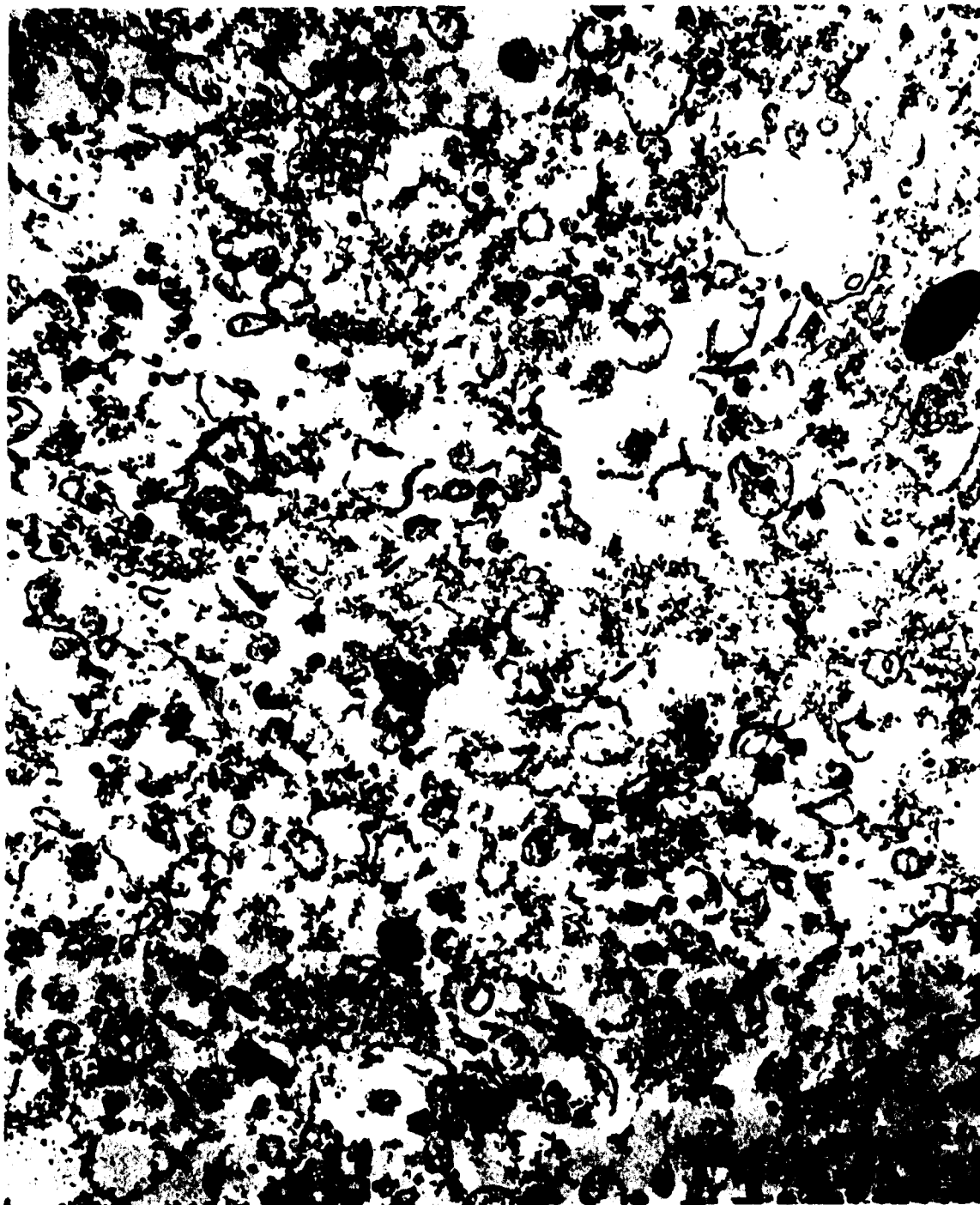
FIGURE 16

Electron micrograph of the microsomal fraction from
rat myometrium. x 14,000
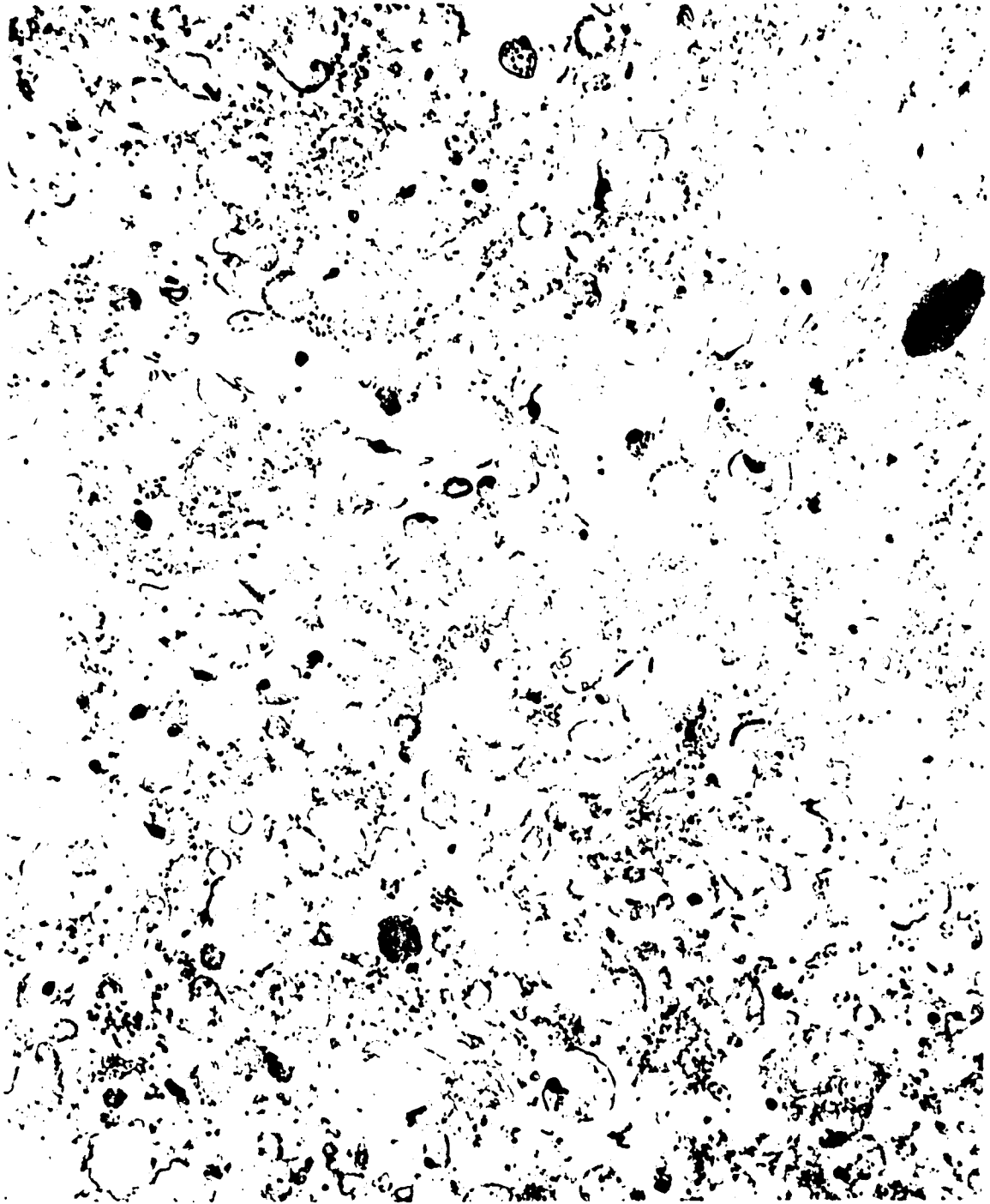
FIGURE 16

Electron micrograph of the microsomal fraction from rat myometrium. x 14,000

FIGURE 17

Electron micrograph of the microsomal fraction from
rat myometrium. x 27,000.
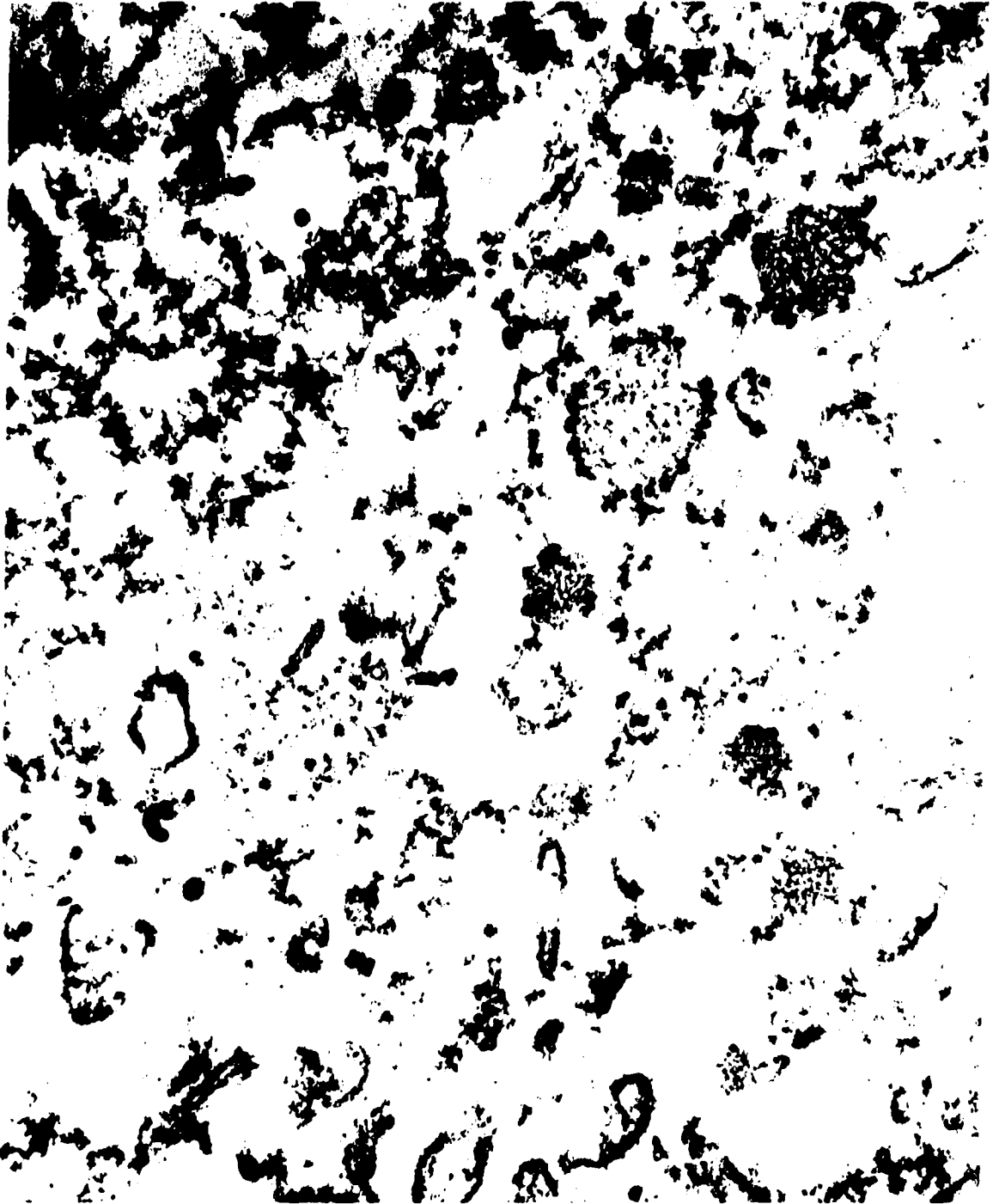
FIGURE 17

Electron micrograph of the microsomal fraction from
rat myometrium. x 27,000.

FIGURE 18

Electron micrograph of the microsomal fraction from
rat skeletal muscle. x 32,000.

FIGURE 18

Electron micrograph of the microsomal fraction from
rat skeletal muscle. x 32,000.

also showed masses of other materials which may be
collagen or myofibrillar material.

Fig. 15 shows an electron micrograph prepared from
the mitochondrial fraction; both whole and broken
mitochondria were found. The other material seen may
be broken nuclei together with collagen material, since
a high percentage (25%) of collagen nitrogen was found
in this fraction by Wakid, Wakid and Needham (144,145).

Fig. 16 and Fig. 17 are the electron micrographs
at two different magnifications of the preparation
from the microsomal pellet. Some round structures were
observed, but, a well defined membrane enclosing these
structures could not be distinguished. Close examination
reveals small particles on the periphery of most of the
larger round structures. These may be ribosomal parti-
cles. An electron micrograph of this fraction of Wakid
(144) also showed that most of the larger round structures
had dense particles attached to their walls, however he was
able to find some smooth walled vesicles in his preparation.
Thin, elongated and smooth structures seen in this electron
micrograph (Fig. 16) may be pieces of the broken walls
of the smooth endoplasmic reticulum. Numerous bits
with no definite shape are seen throughout the prepar-
ation. They may be pieces of membranes.

Microsomal fraction from skeletal muscle isolated
and prepared identically to the microsomal fraction of
myometrium (Fig. 17) as shown in Fig. 18. Here, one
can clearly see the vesicular nature of the material
from this fraction. On close inspection a triple layered
vesicular membrane can be seen as reported recently by
Hasselbach and Elfrin (146), for similar preparations
from skeletal muscle.

Ca Uptake in Mitochondrial and Microsomal Fractions

Table 11 shows Ca uptake and the effect of various
compounds (and inhibitors) on Ca uptake in mitochondria
and microsomes. Total uptake in mitochondria is higher
(25%) than in microsomes. High Na or K (125 mM) reduced
Ca uptake by 90% in mitochondria; neither had an effect
on microsomal Ca uptake. Oxalate and inorganic phosphate
(5 mM) had no effect on microsomal uptake and slightly
lowered uptake in mitochondria. Sodium azide (0.5 mM)
completely inhibited uptake in mitochondria whereas uptake
was only decreased by 12% in microsomes. Mersalyl
(0.5 mM) completely inhibited Ca uptake in both the
mitochondria and the microsomes. Ca uptake in skeletal
muscle microsome was 17 times higher than myometrium
microsomes. Oxalate increased uptake in skeletal
microsomes by 2.8 times and mersalyl inhibited it al-
most completely.

Fig. 19 shows the rate of Ca uptake in mitochondrial

TABLE 11

Ca^{++} Uptake in Mitochondrial and Microsomal Fractions

in Standard Medium Containing 8 μM Ca^{++}. Incubation

Time 10 Minutes

Uptake, μmoles/gm Protein

Additions	Mitochondria		Microsomes		Skeletal microsomes	
	Ca uptake	% control	Ca uptake	% control	Ca uptake	% control
None	5.52 ± 0.31	100 ± 2.81	4.42 ± 0.26	100 ± 2.51	72.73 ± 1.9	100 ± 2.1
+125 mM K$^+$	0.66 ± 0.09	12.2 ± 0.26	4.59 ± 0.32	104 ± 2.60	----	----
+125 mM Na$^+$	0.58 ± 0.57	10.8 ± 0.89	4.55 ± 0.39	103 ± 3.25	----	----
5 mM Oxalate	5.25 ± 0.54	90 ± 1.81	4.38 ± 0.29	100 ± 2.72	203.5 ± 3.4	280 ± 4.2
5 mM Pi	4.85 ± 0.41	88 ± 1.71	4.42 ± 0.31	100 ± 2.69	----	----
0.5 mM Azide	0.00	0.00	3.90 ± 0.32	88 ± 1.9	----	----
0.5 mM Mersalyl	0.00	0.00	0.00	0.00	4.3 ± 0.21	6 ± 0.23

Values are means of 5 determinations ± S.E.M.
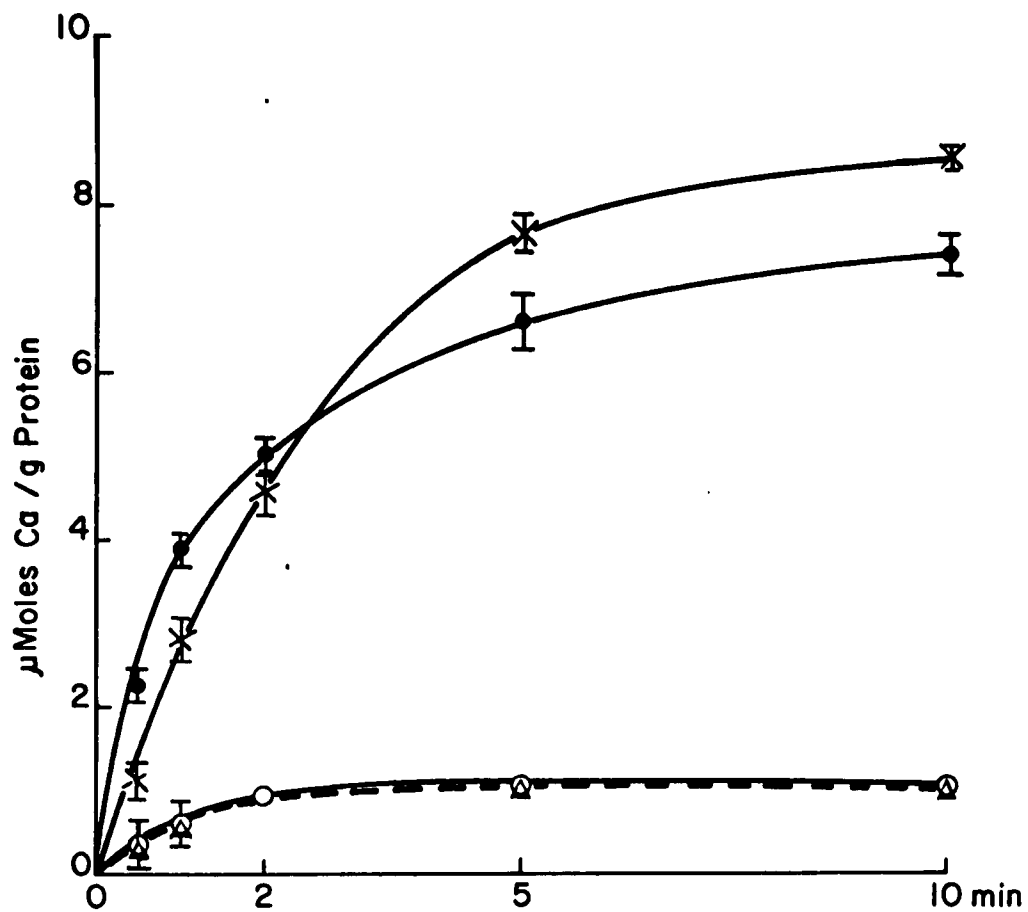
FIGURE 19

Rate of Ca uptake in mitochondrial and microsomal
fractions at 25°C and 4°C. Mitochondria 25°C (X),
Microsomes 25°C (•), Mitochondria 4°C (△),
Microsomes 4°C (O).

Each point is mean of 5 determinations. Vertical bars
on either side of points indicate S.E. In the absence
of a bar S.E. is within the point.
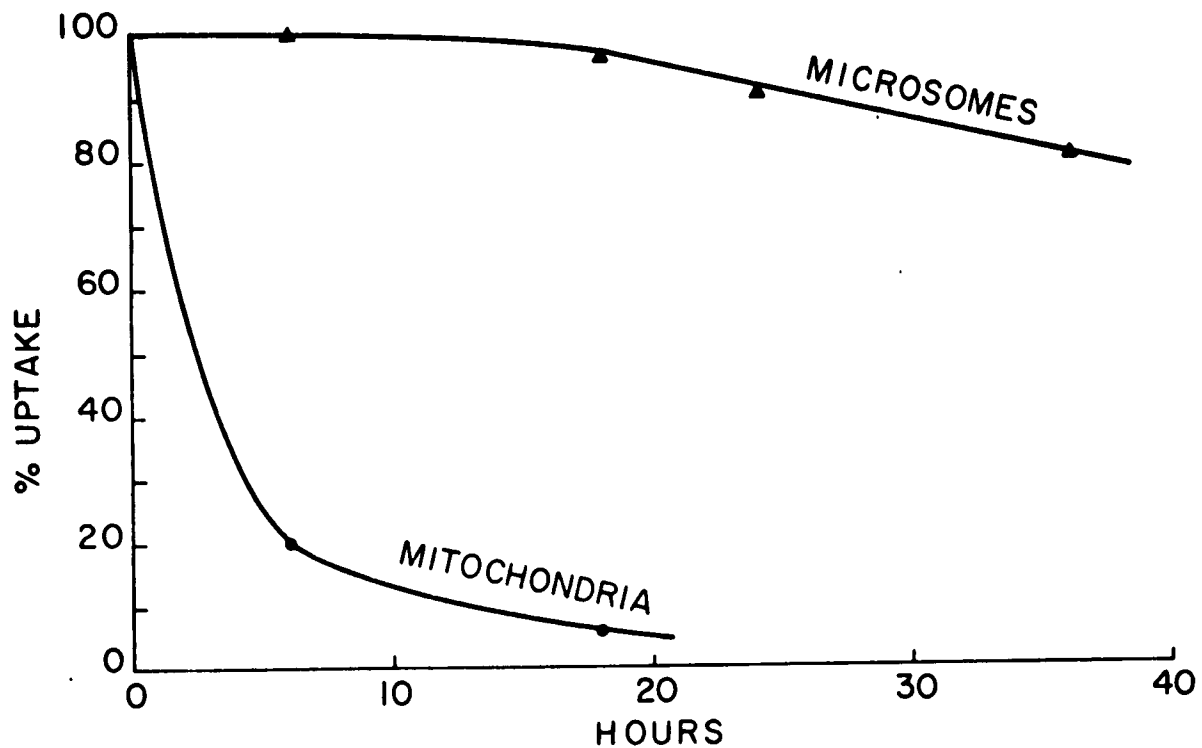
FIGURE 20

Effect of aging on Ca uptake by the microsomes and
mitochondria in standard medium containing 15 μM Ca.
Each point is a mean of 5 determinations.
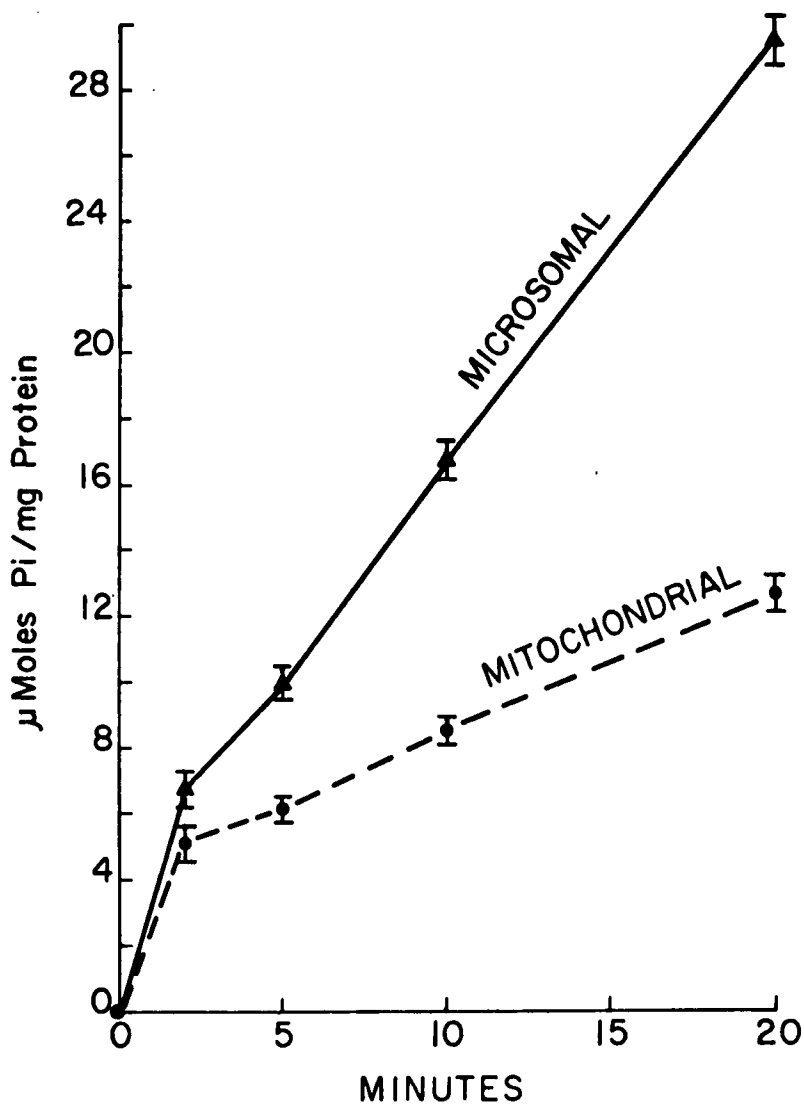
FIGURE 21

Rate of ATP splitting by mitochondrial and micro-
somal fractions.

Each point is mean of 5 determinations. Vertical
bars on either side of points indicate S.E.

and microsomal fractions at 25°C and at 4°C. Although the total Ca uptake in mitochondria is higher than in microsomes, the rate of Ca uptake in the first two minutes is slower. The rate of Ca uptake in microsomes starts to slow down after 2 minutes and only a very small amount is taken up between 5 and 10 minutes. In mitochondria the rate of Ca uptake remains high for 5 minutes. For both types of particles, these curves suggest that uptake of Ca remains linear with time for very short intervals.

Ca uptake at 4°C (bottom curves Fig. 19) was small in the first 2 minutes and negligible after 2 minutes in both the mitochondria and microsomes.

Fig. 20 shows the effect of aging on the Ca uptake activity of the mitochondrial and microsomal fractions. Whereas uptake in mitochondria decreased to 20% in 6 hours, and nearly disappeared in 18 hours, the microsomal fraction retained over 95% of the activity even after 18 hours and 80% of the activity after 36 hours.

ATPase Activity in Mitochondrial and Microsomal Fractions

The rate of ATP hydrolysis in the Ca uptake media by mitochondria and microsomes is shown in Fig. 21. The rate of ATP splitting was considerably higher in the microsomal fraction than in the mitochondrial fraction. After 10 minutes, microsomal ATPase hydrolysed
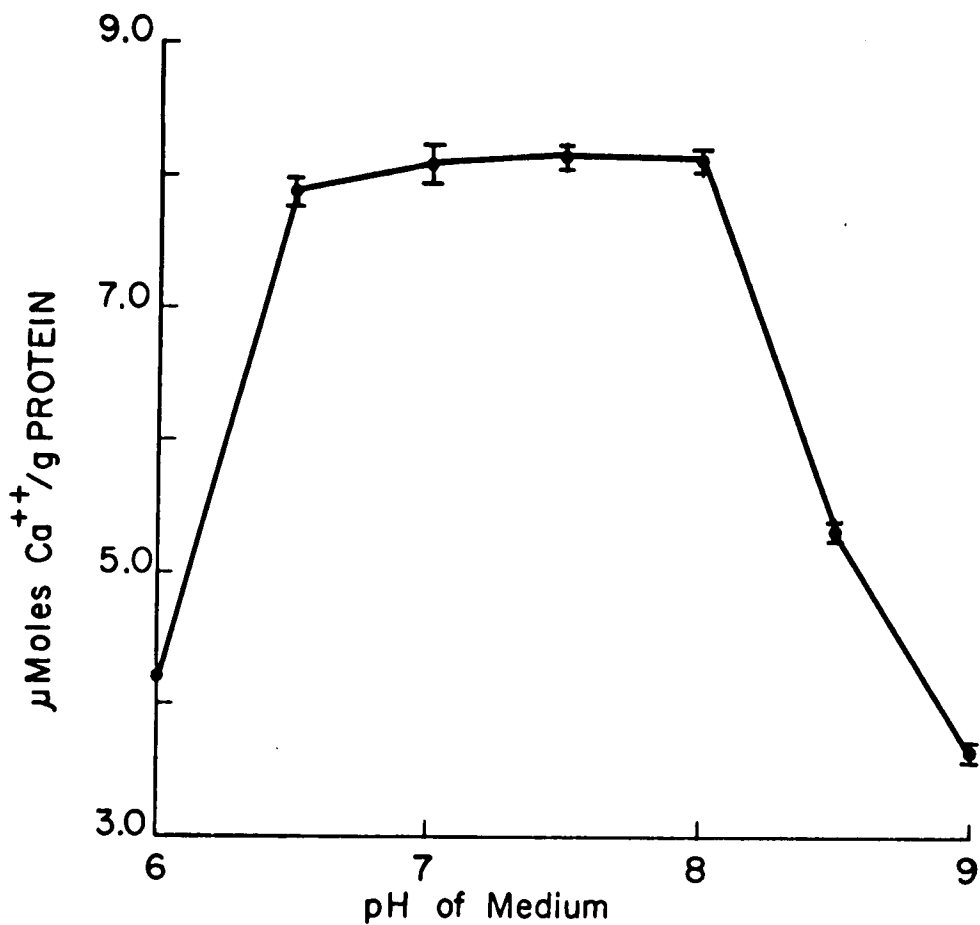
FIGURE 22

Effect of pH on microsomal Ca uptake in standard
medium containing 15 μM Ca.

Each point is mean of 5 determinations. Vertical
bars on either side of points indicate S.E. In the
absence of a bar S.E. is within the point.

TABLE 12

Effect of ATP and Mg on Ca Uptake in Standard
Medium containing 15 μM Ca, but no ATP and Mg.
Incubation Time 15 minutes.

Additions	Ca Uptake μMoles/g Protein
None	0.21 ± 0.084
5 mM ATP	5.63 ± 0.174
5 mM Mg	0.69 ± 0.074
5 mM ATP & Mg	6.32 ± 0.179

Values are means of 5 experiments $\pm$ S.E.M.

16.6 moles whereas mitochondrial ATPase hydrolysed only

8.6 moles Pi/mg protein. When calculated, the inorganic

phosphate liberated after 10 minutes by the microsomal

ATPase was 30% higher than the amount of ATP added.

This may indicate the presence of myokinase. The rate

of ATP hydrolysis by each fraction was very fast in the

first two minutes and then decreased markedly, more for

mitochondrial ATPase than for microsomal ATPase.

After making the above observations in mitochondrial

and microsomal fractions, the microsomal Ca uptake was

studied in more detail. The results of these experi-

ments are shown in the following pages.

The effect of varying pH on Ca uptake is shown in

Fig. 22. The pH was adjusted with Tris-HCl buffer. The

pH of the medium after Ca uptake was also measured, and

changes were negligible. It can be seen that for Ca

uptake, the optimum pH was between 7.5 and 8. There was

however a broad peak obtained between pH 6.5 and 8.

Table 12 shows the effect of the omission of ATP

or Mg or both from the medium on Ca uptake. When there

was no ATP and Mg in the medium, there was no uptake.

When ATP alone was added, the uptake was 89% of when

both ATP and Mg were present. When Mg alone was present

without ATP, there was only about 11% uptake compared

to when both ATP and Mg were present. This means that
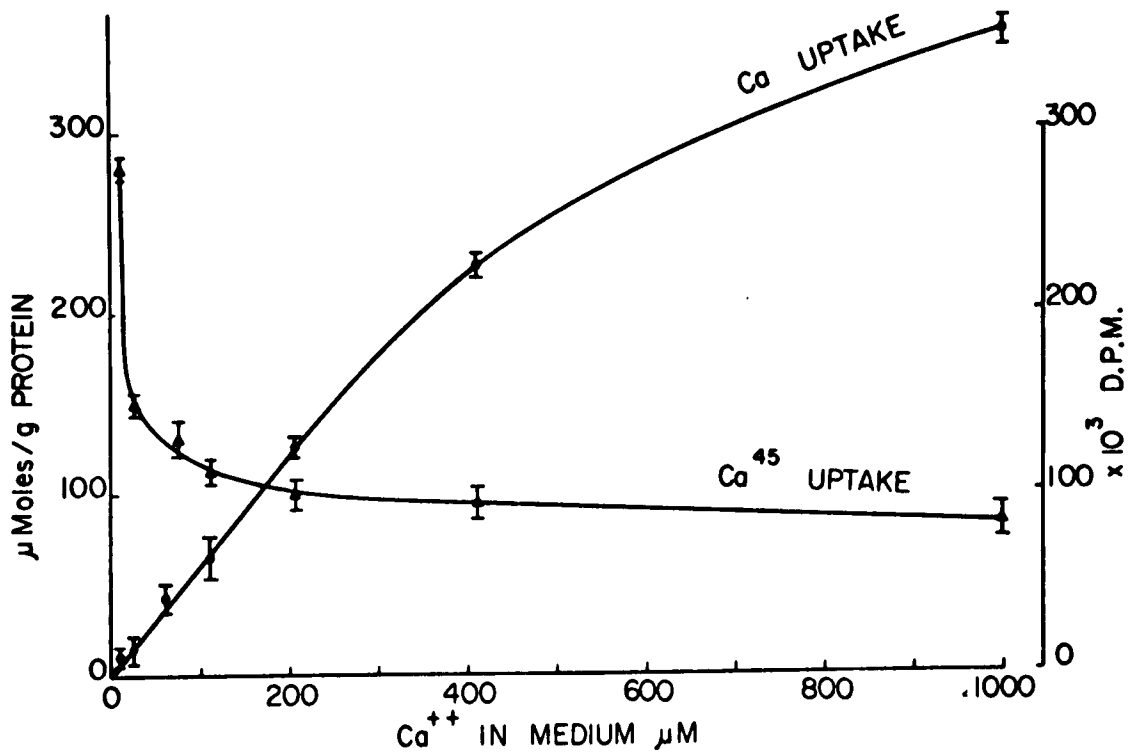
FIGURE 23

Effect of increasing concentration of Ca in the medium on Ca and Ca^{45} uptake in standard medium.

Each point is mean of 3 determinations. Vertical bars on either side of points indicate S.E.

for Ca uptake ATP requirement is absolute whereas the presence of added Mg increases uptake only to a small degree.

Medium Ca concentration and Uptake

The effect of increasing Ca concentration in the medium from 10 μM to 1 mM on Ca uptake and Ca^{45} uptake is shown in Fig. 23. Ca^{45} (dpm) in the media was kept constant while the total Ca was raised by adding Ca. It can be seen from the curve of Ca uptake that it increased with the increase in the external Ca concentration in nearly a straight line relationship up to 400 μM Ca in the medium when 225 μmoles Ca per mg protein were taken up. When the external Ca was further increased from 400 μM to 1 mM, Ca uptake increased from 225 to 355 μmoles/mg protein. The uptake of Ca^{45} decreased very sharply when external Ca concentration was increased up to 100 μM. Thereafter Ca^{45} uptake decreased slowly when the external Ca was increased from 100 μM to 1 mM. The curve of Ca^{45} uptake has an inverse asymtotic shape. This result shows that unlabelled Ca competed with Ca^{45} for uptake by microsomes as expected if a limited number of binding sites were available. However, the curve for Ca^{45} did not tend to reach zero even when Ca was increased by 100 fold in the medium. These and other aspects of Ca
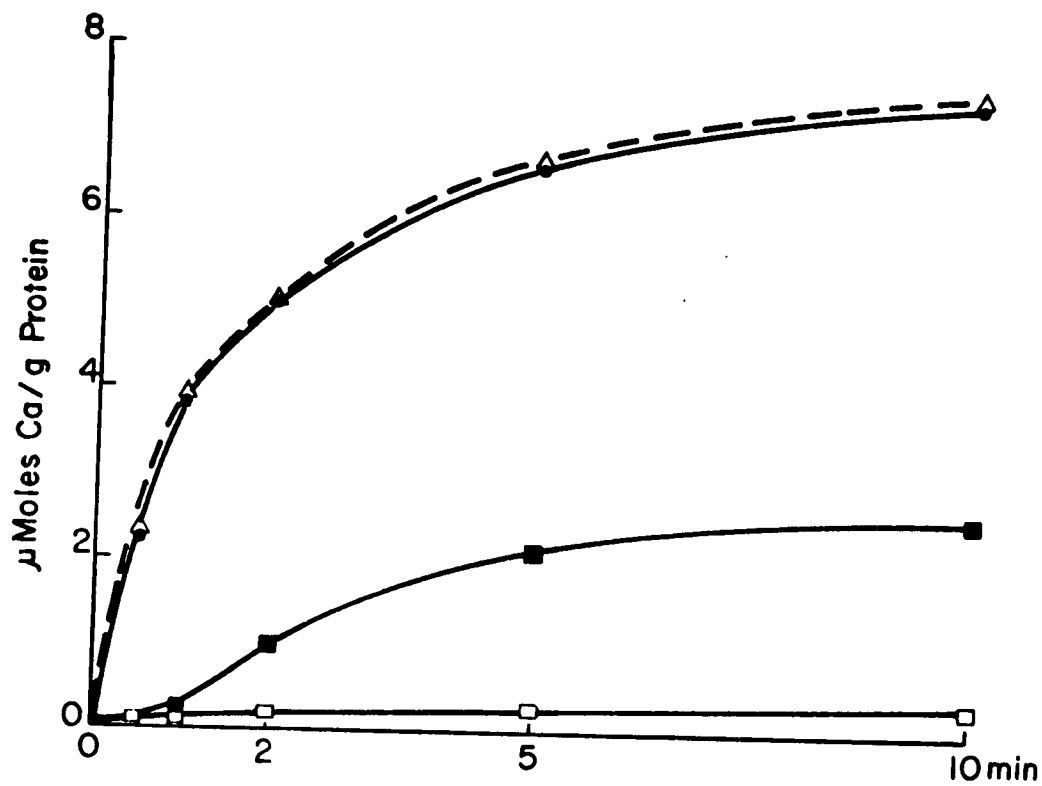
FIGURE 24

Effect of the addition of Strontium in standard medium
containing 12 µM Ca on Ca uptake. Contol (●), 0.05
mM $SrCl_2$ (△), 0.25 mM $SrCl_2$ (■), 1 mM $SrCl_2$ (□)
Each point is mean of 5 determinations.
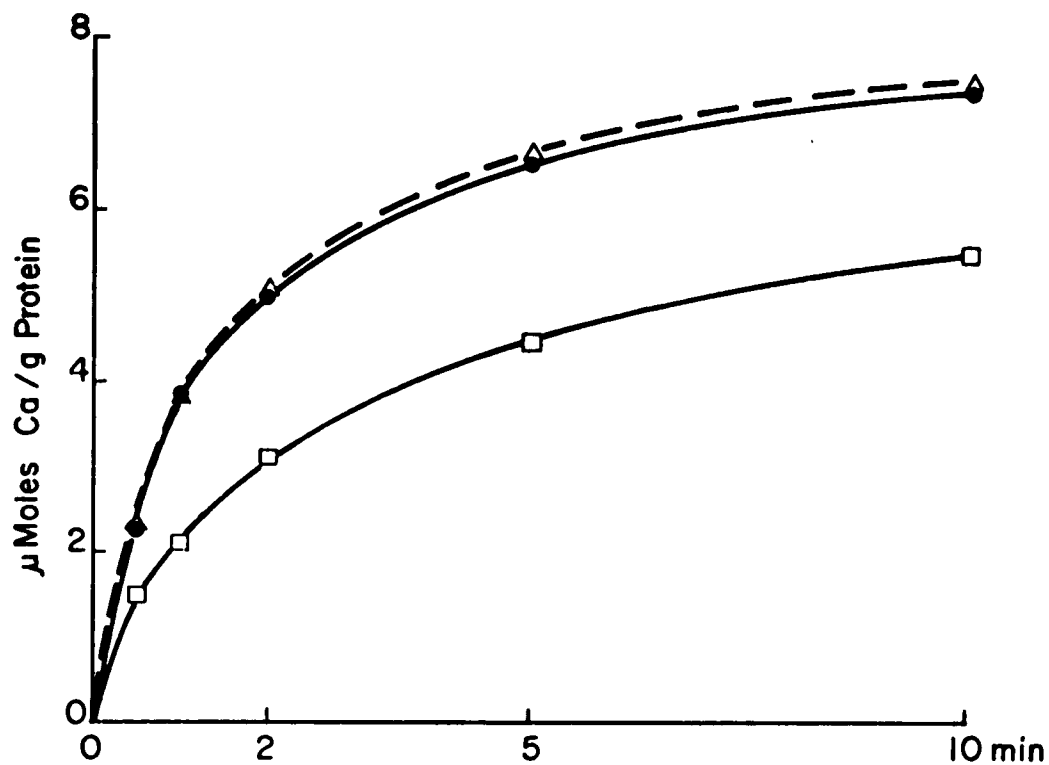
FIGURE 25

Effect of the addition of Barium in standard
medium containing 12 µM Ca on Ca uptake. Control
(●), 0.05 mM BaCl$_2$ (△), 1 mM BaCl$_2$ (□).
Each point is mean of 4 determinations.

binding will be dealt with in the discussion.

Effect of Multivalent Cations

Fig. 24 shows the effect of the addition of three different concentrations of strontium on Ca uptake. When the Ca uptake medium contained 0.05 mM Sr which was more than 4 times the concentration of medium Ca, there was no effect on either the rate or the total Ca uptake. However with higher concentration (0.25 mM), both the rate, and total Ca uptake in 10 minutes were decreased. The total uptake in 10 minutes was lowered by 60% and the rate also roughly to the same extent. When still higher concentrations (1 mM) of Sr was used Ca uptake was almost completely inhibited. If one computes the inhibition of Ca^{45} uptake by Ca from the results shown in Fig. 23 one finds that by increasing Ca from 12 μM to 50 μM, Ca^{45} uptake was reduced by 40%. When Sr, 50 μM was added in the Ca uptake medium containing 12 μM Ca as pointed out above, there was no change in the total Ca uptake. Since total Ca uptake was measured by the uptake of Ca^{45}, it follows that with the addition of 50 μM Sr there was no change in Ca^{45} uptake. These results show that Sr is much less effective than Ca in competing for the sites which bind Ca^{45}.

Fig. 25 shows the effect of 0.05 mM and 1 mM $BaCl_2$ in the medium on Ca uptake. The lower concentration of
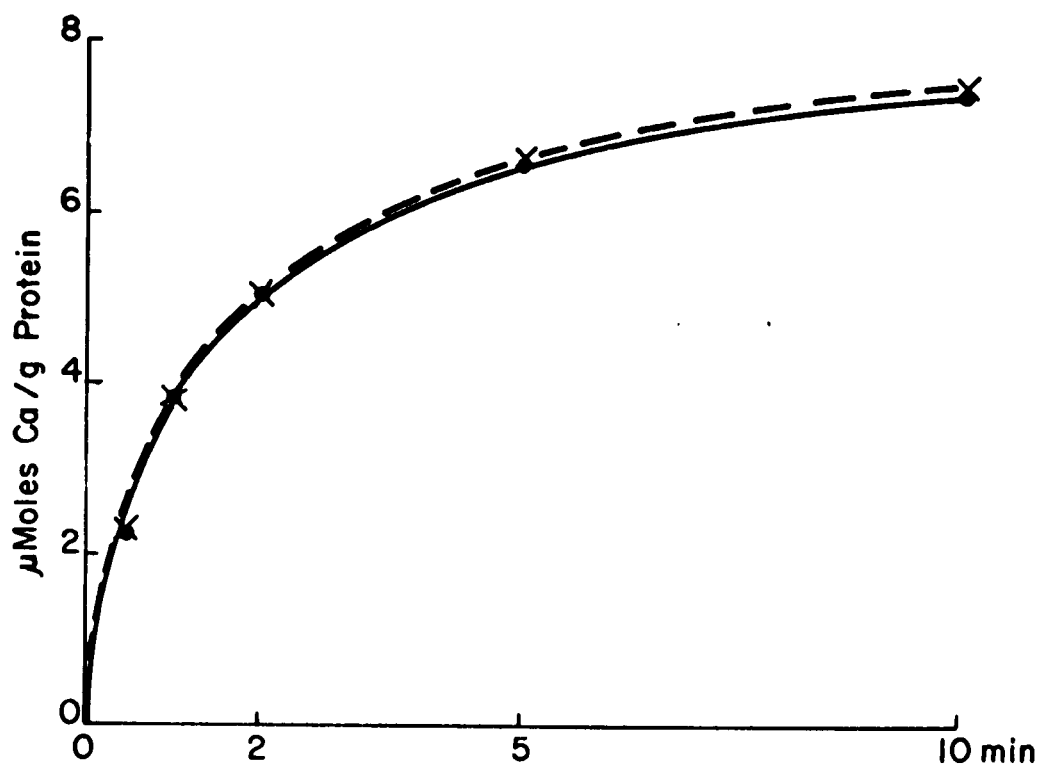
FIGURE 26

Effect of the addition of Lanthanum in standard
medium containing 12 µM Ca, on Ca uptake by
the microsomal fraction: control (●), 0.05
mM LaCl$_3$ (✕).
Each point is mean of 5 determinations.

Ba (0.05 mM) like Sr, had no effect. With 1 mM Ba in the medium, Ca uptake was reduced by only 28%, whereas this concentration of Sr completely inhibited the uptake (Fig. 24). Thus Ba is even less effective than Sr in its binding to the Ca^{45} binding sites.

In Fig. 26 the effect of 0.05 mM lanthanum (La) on Ca uptake is shown. Ca uptake was not affected with this concentration of La. Higher concentration of La were found to give erroneous results which was discovered later as due to some interaction of Ca, La, and phosphate resulting in the retention of Ca on the filters. This was clarified by measuring the Ca uptake under the following conditions.

Uptake Measured at 25°C

1. Calcium uptake in standard medium.

2. Calcium uptake in standard medium containing 1 mM $LaCl_3$.

3. Calcium uptake in standard medium without ATP.

4. Calcium uptake in standard medium without ATP containing 1 mM $LaCl_3$.

5. Calcium uptake in standard medium without ATP containing 6 mM Pi.

6. Calcium uptake in standard medium without ATP containing 6 mM Pi and 1 mM $LaCl_3$.
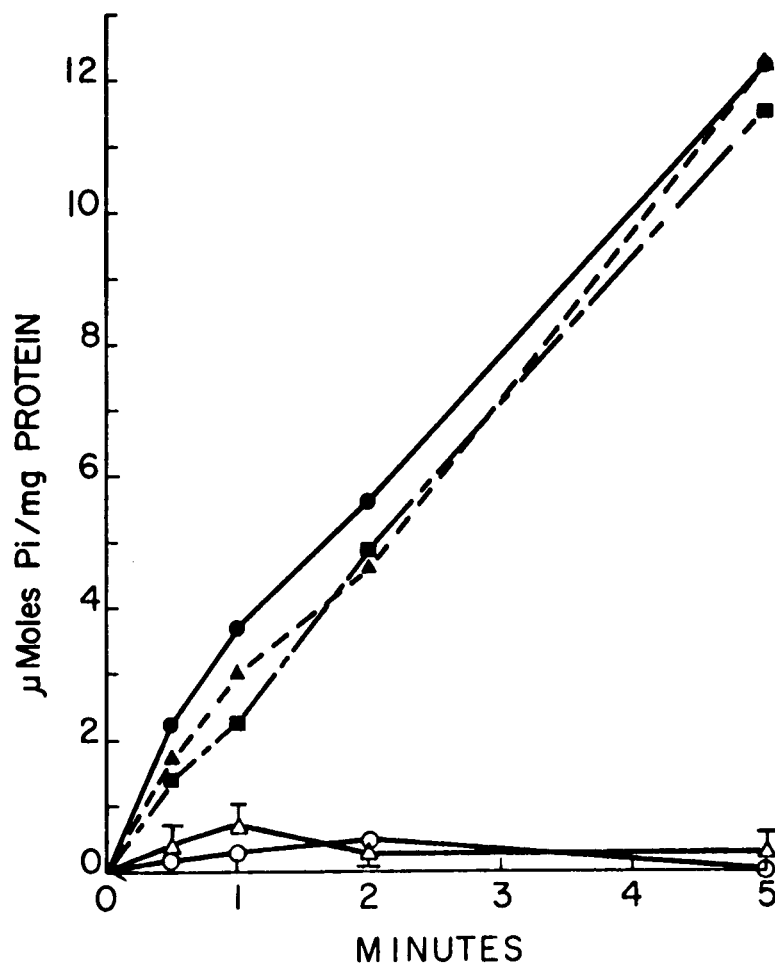
FIGURE 27

Rate of ATP splitting by the microsomal fraction. Stand-
ard medium with no calcium and 1 mM EGTA (▲), 10 μM
Ca (●), and 10 μM Ca with 0.5 mM mersalyl added (■),
difference between Ca and no calcium curve (△), differ-
ence between Ca without and with mersalyl (○).
Each point is mean of 4 determinations. Vertical bars on
either side of points indicate S.E. In the absence of a
bar S.E. is within the point.

Uptake measured at 4°C

7.   Ca uptake in standard medium

8.   Ca uptake in standard medium containing 1 mM $LaCl_3$.

9.   Ca uptake in standard medium containing 1 mM $LaCl_3$ and 6 mM Pi.

10. Ca uptake in standard medium without ATP containing 6 mM Pi.

The results obtained showed that Ca uptake in 2, 6 and 10 was five times that of 1. There was no uptake in any of the other media. It is reasonable to deduce from the above that the apparent Ca uptake found with higher concentration of La in the medium was due to some interaction (precipitation reaction) of Ca, La and phosphate.

Ca Activated ATPase

The effect of the presence and absence of Ca, and the effect of mersalyl with Ca on the ATP hydrolysis is shown in Fig. 27. In medium containing 0 Ca, 1 mM EGTA was added to complex any Ca as a contaminant in the medium. The difference in the ATPase activity with and without Ca in the medium and also the difference with and without mersalyl in Ca medium are also shown in Fig. 27. These differences are very small and the difference in the activity with and without Ca is not significant. Inhibition of activity with mersalyl at 2 minutes is

TABLE 13

Effect of the addition of various drugs on Ca uptake by microsomes and standard medium containing 12 µM of Ca.

Concentration of each drug used is shown after the drug.

moles Ca/g Protein

Time (Minutes)	Control	Oxytocin 10 mU/ml	Ertotamine tartarate 50 µM	Ergonovine maleate 50 µM	Caffeine 8 mM	Cyclic AMP 1µM or 1mM
1	2.41 ± 0.112	2.45 ± 0.101	2.25 ± 0.113	2.50 ± 0.045	2.55 ± 0.059	2.60 ± 0.076
2	3.58 ± 0.085	3.48 ± 0.095	3.65 ± 0.123	3.55 ± 0.098	3.65 ± 0.079	3.75 ± 0.146
5	5.23 ± 0.073	5.35 ± 0.182	5.20 ± 0.096	5.15 ± 0.128	5.25 ± 0.155	5.35 ± 0.179
10	5.87 ± 0.297	6.40 ± 0.195	5.45 ± 0.18	5.60 ± 0.286	5.80 ± 0.232	5.75 ± 0.240

Values are means of 4 experiments ± S.E.M.
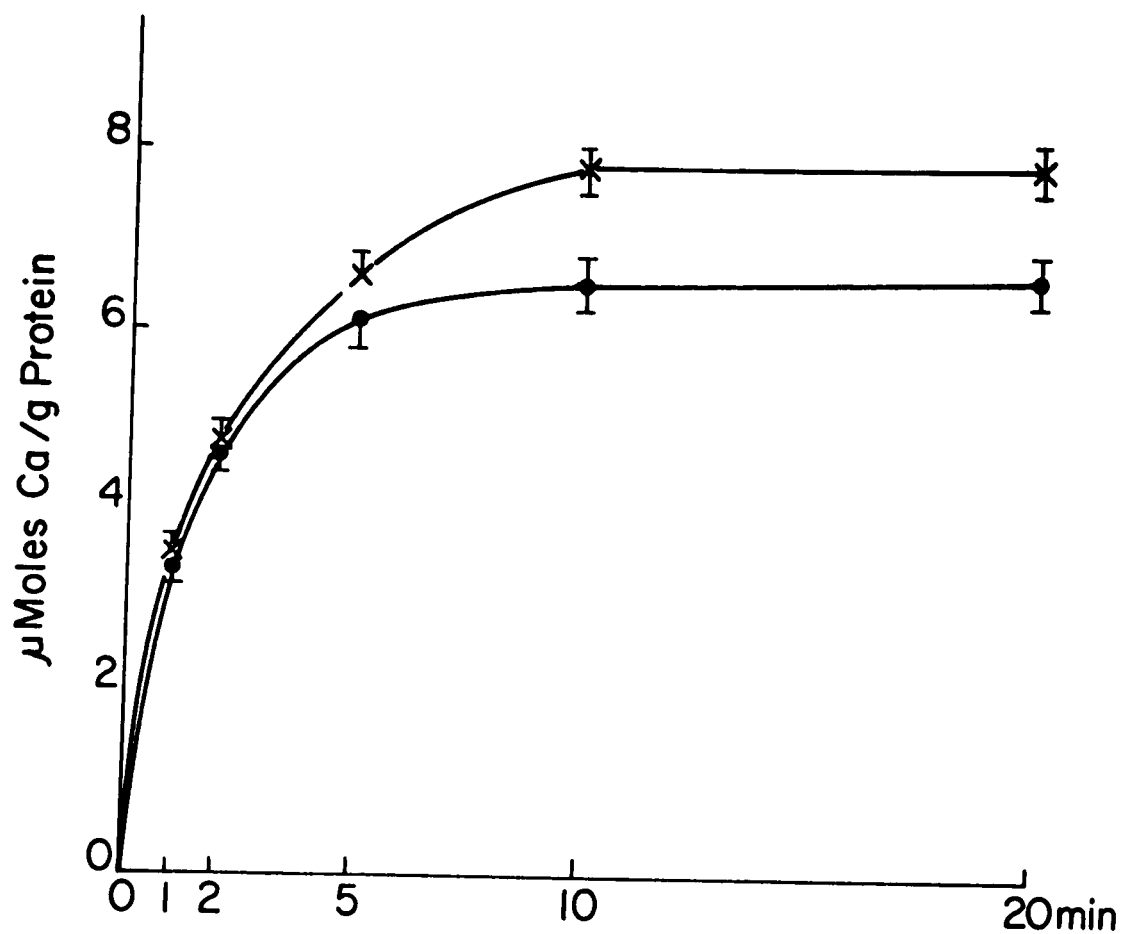
FIGURE 28

Effect of Epinephrine on Ca uptake by the microsomal
fraction, in standard medium containing 15 µM Ca control
(●), 1 ng/ml Epinephrine (X).
Each point is mean of 5 determinations. Vertical bars
on either side of points indicate S.E.

FIGURE 28

Effect of Epinephrine on Ca uptake by the microsomal
fraction, in standard medium containing 15 μM Ca control
(●), 1 ng/ml Epinephrine (×).
Each point is mean of 5 determinations. Vertical bars
on either side of points indicate S.E.

TABLE 14

Effect of epinephrine on the rate of ATP splitting in Ca and Ca free standard media

μmoles Pi/mg Protein

Additions	Time in minutes			
	1	2	5	10
EGTA 1 mM	3.7 ± 0.15	5.6 ± 0.21	12.2 ± 0.22	20.5 ± 0.16
EGTA 1 mM + Epinephrine 10 mg/ml	3.6 ± 0.16	5.8 ± 0.09	12.1 ± 0.13	21.0 ± 0.15
10 μM Ca	3.75 ± 0.12	5.7 ± 0.12	11.9 ± 0.21	22.3 ± 0.10
10 μM Ca + Epinephrine 10 mg/ml	3.5 ± 0.21	5.9 ± 0.18	12.0 ± 0.18	21.8 ± 0.08

Values are means of 5 experiments ± S.E.M.

marginally significant, but at 5 minutes the difference
becomes negligible. Therefore it is doubtful that
mersalyl had any effect on the ATPase activity. There
does not seem to be any parallelism between the rate
of Ca uptake (Fig. 19) and the curves shown in this
figure for ATPase activity with or without Ca. Although
there was only negligible depression of the ATPase
activity with mersalyl, this compound inhibited the Ca
uptake nearly completely (Table 11).

Effect of Drugs

Table 13 shows the effect of certain drugs which
have been shown to modify the contractile responses of
uterine and skeletal muscle. None of these compounds
(oxytocin, ergots, caffeine and cyclic AMP) in the
concentrations shown (Table 13) affected microsomal Ca
uptake. Cyclic AMP was also included in this list,
since there is a great deal of speculation about its
role in Ca binding to membranes (147). In one experiment
cyclic AMP was used with and without caffeine in the
absence of ATP from the medium. There was no Ca uptake
in either case.

Epinephrine (10 ng/ml) was found to increase Ca
uptake as shown in Fig. 28. There was significantly
more Ca uptake between 5 and 10 minutes in the presence
of epinephrine compared to the control. The total

TABLE 15

Comparison Between Two Different Methods Used to Measure the Ca Uptake By

Microsomes in Standard Medium Containing 15 µM Ca.

Incubation Time: 10 Minutes

System	Ca Uptake µMoles/g Protein* Radioactivity Method			Total Ca µMoles/g Protein Atomic Absorption Method			Total Mg µMoles/g Protein Atomic Absorption Method		
	4°C	25°C	Difference	4°C	25°C	Difference	4°C	25°C	Difference
Pellet	1.52	7.4	5.88 ± 0.61	66.0	71.97	5.97 ± 0.69	174.2	168.1	6.1 ± 0.41
Supernatant	0.25	6.46	6.21 ± 0.52	29.35	22.82	---	---	---	----
Filtrate	0.13	6.21	6.08 ± 0.39	---	---	----	---	---	----

*Ca uptake in the pellet was calculated from the dpm in the pellet and the specific activity of the medium for uptake. The amount of Ca calculated was divided by the protein content of the pellet. Calcium uptake from the supernatant (after centrifugation) or the filtrate was calculated by dividing the difference in dpm of the filtrate or the supernatant by the specific activity of the medium. The amounts of Ca calculated were divided by the microsomal protein added in the reaction mixture.

Values are means of 3 determinations ± S.E.M. (Paired controls were used).

uptake with and without epinephrine after 5 minutes was
6.4 and 6.1 μmoles/g protein respectively and after 10 or 20
minutes it was 7.8 and 6.3 μmoles/g protein respectively.
In a separate experiment when Ca uptake without ATP in
the medium but in the presence of epinephrine was
measured, there was no uptake of Ca.   I also measured
the rate of ATP hydrolysis with epinephrine   and with
and without Ca in the medium.   No difference in the
rate of ATP splitting was found in any of these situations.
These results are shown in Table 14.

Net Ca Uptake

Ca uptake measured by the filtration method may
represent only an exchange of Ca^{45} with Ca already pre-
sent in the microsomal fraction. Any such exchange would,
of course, have to be ATP and temperature dependent,
since there was virtually no uptake without ATP or at
4°C.

Table 15 shows the results of two experiments in
which microsomal fraction was separated by centrifugation
after incubation in Ca uptake medium (see Methods).

Total Ca in the supernatant and the pellet was
measured.   In order to measure Ca in the pellet it was
suspended in approximately 3 ml de-ionized water, 0.2
ml taken for protein determination and 2 ml of the

remaining suspension dried and wet ashed for electrolyte analysis by atomic absorption (see Methods). Ca in the supernatant was measured directly.

Radioactivity was also measured in the supernatant and in the ashed pellet solution. From the radioactivity measurements, Ca uptake was computed in the usual manner from the known specific activity of the medium (see Methods). For comparison, Ca uptake was also measured in the above preparation with the usual filtration methods.

The results show firstly that there was a good agreement between Ca uptake by measuring radioactivity in the filtrate by the usual method or in the supernatant using the centrifugation method.

Secondly the difference in total Ca in the supernatant at 4°C and 25°C corresponded with the difference in Ca measured with radioactive method. The amount of microsomal protein added in the reaction mixture before centrifugation was used to calculate the value of Ca per gram protein removed from the supernatant.

Finally there was an agreement between the amounts of Ca taken up whether it was measured by the difference in the total Ca content of the pellets or by difference in their radioactivity (Radioactivity Method).

Magnesium was also measured in the pellet and as seen in Table 15 the pellet with lower total Ca (4°C)
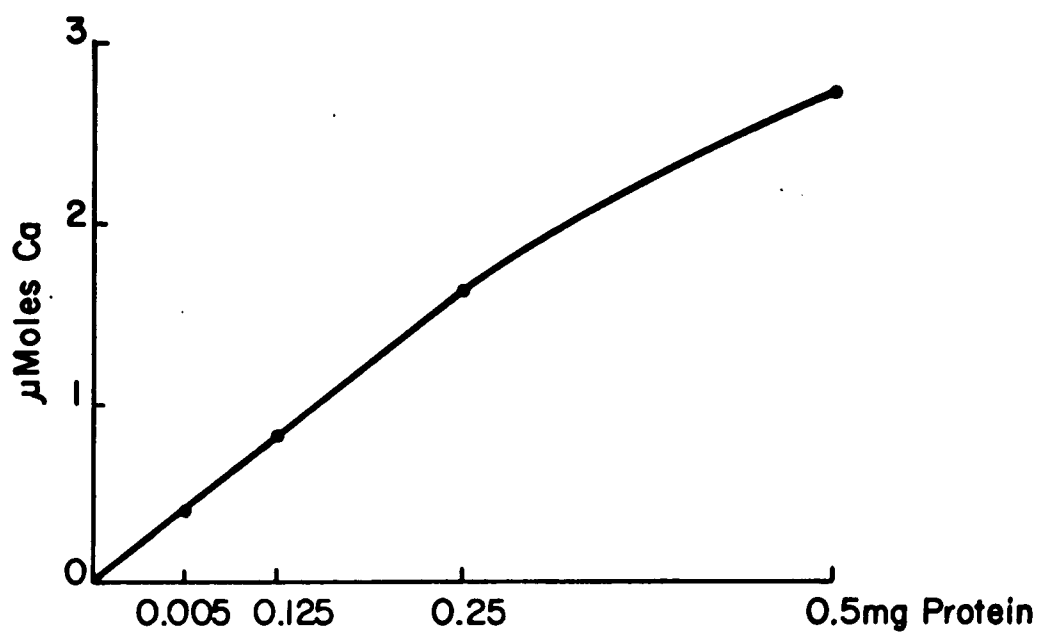
FIGURE 29

Relation between microsomal protein and Ca uptake in standard medium containing 12 µM Ca.

Each point is mean of 4 determinations.

had higher total Mg content whereas pellets with higher total Ca (25°C) had lower total Mg, and in fact the amount of Ca gained by the pellet was equal to the Mg lost by it.

Ca Uptake and Amount of Microsomes

Fig. 29 shows that there was near linear relationship between the amount of Ca taken up and the amount of microsomal protein up to 0.5 mg/ml of reaction mixture. Concentrations higher than shown here were also used, but they presented a difficulty with the filtration method as they tended to clog the filters.

Ca Uptake in Ca Buffers

Ca-EGTA buffers were prepared (see Methods) to achieve the desired free Ca concentration. Fig. 30 shows Ca uptake with three different concentrations (3×10^{-7} to 8.6×10^{-6} M) of free Ca in the medium. When free Ca concentration in the medium was 3×10^{-7} M, there was negligible uptake from this medium. When free Ca ion concentration was raised to 6×10^{-7}, an uptake of 1.3 µmoles of Ca/g protein which is about 20% of the mean value of Ca uptake obtained in a number of experiments shown in previous figures. However in these experiments total Ca concentration varied between 10 to 15 µM (or 1 to 1.5×10^{-5} M), and it was not buffered with EGTA. When free Ca concentration
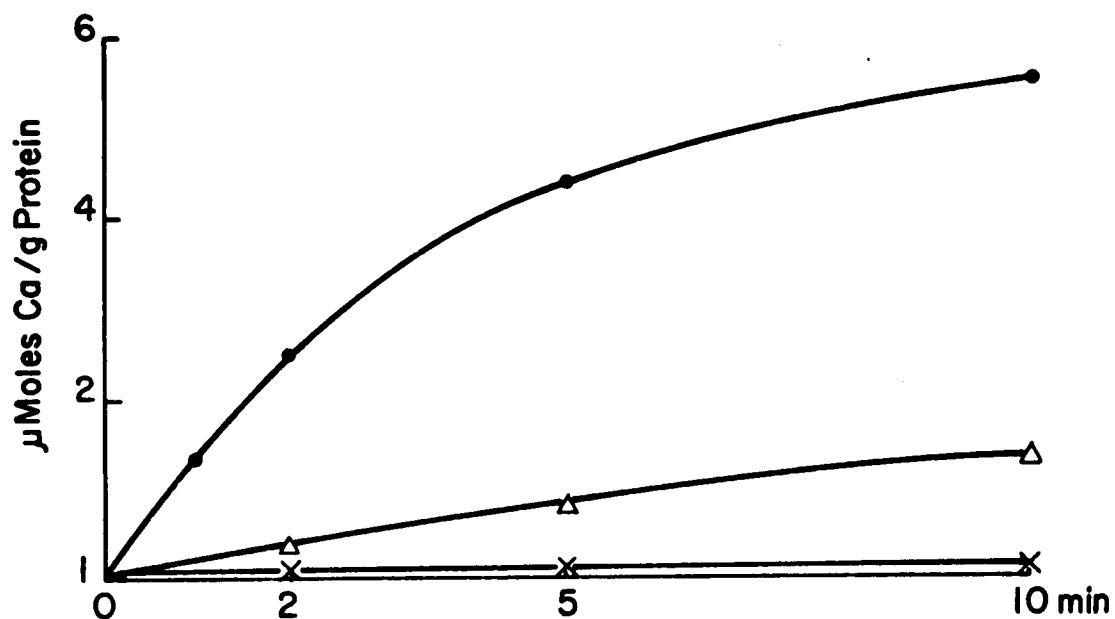
FIGURE 30

Ca uptake by microsomes in standard medium containing
Ca-EGTA buffers with precalculated concentration of
free Ca^{++}. $3 \times 10^{-7}M$ Ca^{++}($\times$), $6 \times 10^{-7}M$ Ca^{++}($\triangle$),
$8.6 \times 10^{-6}M$ Ca^{++}($\bullet$).
Each point is mean of 4 determinations.

was adjusted to 8.6×10^{-6} M, Ca uptake increased to
5.5 μmoles/g protein.

D I S C U S S I O N

Protein Distribution

The protein distribution I observed in various subcellular fractions from rat myometrium (Table 9) is in agreement with the results of Wakid, and Wakid and Needham (144,145) and Wichmann (137). These workers found the bulk of the total homogenate protein in the nuclear and supernatant fraction. Microsomal protein content was found to be greater than mito-chondrial protein content in both these studies. Wakid and Needham (145) reported values (expressed as per cent of the total protein in the homogenate) of 7.6 and 3.2 for the pregnant rat and 10.8 and 7.1 for the ovariectomized rat in microsomal and mitochondrial fractions respectively. Wichmann (137) found 7% and 3.6% of total homogenate protein from the myometrium of pregnant rat, in the microsomal and mitochondrial fractions respectively. In both these studies a Potter-Elvehjem ground glass homogenizer was used. My values of protein as percent total (Table 9) were respectively for microsomal and mitochondrial fractions 10 and 6.6 when the Polytron homogenizer was used and 10.9 and 10.5 when the Teflon homogenizer was used.

Carsten (136) in contrast recently obtained a very low yield of mitochondria and microsomes from cow myometrium after homogenization in a Waring-Blendor.

She reported values of 0.41 and 0.80 mg protein per gram wet weight of the tissue for mitochondrial and microsomal fractions respectively. These values are 10% of the microsomal protein I found, and less than 3% of the mitochondrial protein measured in my study (Table 9). I have no explanation for this large difference.

The protein content of the microsomal fraction from the skeletal muscle was found to be lower by 65% as compared to the myometrium microsomes. This shows that there is considerable variation in the amount of cytoplasmic particles from different tissues in the same animal. This value is however comparable to those reported by other workers (35,78), for similar preparations from skeletal muscle.

Characterization of Subcellular Fractions

The detection limit of the succinic dehydrogenase assay method used was not known. However, there was no detectable activity in the microsomal fraction but the activity was present in mitochondria. Using a similar method, Wakid and Needham (145) found definite succinic dehydrogenase activity in the microsomal fraction but there was more activity in the mitochondrial fraction. However he used a Potter Elvehjem ground glass homogenizer whereas either a Teflon or Polytron homogenizer was used in my experiments. Mitochondria obtained with

these two homogenization techniques showed a difference in activity per mg protein basis; mitochondria of Teflon homogenized tissues had lower succinic dehydrogenase activity by 29%. The lower activity may be due to a greater content of non mitochondrial material contributing to the protein in this fraction since a higher protein content (37%) was found in the mitochondria from the Teflon homogenized tissues.

Electron Microscopy

Consistent with the results of succinic dehydrogenase assay, electron micrographs revealed that microsomal fractions obtained by the procedures used were relatively free of mitochondrial contamination. In nuclear fractions, a number of mitochondria and broken nuclei could be seen. It should be noted that while sucrose is the best isolation medium for preserving both the microsomes and mitochondria, this medium disrupts intact nuclei (148). This point however was not of major concern, since the nuclear fraction was not used for any of the experiments. In the electron micrograph (Fig. 15), of the mitochondrial fraction besides the clear presence of mitochondria, round structures that had smooth walls could be seen. Electron micrographs of the microsomal fraction have a striking similarity to that of Wakid

(144) who also found that a large majority of the large particles had denser small particles attached to their walls. Palade and Siekevitz (149) have reported similar observations with liver and pancreas microsomes, and they presented evidence that the dense bodies consisted of ribonucleoproteins. Collagen fibers could also be distinguished in our micrographs. Wakid found that 5% of the protein in the microsomal fraction was collagen. Carsten (136) in her recent report showed vesicles in electron micrographs prepared from the microsomal fraction of cow myometrium. A triple layered membrane enclosed these vesicles. She concluded that these vesicles were similar to the vesicles obtained from skeletal muscle microsomal fraction. As mentioned in the results, no vesicles with unit membranes were seen in my preparation of microsomal fraction. Whether structural differences exist in vivo between the cow cytoplasmic particles and those of the rat myometrium, or whether these differences were caused as a result of the isolation procedure cannot be answered with certainty.

From the combined evidence from succinic dehydrogenase assay and electron microscopic observations, it may be concluded that the microsomal fraction was relatively free of mitochondrial contamination, and

that the mitochondrial fraction was also relatively
pure (see p.144 for discussion on inhibitors of Ca
uptake).

Net Ca Uptake

Using two different techniques for measuring Ca
uptake (radioactive method or total Ca measurement)
firstly it was shown that there was remarkable agree-
ment in the values obtained by these methods. Secondly
it was demonstrated that there was a net uptake of Ca,
and not an exchange of Ca^{45} with Ca. Another important
observation made from these experiments was that when
Ca was taken up Mg was lost, although the concentration
of Ca in the medium was only 15 µM as compared to 4 mM
of Mg. Since without ATP there was negligible uptake
of Ca, it is suggested that ATP selectively increases
Ca binding which results in displacing another cation
as Mg as shown in this case. Carvalho and Leo (150) also
demonstrated this ATP dependent preferential binding
of Ca in skeletal microsomes. All the Ca taken up could
be accounted for by the amount of Mg lost (Table 15).
Hereafter we will consider Ca^{45} uptake in microsomes as
an indication of net uptake.

Ca uptake in Mitochondria and Microsomes

Total Ca uptake by the mitochondria per mg protein

TABLE 16

Ca binding capacities of subcellular fractions in standard medium

Incubation time - 10 minutes

Amount of Ca added	Uptake mμmoles/g muscle		
	Mitochondria	Microsomes	Skeletal microsomes
8 μM	29.0	36.0	208.0
12 μM	46.99	56.56	---

Calculated from protein value in each fraction per gram tissue and the Ca uptake values.

was 25% higher than the Ca uptake by the microsomes.

However per gram tissue there was 37% more microsomal

protein than mitochondrial protein. Thus the Ca binding

capacity of the microsomes per gram tissue was higher

than the mitochondria (Table 16). Differences between

these fractions were also found in the characteristics

of their Ca uptake (Table 11). Ca uptake by mitochondria,

but, not by microsomes was inhibited by high Na or K.

This inhibition may be due to a competition between these

cations and Ca for transport systems or energy supply

since an ATP dependent uptake and a passive binding of K

and Na has been demonstrated in mitochondria (151,152),

whereas no such transport of Na or K has been shown in

microsomes. If the inhibition of mitochondrial Ca

uptake by Na or K observed in the present experiments is

effective in vivo, Ca uptake by mitochondria can be ruled

out as a mechanism for relaxation of this tissue. There

are contradictory reports (35,107) in the literature on the

effect of Na and K on Ca uptake by skeletal microsomes.

Both inhibition (107) and potentiation (153) have been

reported.

Azide inhibited Ca uptake in mitochondria by 88%

and only by 12% in microsomes. This result lends further

support to the argument that these fractions were relatively

pure, as azide has been known to inhibit Ca uptake and

ATPase activity in mitochondria, but not the ATPase in

microsomes (154).

Mersalyl which has been shown to inhibit Ca uptake in skeletal microsomes (78), a result confirmed in these experiments, completely inhibited Ca uptake in microsomal and mitochondrial fractions of rat myometrium. Carsten (136) found that Ca uptake by the microsomes from cow myometrium was inhibited by 40% by mersalyl.

Neither oxalate nor inorganic phosphate had any effect on microsomal Ca uptake. Both these compounds have been shown to increase Ca uptake in skeletal and cardiac microsomes. In the present experiments on skeletal microsomes, oxalate increased Ca uptake by 2.8 times. A similar enhancement of Ca uptake by oxalate has been shown by other workers (107,115). In heart microsomes oxalate is even more effective in potentiating Ca uptake. Increases of 50 (115) to 100 (155) fold have been reported.

The failure of oxalate and phosphate to enhance Ca uptake in myometrial microsomes may have been due to the non-vesicular nature of the fraction or an incomplete sealing of the vesicle membrane. The former explanation is supported by the electron microscopic observations mentioned above (Figs. 16 and 17). Whereas skeletal muscle preparation contained vesicles with well defined triple layered membranes, myometrial microsomes lacked such vesicles. The potentiating effect of oxalate and phosphate on Ca uptake by skeletal microsomes has been explained on the basis of the ability of these agents to

precipitate Ca, thus trapping it inside the vesicles. In the absence of vesicles or in the presence of a leaky membrane, this effect cannot be obtained. There was a slight lowering of Ca uptake by oxalate and inorganic phosphate in mitochondria. The slight decrease in Ca uptake by myometrial mitochondria may be due to the leakage of Ca as was recently shown by Haugaard et al (156) in liver and heart mitochondria. They found that although Pi in similar concentration as used in the present experiments increased the rate of ATP dependent Ca uptake by mitochondria, Ca was not retained by the mitochondria and a considerable amount of Ca had leaked out after 1 minute. Oxalate may be acting in the same manner. On the other hand, in skeletal muscle mitochondria oxalate was shown to increase Ca uptake (107). In heart mitochondria, oxalate failed to increase Ca uptake (154).

Although total Ca uptake per gram protein was higher in mitochondria as shown in the results, the rate of Ca uptake was slower than in microsomes. The rate of Ca uptake by skeletal muscle microsomes is reported to be much higher than myometrial microsomes; Ca uptake being complete between 1 and 2 minutes at 25°C (107). At the same temperature Ca uptake in myometrial microsomes as shown in the present results was complete between 5 and 10 minutes. This would be compatible with slower speeds

of contraction and relaxation of smooth muscle. Carsten
(136) in her preparations of cow myometrium found that Ca
uptake was virtually complete in one minute at room temp-
erature. The capacity for Ca uptake per gram tissue found
by Carsten (136) was only 2% of the values obtained in
the present results (Table 16). Therefore the rate of
Ca uptake expressed as μmoles of Ca per gram protein
per minute is higher in my preparations than Carsten's
(136). The capacity for Ca uptake by myometrial micro-
somes was only 17% of that of skeletal microsomes. The
value for the capacity of Ca uptake found in this study
falls within the range of values reported for cardiac
microsomes (154,155). Ca uptake was only 12 - 15% in
both mitochondria and microsomes at 4°C as compared to
25°C, and a part of it (2 to 5%) may represent passive
binding as shown in Table 12. The extreme dependence of
Ca uptake on temperature in the presence of ATP and a
virtual absence of Ca uptake in the absence of ATP
suggests an enzymatic reaction and an ATPase as the
likely enzyme.

Mitochondrial Ca uptake was found to be extremely
unstable with time, in complete agreement with Carsten
(136). Microsomal fractions from myometrium were relat-
ively stable as compared to the microsomal fractions from
cardiac or red skeletal muscles which have been shown
to be extremely unstable (157,158). The stability

of myometrial microsomes was similar to that of skeletal
microsomes (95).

ATPase Activity

In agreement with Wakid and Needham (145) and the
recent report of Carsten (136), Mg ATPase activity was
nearly twice as much in microsomal as in the mitochondrial
fraction. Ca uptake per mg fraction protein however, as
mentioned earlier was higher in mitochondrial than in the
microsomal fraction. The reason for a very high ATPase
activity in the myometrial microsomes compared to a
number of tissues studied (159-164) is not clear.

pH Dependence

The pH and microsomal calcium uptake activity curve
was very broad, the optimum pH being 7.5 to 8. Recently
Chimoskey and Gergely (165) found the optimum pH for
total Ca uptake in dog heart microsomes was 7 and that
the Ca uptake decreased to less than 25% at pH 7.4. How-
ever, the optimum pH probably varies from species to
species; e.g. good Ca uptake by calf heart microsomes at
pH 7.4 was reported by Fanburg and Gergely (154). The
absence of a narrower peak for optimum pH in the present
results may be due to the presence of other reaction or
reactions which become rate limiting at near optimal
pH in our experiments. The rate of uptake was not

measured in these experiments so it was also possible
that the total binding capacity of the system was
exceeded in the pH range from 7.5 to 8 so that total
uptake was constant. However, the rates of uptake may
have differed.

ATP and Mg Dependence

ATP was found to be essential for Ca uptake whereas
the addition of Mg with ATP increased uptake by only 11%.
Ca uptake in the absence of added Mg may be due to the
presence of Mg bound to the microsomes. A considerable
amount of bound Mg (170 μmoles/mg protein) was found in
the microsomal pellet. Ebashi and Lipmann (95) obtained
50% Ca uptake by skeletal microsomes in the absence of
Mg compared to the uptake with added Mg. When they re-
moved the bound Mg by washing the microsomes with EDTA,
they were able to get only 10% Ca uptake. With the
addition of Mg in the EDTA treated microsomes, the Ca
uptake did not reach the original value, but was 30%
lower. Martonosi and Feretos (107) also found a con-
siderable Ca uptake by skeletal microsomes in the
absence of added Mg. In the absence of both ATP and Mg,
Ebashi and Lipmann (95) found Ca uptake reduced to 8%, in
our experiments it was reduced to 5%.

Effect of Medium Ca Concentration

The increase in Ca uptake with increase in the con-
centration of Ca in the medium (Fig. 23) is in agreement

with Martonosi and Feretos (107) and Ebashi and Lipmann

(95). Martonosi and Feretos obtained a straight line

when Ca uptake and Ca in the medium (between 10^{-7} to

10^{-5}) were plotted. They did not report experiments

with higher concentration of Ca in the medium. Ebashi

and Lipmann (95) showed an increase in Ca uptake with

increasing Ca in the medium from 0.001 to 0.1 mM. At

0.1 mM the system appeared to be saturated as increasing

the Ca concentration from 0.1 to 1.0 mM slightly lowered

the total Ca uptake. In the present results the curve

(Fig. 23) starts to bend at a concentration of 1 mM.

The question arises whether the increase of Ca

uptake by increasing Ca in the medium shown in these

results and by others for skeletal microsomes (95,107)

is wholly an ATP dependent uptake, or if it is in part

due to a passive binding of Ca when Ca concentrations

increased. Carvahlo and Leo (159) have recently presented

evidence that the uptake of Ca observed in skeletal

microsomes was in fact a selective binding of Ca

induced by ATP in exchange for other cations (H, K, or

Mg). In the absence of ATP, Ca was still able to exchange

with other cations. However, the concentration of Ca

required to displace the same amount of Mg was 1000 fold

higher than that needed in the presence of ATP. He

showed that 10^{-6} M Ca in the presence of ATP displaced

50% of the bound Mg, whereas 2 mM Ca was required to

displace the same amount of Mg in the absence of ATP.
Mg concentration in the medium in their experiments
(150) was 3.8 mM. Results showing (Table 15) that Mg
was displaced when Ca was taken up in the presence of
ATP suggest that Ca taken up by myometrium microsomes
was also in exchange for Mg.

The shape of the curve of Ca^{45} uptake with
increasing Ca in the medium (Fig. 23) is difficult to
explain on the basis of a single type of binding of
calcium to the microsomes. Uptake of Ca^{45} falls very
rapidly to a value of 46% of original when the Ca con-
centration in the medium was raised from 10 to 50 μM.
Thereafter it fell very slowly when the external con-
centration increased from 50 μM to 1000 μM. At 1 mM
there was still 30% of Ca^{45} taken up, and at this point
the curve was already asymptotic. Despite a twenty fold
decrease in the specific activity of the medium, Ca^{45}
uptake was reduced by only 16%.

One of the explanations that can be offered for
this behaviour is that there are two types of uptake of
Ca. One that is relatively selective, ATP dependent and
saturable (active), and the other that is assumed to be
in series with the first is unselective, nonsaturable
and not ATP dependent (passive). When the external Ca
increased, Ca uptake in the first type of sites increased;
and Ca^{45} uptake decreased owing to the decrease in the

specific activity of the medium. At the same time, at some point during the increased Ca uptake, these active sites for selective Ca uptake become saturated. After the saturation of these Ca selective (active) sites, passive uptake still occurred and was capable of taking up passively higher amounts of Ca as the concentration of Ca is increased, probably by displacing other cations according to the law of mass action. Thus as the specific activity of the medium decreased with increasing Ca, passive Ca uptake also increased to the same degree, so that no marked fall in Ca^{45} uptake occurred. This passive uptake might consist of accumulation of Ca in a membrane region or of binding at anionic sites.

Effect of Multivalent Cations

Strontium in low concentrations was shown to be less effective in competing with Ca^{45} than Ca. However, at very high concentrations (1 mM) Sr completely inhibited the Ca^{45} uptake, while Ca only inhibited it by 60%. This may have resulted if the Ca binding was not at a single site; but two types of binding of Ca occurred as discussed earlier.

According to the explanation of two types of binding, Sr in lower concentrations was ineffective since the sites of active binding were relatively selective for Ca. Sr could only bind to the passive sites which I have postu-

lated to be in series with the sites at which Ca is actively bound. Therefore Sr would not have access to the passive binding sites until concentrations (100 fold of Ca) were used to compete with Ca at the active sites. Thereafter, increased Sr concentrations would interfere with Ca uptake at both sites and eventually inhibit it completely. I would like to stress that this is not the only model consistent with the data and that it remains to be tested. However, I have been unable to explain the Ca - Sr interaction on a single binding site model.

Martonosi and Feretos (107) showed that Sr and Ba were relatively ineffective in competing with Ca for ATP dependent uptake in rat or rabbit skeletal muscle microsome. They used concentrations of Sr and Ba up to 40 times that of the Ca concentration in the medium. Van der Kloot (166) however, using a method similar to Martonosi and Feretos, and also using Sr^{89} showed that microsomes from lobster muscle were able to transport Ca and Sr with almost equal facility. Weber (47) has recently showed that transport of Sr had similar maximal rate as did Ca in frog and rabbit reticulum; however a considerably lower affinity for Sr than for Ca by the reticulum was found. Clearly more work needs to be done to establish the relative effectiveness of Sr, Ba and Ca for passive binding and an ATP dependent binding.

Barium was found to be an even poorer competitor

than Sr for binding, as even at 1 mM concentration it
decreased Ca uptake by only 30%. These effects of Sr
and Ba are compatible with their effects on contraction
when substituted for Ca. Strontium and Ba were shown
by Daniel (60) to be able to restore contractions of
Ca depleted uteri, Sr restoring them completely and Ba
partially. In contrast to Ca, Sr and Ba supported
responses disappeared very rapidly when these ions were
removed from the bathing medium. From these and other
results, Daniel postulated that Sr and Ba were much less
tightly bound at the muscle cell surface than Ca, and
Ba less tightly than Sr. This was further extended
to apply to their binding by a hypothetical relaxing
factor. Barium in his experiments produced irreversible
contractures, which would be in keeping with these results
in that Ba was almost completely ineffective in competing
with Ca for uptake; Ca uptake being reflected as relax-
ation in vivo. Uterine muscle contraction supported by Sr
in the absence of Ca was followed by relaxation which was
slower in its onset and less frequent. This would mean
that some binding of Sr must occur although it is much
smaller than binding of Ca.

The effect of La (0.05 mM) was similar to that of Sr
and Ba. It also did not affect Ca uptake. A report by
van Breeman (167) demonstrated that trivalent cation La

was capable of reversibly destroying the permselectivity of the membrane for Ca ions and reversing the membrane polarity. This was done presumably by chelate binding of trivalent cation to nucleophillic sites on the membrane, thus imparting the remaining positive charge to these sites and reversing their polarity. In view of this, it was considered important to know if simple electrostatic binding was the mechanism of calcium accumulation in the myometrial microsomes. This was not found to be the case since La in concentrations more than four times of Ca concentrations in the medium had no effect on Ca uptake. Ineffectiveness of La at these concentrations can be explained again on the basis of relatively selective binding of Ca at the active sites. This is in agreement with the recent finding of Entman et al (168) who showed that La 10^{-5} M had no effect on either ATP dependent Ca uptake by cardiac microsomes or on the exchange of Ca with Ca^{45} in this preparation. The Ca concentration in their medium was however higher (2.5×10^{-5} M) than the amount of La (10^{-5} M) added.

In a very recent report (169) on the effect of La on Ca distribution in intestinal smooth muscle, evidence was presented to suggest that La replaced Ca at superficial binding sites, decreased the mobility of Ca located at other less superficial membrane sites, and prevented uptake

of Ca^{45} to various subcellular sites. Using radioauto-

graphic techniques, Laszlo et al (170) found that La

occupied only the extracellular tissue space, and in

ionic form did not penetrate into the cells. Assuming

despite the above report that some La may have penetrated

into the cells of intestinal smooth muscle in the above

study, it may have then prevented the uptake of Ca^{45} by

the mitochondria. It has recently been shown by Mela

(171) that the energy dependent Ca accumulation in mito-

chondria was strongly inhibited by La.

Ca and ATPase

The results showed that there was no significant

difference in the rate of ATP splitting with and without

Ca in the medium. However, the very rapid rate of ATP

splitting in microsomal material from myometrium may have

obscured a small "extra" ATP splitting. In skeletal and

cardiac muscle a calcium stimulated ATPase "extra ATPase"

has been demonstrated during the Ca uptake by the

reticulum by a number of workers (47,78,95). There are

however reports at variance with it (107,153). There is

no direct evidence that the extra ATPase is in fact a Ca

transport ATPase. Furthermore there are a number of

inhibitors such as oleic acid, (101) fluorodinitrobenzene,

alkaline pH, (172) rutamycin, (103) and caffeine (48),

that inhibit Ca uptake and do not inhibit ATPase activity.

This may be due to the effect of these agents on the membrane, rendering it freely permeable to Ca but this has not been demonstrated. So far no one has demonstrated clearly that Ca uptake can occur without splitting of ATP.

Carsten (136) presented evidence for an "extra ATPase" which might be associated with Ca uptake in the microsomal fraction of cow myometrium. Since a very high rate of ATP splitting was observed in the myometrial microsomes, it is tempting to speculate that in the presence of high basic rate of ATP splitting, there is no need for an extra splitting for Ca transport. The energy needed for Ca uptake may either result from a more efficient use of the basic ATPase or through directing the energy being used for another operation to the Ca transport system depending on the sequence of priorities in the cellular reactions in vivo. Calculation using a ratio of 2 Ca bound per ATP split as found in skeletal microsomes (35,78) suggested that the amount of extra ATP that would be split as compared to the basic ATP splitting would be so small (1.5 μmoles/g protein/min) as to be not detectable.

There is yet another possibility, that the usual methods used for ATPase assay which only measured Pi in the medium are unable to detect the Pi which has been split and incorporated into the particulate material. Thus there may have been an extra splitting of ATP with Ca and the extra Pi split was incorporated into the microsomes,

and thus not detected with the assay method. In fact, recently (173) such a Ca dependent incorporation of Pi in renal membranes has been demonstrated.

Effect of drugs

The failure of oxytocin to affect Ca uptake was not surprising, in view of the general conclusion from a number of studies on the action of this hormone on the excitatory process of the myometrium. In most situations oxytocin caused depolarization and thereby increased the frequency of spike discharges and hence increased con- tractile activities (174-176). Thus the effect of oxytocin was attributed to the changes in membrane permeability to cations. However, oxytocin has been shown to stimulate the depolarized uterus (70). This means that oxytocin may not be able to release Ca from the microsomes directly. However the conditions required for Ca release by oxy- tocin may not have been met by our experimental conditions. In the living cell, when oxytocin causes contraction, there is conceivably more Ca bound by the microsomes (loaded) and a minute amount of Ca (10^{-6}M) in the cytoplasm, whereas in the Ca uptake experiments there were higher concentrations of Ca (10^{-5}M) in the medium, and the microsomes were not loaded. Secondly ionic environment of the cell (high K and low Na) for oxytocin action is again different from that used for Ca uptake experiments by isolated micro- somes (isotonic sucrose). Thirdly the levels of Mg and ATP may be quite different in the living cell than used in the

present experiments. If oxytocin cannot in fact release Ca from the microsomes directly, then there is either the possibility that oxytocin may release Ca from another binding site in the cell or cell membrane, or the receptor mechanism for oxytocin to trigger Ca release may have been damaged in the isolated microsomal fraction.

Ergot alkaloids also did not affect Ca uptake, although they have been known to stimulate the uterine muscle and have in fact been in the past called 'oxytocics'. Innes (177) has however shown that ergot alkaloids, which have been widely used as oxytocin were in fact acting on epinephrine receptors and probably not on oxytocin receptors at all. Whatever the mechanism of their stimulatory effect on the uterine muscle may be, it seems unlikely from the present results that ergot alkaloids acted by altering the ATP dependent Ca binding to the smooth muscle reticulum. There is a single report (178) in the literature that showed that both these alkaloids (ergotamine tartrate and ergonovine maleate) in the concentrations used in the present investigation inhibited Ca uptake from skeletal microsomes. Full details of the method were not given, but the filtration method for measuring Ca uptake was used. If the blanks containing these compounds were not used simultaneously, it is possible that the inhibition of Ca binding observed in the above study may have been merely a complexing of Ca by tartrate and maleate ions which would result in higher Ca in the filtrate. Formation of Ca

complex in solution with the anions of carboxylic acids has been shown (179). In our experiments appropriate blanks were always run simultaneously. The limitations mentioned above in the interpretation of the lack of affect of oxytocin also apply in the interpretation of these results.

Caffeine had no effect on Ca uptake by myometrial microsomes in a concentration which is recently been shown by Weber (48) to inhibit Ca uptake by the skeletal microsomes. Carvalho (123) however found that caffeine did not release the fraction of Ca bound actively or passively by the reticulum of skeletal muscle. Nayler and Hasker (180) have shown that heart mitochondria incubated in caffeine medium had lower Ca than those not incubated in caffeine free medium.

Although there is no conclusive evidence, cyclic 3'5'AMP has been suggested to be involved in Ca transport in several tissues such as kidney, intestines and bones (147,182). By injecting dibutyryl cyclic 3'5'AMP in parathyroidectomized rats Rasmussen and Tennenhouse (147) showed an increase in intracellular Ca in the rat kidney. Recently the presence of adenyl cyclase, the enzyme which forms cyclic 3'5'AMP from ATP, in the canine sarcoplasmic reticulum has been demonstrated (183) and it was suggested that the enzyme may be in-volved in the transport of Ca shown to be present in

these particles. Carsten and Mommaerts (103) however
were unable to show any effect of cyclic 3'5'AMP on
the Ca uptake by skeletal microsomes. Cyclic 3'5'AMP
in concentrations (1 μM) recently shown to be present
in this tissue (181) and at a much higher concentration,
(1 mM), had no effect on Ca uptake. If cyclic 3'5'AMP
were being formed from ATP in the microsomal fraction
and played a role in Ca uptake, caffeine might have
been expected to affect the process by increasing cyclic
AMP levels.

The present results do not establish or disprove
that there is no link between the cyclic AMP levels and
Ca uptake in the microsomes. Study of the effect of
cyclic AMP on microsomal Ca uptake under a variety of
conditions would be required to establish its role with
respect to Ca binding in the intact cell.

Epinephrine (10 ng/ml) increased total Ca uptake
by 20% (after 10 minutes). This is in keeping with its
inhibitory effect on the uterine smooth muscle. One
study by Marshall (175) on the action of drugs on myo-
metrium suggested that the inhibitory action of epine-
phrine may partially be due to its effect on Ca binding
although the primary effect was thought to be due to a
selective increase in the membrane permeability to
potassium. More recently Diamond and Marshall (184,185)
suggested that the depressant action of epinephrine on
the rat uterus may be associated with Ca binding to the

superficial binding sites and was not related to potassium permeability.

Daniel et al using K^{42} have recently provided a more conclusive evidence that catecholamines acting on beta receptors (for example, the inhibitory effect of epinephrine on the rat uterus) did not seem to alter the membrane permeability to K. Schild (187) from his recent experiments on depolarized uterus concluded that isoprenaline may be acting by stimulating an intercellularly located, Ca accumulating mechanism.

My results showing an increase in Ca uptake by myometrial microsomes are consistent with the above observations; however the time taken for the observed increase in Ca uptake with epinephrine (5 to 10 minutes) seems too long to be consistent with the speed of relaxation caused by epinephrine which is known to take only a few seconds (59,70,187). This does not exclude the possibility that in the intact cell, this time taken for Ca uptake is greatly reduced by an appropriate and more efficient chain of reactions. Also the conditions used in the present experiments may not correspond to those controlling Ca uptake in the cell; levels of ATP, Mg, cyclic AMP and Ca may be different during the action of epinephrine in the living cell from those present in the Ca uptake experiments. Therefore the rate limiting steps may be different in the two situations. There is also the possibility that there are reactions

preceeding Ca uptake that are required for the action of epinephrine in the living cell. If this is correct the isolated microsomal fraction is only a part of the machinery required for the relaxant effect of epinephrine.

The increase of Ca uptake observed in the present experiments was not accompanied by an increase in the rate or the amount of ATP split. Furthermore epinephrine did not affect passive binding. These findings lead to the suggestion that an increase in Ca uptake by epinephrine may increase the efficiency of ATP coupling to Ca uptake or that epinephrine in the presence of ATP may increase the binding of Ca; or in turn epinephrine may expose additional binding sites for an ATP dependent Ca binding. Epinephrine has recently been shown to increase cyclic AMP levels by 2 to 5 times in this tissue (181) but cyclic AMP as shown in these results had no effect on Ca uptake. From this it seems reasonable to conclude that the two actions of epinephrine i.e. an increase on Ca uptake and an increase in the levels of cyclic 3'5'AMP are not directly related.

Amount of Microsomes and Ca Uptake

Ca uptake increased with the increase in the amount of microsomes. Hasselbach (144) from his studies on skeletal muscle suggested that each vesicle could take up a fixed amount of Ca, therefore the capacity and the number of vesicles determined how much Ca was taken up.

Ebashi and Lipmann (95) also found an increased Ca uptake
with increasing microsomal protein. However, van der
Kloot (166) found no obvious relation between the protein
concentration and the amount of Ca taken up in the
lobster muscle microsomes. He found that as the con-
centration of protein was decreased, there was only a
slight fall in the total amount of Ca taken up. This may
be due to species differences.

Sensitivity of Ca Uptake System

Experiments using Ca-EGTA buffers showed that the
Ca uptake system was insensitive to a Ca concentration
of 3×10^{-7} M in the medium. This is in surprisingly
good agreement with the threshold concentration for con-
tractions in smooth muscle shown by Filo et al (37) and
recently by Bozler (38). These investigators reported
a concentration of 1.8×10^{-7} and 4×10^{7} M for vascular
and stomach smooth muscle respectively. In the present
experiments when the buffered concentration of Ca was
increased to 6×10^{-7}, Ca uptake became sensitive and
Ca was taken up. With higher concentration (8.6×10^{-6} M)
considerably more Ca was taken up. These results are
similar to those of Weber et al (188) who used Ca-EGTA
buffers in similar experiments on skeletal muscle micro-
somes.

In order to judge whether the microsomal Ca uptake

system studied in vitro is able to control the calcium

ions activity compatible with contraction and relaxation

in this muscle, the following questions should be con-

sidered. Do the microsomal particles in the muscle have

a sufficient capacity to bind the amount of Ca that must

be removed to induce relaxation? Can the rate of Ca up-

take by the microsomes account for the speed of relaxa-

tion?

In order to answer the first question, a number of

previous workers have used calculations based on the

actomyosin content of the muscle, molecular weight of

myosin, and the number of molecules of Ca required per

molecule of myosin for activation in skeletal muscle

(35,155). Recently such calculations were used to

determine the Ca uptake capacity of sarcoplasmic reticulum

isolated from cow uterine muscle (136). These calculations

in my opinion cannot justifiably be applied to smooth

muscle, since, certain important differences in the

properties such as solubility, and ATPase activity of

smooth muscle actomyosin and skeletal muscle actomyosin

have been found (189). Secondly even rough estimates of

molecular weight of myosin of smooth muscle are not

available in the literature. Thirdly the stoichiometry

of combination of Ca and myosin of smooth muscle

is not known and to use figures from data on skeletal

muscle could be misleading. Finally it is clear

166.

from the results of this study, that a number of differences exist in Ca uptake by myometrial microsomes and skeletal microsomes.

I used a simple calculation which includes parameters of this tissue that are known with reasonable certainty. This is as follows. From the results of the experiments with Ca-EGTA buffers discussed above, it is shown that Ca uptake system was insensitive to Ca ion concentration of 3×10^{-7} M and becomes sensitive when the concentration of Ca was raised to 6×10^{-7} M. From these observations and results of others on Ca threshold for contraction in smooth muscle (37,38) it is safe to say that in order to reverse the state of contraction to relaxation in this muscle, the system should be capable of removing the amount of Ca which will lower the cytoplasmic Ca concentration from 6×10^{-7} to 3×10^{-7} M. In other words 3×10^{-10} moles of Ca should be removed per ml. of cell water. However the amount of Ca required for maximal contraction in vascular and stomach smooth muscle was found to be 2×10^{-6} M. Using this figure we get ($20 \times 10^{-10} - 3 \times 10^{-10}$) 17×10^{-10} moles of Ca that must be removed per ml of cell water to cause relaxation from a maximal contraction.

Total tissue water is known to be 840 ml/kg (190). The extracellular space as measured by sucrose is 430 ml/kg (191). Thus the cell water in this tissue will

be (840 - 430) 410 ml/kg or 0.41 ml/g. From 1 gram muscle (17 x 0.41) 6.8 x 10^{-10} moles of Ca should be removed to cause relaxation. From the results with Ca-EGTA buffers mentioned above, it was found that 1.3 μmoles/g microsomal protein was the maximal amount of Ca taken up. From 1 gram of muscle a yield of 8.08 mg microsomal protein was obtained and this amount of microsomes will be capable of taking up 10.5 mμ moles or 105 x 10^{-10} moles Ca. This amount is over 15 times the above calculated Ca that should be removed to cause relaxation from a maximal contraction. This is to be expected because the Ca accumulating structures in living muscle may not be filled to capacity during rest. This is suggested by the observations that contraction followed by relaxation could be elicited by Ca injection several times in skeletal muscle before the system became saturated (7). No evidence of this kind is available for smooth muscles.

To answer the second question further calculation deduced from the above can be made. From the plot of Ca-EGTA results (Fig. 30) used in the above calculations, one can see the rate of Ca uptake (with 8.6 x 10^{-6} Ca in the medium) is linear at least for the first 2 minutes. Although, the Ca concentration in the medium (8.6 x 10^{-6}) was four times the value used above for maximal contraction, one can still use the curve to cal-

culate roughly the time taken to remove 1.2×10^{-10}

moles of Ca/g protein assuming Ca uptake is linear with

time at intervals shorter than measured in the present

experiments. If one assumes that the rate of Ca uptake

is linear with the concentration of microsomal protein,

an assumption that may not be far from true since the

amount of Ca uptake showed a linear relation to the con-

centration of microsomal protein (Fig. 29), one can

calculate the time taken by microsomes from 1 gm of

muscle (8.08 mg) to remove 6.8×10^{-10} moles Ca. This

computation gives us a figure close to 7 seconds. Daniel

(192) has found that the maximum rate of fall of tension

in stretched induced tension in estrogen dominated rat

uterus was 0.8 g/sec. The average maximum tension

developed was 5.8 gm and the mean time for relaxation

under these conditions was 13 seconds. However the Ca

uptake system if responsible for relaxation should remove

the appropriate amount of Ca in less than the time that

it takes for relaxation, since relaxation time represents

the total time for the over all process, and Ca removal

is just one of the steps. However, considering the

difficulties in comparing the rates of Ca uptake in

isolated microsomes and in living cells our data are con-

sistent with the conclusion that relaxation is mediated

by this binding process.

A definite answer to the question can only be given

therefore after direct measurement of Ca uptake at very

short intervals have been made. This is not possible within the limits of accuracy of the present techniques.

There are two other factors that should be considered since they will tend to lower the values of rate of Ca uptake measured in vitro. The first is the purity of the relaxing particles, since the microsomal fraction isolated with the present technique may not be the most active preparation. Hasselbach and Makinose (78,118) were able to get increased rates of Ca uptake by further purifying the microsomal fraction from skeletal muscle. Secondly, the rate of mixing with ordinary stirring method used in these experiments, may not be rapid enough as shown by Ohinshi and Ebashi (193) who by using a rapid mixing technique achieved Ca uptake rates in skeletal microsomes many times higher than the rate observed by Weber et al (47) who used the usual magnetic stirring on similar preparations. Weber et al (47) further noticed that the speed of stirring affected the rate measurements. This may be due to the aggregation of vesicles as pointed out by Weber (35).

On the other hand in all the above calculations of the speed and of the capacity for Ca uptake, it was assumed that all the Ca binding sites available in the isolated fractions were involved in the relaxation process. If this is not correct the uptake observed overestimates the possible role of the ATP dependent Ca binding in relaxation.

Before concluding this study it is worthwhile to
list the similarities and differences that can be found
in the Ca uptake by the sarcoplasmic reticulum of
skeletal and smooth (myometrium) muscle. These were
pointed out wherever appropriate throughout this thesis.

Similarities

(1) Ca uptake in the two systems is similar with
respect to their requirement of ATP and Mg.

(2) Ca uptake increases with the increasing Ca con-
centration in the medium.

(3) Ca uptake increases with increasing protein con-
centration in the medium.

(4) Ca uptake is inhibited by mersalyl.

(5) Ca uptake is not inhibited by azide.

Differences

(1) Myometrial microsomal preparation was not
vesicular in nature unlike the skeletal micro-
somal preparation.

(2) Oxalate or inorganic phosphate are ineffective
in potentiating Ca uptake in myometrial micro-
somes.

(3) The rate and capacity of Ca uptake are much
lower in myometrial system than in skeletal
muscle system.

(4) There is no detectable extra splitting of ATP
accompanying Ca uptake in myometrial microsomes.

The above comparison is also generally valid between cardiac and smooth muscle since the observed characteristics of cardiac microsomal Ca uptake are similar to skeletal microsomal Ca uptake except some quantitative differences, that are listed below.

(1) The rate and capacity of Ca uptake in cardiac system are much lower than the skeletal system. Cardiac system in this regard is closer to the myometrial system.

(2) Whereas oxalate increases Ca uptake by 2 to 5 times in skeletal microsomes, it increases 50 to 100 times in cardiac microsomes.

(3) Cardiac microsomes are considerably more unstable than the skeletal or the myometrial microsomes.

C O N C L U S I O N S

Part II

From the results of Part II of this study, the
following conclusions are drawn:

(1) Mitochondrial and microsomal fractions as isolated
and characterized by the present techniques are
able to accumulate Ca in the presence of ATP and
Mg in the medium.

(2) Characteristics of mitochondrial Ca uptake are
found to be different from the microsomal Ca
uptake. The most important of these differences
was the inhibition of Ca uptake by Na or K in
mitochondria and not in microsomes. This obser-
vation implies that mitochondrial Ca uptake is
unimportant in contraction relaxation cycle in
this muscle.

(3) Properties of microsomal Ca uptake, such as threshold
of Ca sensitivity, speed of Ca removal, and the cap-
acity to remove Ca, are consistent with these para-
meters of contraction-relaxation in this muscle,
with certain qualifications. These arise from the
limitations in the accuracy of the method used and
in our ability to relate the living cell to the
isolated systems.

(4) The small increment of Ca uptake observed with epine-
phrine appears to be unlikely to account for its
relaxant effect in this tissue. The time taken for
the observed increase for Ca uptake was too long to

compare with the time taken for relaxation induced by epinephrine. This result does not eliminate the possibility that epinephrine may cause relaxation by affecting Ca binding in the intact cell.

(5) There were several striking differences in the structural and Ca uptake properties of the microsomal fraction of myometrial and its identical fraction from the skeletal muscle. The slower speed of Ca uptake by myometrial microsomes as compared to the skeletal microsomes is consistent with the slower speed of contraction and relaxation of smooth muscle in comparison with skeletal muscle. No explanation can be given for the absence of vesicular structures in myometrial microsomal preparation as opposed to the presence of vesicles in skeletal microsomal preparation.

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